ADVERSE EFFECTS OF ENGINEERED NANOMATERIALS

EXPOSURE, TOXICOLOGY, AND IMPACT ON HUMAN HEALTH

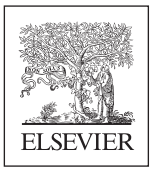
SECOND EDITION

Edited by

BENGT FADEEL
Institute of Environmental Medicine, Karolinska Institutet
Stockholm, Sweden

ANTONIO PIETROIUSTI
University of Rome 'Tor Vergata', Rome, Italy

ANNA A. SHVEDOVA
National Institute for Occupational and Safety Health
Morgantown, WV, United States





ACADEMIC PRESS
An imprint of Elsevier
elsevier.com

Academic Press is an imprint of Elsevier
125 London Wall, London EC2Y 5AS, United Kingdom
525 B Street, Suite 1800, San Diego, CA 92101-4495, United States
50 Hampshire Street, 5th Floor, Cambridge, MA 02139, United States
The Boulevard, Langford Lane, Kidlington, Oxford OX5 1GB, United Kingdom

Copyright © 2017 Elsevier Inc. All rights reserved.

No part of this publication may be reproduced or transmitted in any form or by any means, electronic or mechanical, including photocopying, recording, or any information storage and retrieval system, without permission in writing from the publisher. Details on how to seek permission, further information about the Publisher's permissions policies and our arrangements with organizations such as the Copyright Clearance Center and the Copyright Licensing Agency, can be found at our website: www.elsevier.com/permissions.

This book and the individual contributions contained in it are protected under copyright by the Publisher (other than as may be noted herein).

Notices
Knowledge and best practice in this field are constantly changing. As new research and experience broaden our understanding, changes in research methods, professional practices, or medical treatment may become necessary.

Practitioners and researchers must always rely on their own experience and knowledge in evaluating and using any information, methods, compounds, or experiments described herein. In using such information or methods they should be mindful of their own safety and the safety of others, including parties for whom they have a professional responsibility.

To the fullest extent of the law, neither the Publisher nor the authors, contributors, or editors, assume any liability for any injury and/or damage to persons or property as a matter of products liability, negligence or otherwise, or from any use or operation of any methods, products, instructions, or ideas contained in the material herein.

Library of Congress Cataloging-in-Publication Data
A catalog record for this book is available from the Library of Congress

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library

ISBN: 978-0-12-809199-9

For information on all Academic Press publications visit our website at https://www.elsevier.com/books-and-journals



Publisher: Mica Haley
Acquisition Editor: Erin Hill-Parks
Editorial Project Manager: Tracy
Production Project Manager: Karen East and Kirsty Halterman
Designer: Victoria Pearson

Typeset by Thomson Digital

Contributors

Harri Alenius University of Helsinki, Helsinki, Finland; Systems Toxicology Unit, Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden

Don Beezhold National Institute for Occupational and Safety Health, Morgantown, WV, United States

Enrico Bergamaschi University of Turin, Torino, Italy

Diana Boraschi Institute of Protein Biochemistry of the National Research Council, Napoli, Italy

Hans Bouwmeester RIKILT—Wageningen UR; Wageningen University, Wageningen, The Netherlands

Luisa Campagnolo University of Rome 'Tor Vergata', Rome, Italy

Chunying Chen National Centre for Nanoscience and Technology, Beijing, China

Wim H. de Jong National Institute for Public Health and the Environment, Bilthoven, The Netherlands

Shareen H. Doak Institute of Life Science, Swansea University Medical School, Swansea, Wales, United Kingdom

Dominic Docter University Medical Center of Mainz, Mainz, Germany

Ken Donaldson MRC/University of Edinburgh Centre for Inflammation Research, Queen's Medical Research Institute, Edinburgh, United Kingdom

Rodger Duffin Centre for Inflammation Research, Edinburgh University, Edinburgh, United Kingdom

Albert Duschl University of Salzburg, Salzburg, Austria

Maria Dusinska NILU-Norwegian Institute for Air Research, Kjeller, Norway

Vidana Epa CSIRO Manufacturing, Parkville, VIC, Australia

Bengt Fadeel Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden

Francesca Larese Filon University of Trieste, Trieste, Italy

Irina Guseva Canu Public Health France, Direction of Noncommunicable Diseases and Traumatisms, Saint Maurice, France

Maureen R. Gwinn US Environmental Protection Agency, Washington, DC, United States

Akihiro Hirose The National Institute of Health Sciences, Kamiyoga, Setagaya-ku, Tokyo, Japan

Karin S. Hougaard National Research Center for the Working Environment, Copenhagen, Denmark

Mark A. Jepson University of Bristol, Bristol, United Kingdom

Jun Kanno Japan Bioassay Research Center, Japan Organization of Occupational Health and Safety, Hirasawa, Hadano, Kanagawa; The National Institute of Health Sciences, Kamiyoga, Setagaya-ku, Tokyo, Japan

Shirley K. Knauer Institute for Molecular Biology, CENIDE, Mainz Scientific Screening Center UG&Co. KG, University Duisburg-Essen, Essen, Germany

Robert Landsiedel BASF SE, Experimental Toxicology and Ecology, Ludwigshafen am Rhein, Germany

Tu C. Le CSIRO Manufacturing, Clayton, VIC, Australia

Ying Liu National Centre for Nanoscience and Technology, Beijing, China

Xuefei Lu University of Science and Technology of China, Hefei, Anhui, China

Andrea Magrini University of Rome 'Tor Vergata', Rome, Italy

Mats-Olof Mattsson AIT Austrian Institute of Technology GmbH, Tulln, Austria

Agnieszka Mech Institute for Health and Consumer Protection, European Commission, Joint Research Centre, Ispra, Italy

Nicholas L. Mills Centre for Cardiovascular Science, Edinburgh University, Edinburgh, United Kingdom

Nancy A. Monteiro-Riviere Nanotechnology Innovation Center, Kansas State University, Manhattan, KS, United States

Antonio Pietroiusti University of Rome 'Tor Vergata', Rome, Italy

Craig A. Poland Institute of Occupational Medicine; MRC/University of Edinburgh Centre for Inflammation Research, Queen's Medical Research Institute, Edinburgh, United Kingdom

Adriele Prina-Mello School of Medicine and CRANN Institute of Molecular Medicine, Trinity Centre of Health Sciences, Dublin, Ireland

Jennifer B. Raftis Centre for Inflammation Research, Edinburgh University, Edinburgh, United Kingdom

Kirsten Rasmussen Institute for Health and Consumer Protection, European Commission, Joint Research Centre, Ispra, Italy

Hubert Rauscher Institute for Health and Consumer Protection, European Commission, Joint Research Centre, Ispra, Italy

Elise Rundén-Pran NILU-Norwegian Institute for Air Research, Kjeller, Norway

Ursula G. Sauer Scientific Consultancy - Animal Welfare, Neubiberg, Germany

Kai Savolainen Nanosafety Research Centre, Finnish Institute of Occupational Health, Helsinki, Finland

Jürgen Schnekenburger Biomedical Technology Center, Westfälische Wilhelms-Universität Muenster, Muenster, Germany

Galina V. Shurin University of Pittsburgh Medical Center, Pittsburgh, PA, United States

Michael R. Shurin University of Pittsburgh Medical Center, Pittsburgh, PA, United States

Anna A. Shvedova National Institute for Occupational and Safety Health, Morgantown, WV, United States

Myrtill Simkó AIT Austrian Institute of Technology GmbH, Tulln, Austria

Birgit Sokull-Klüttgen Institute for Health and Consumer Protection, European Commission, Joint Research Centre, Ispra, Italy

Roland H. Stauber University Medical Center of Mainz, Mainz, Germany

Lang Tran Institute of Occupational Medicine, Edinburgh, United Kingdom

Dana Westmeier University Medical Center of Mainz, Mainz, Germany

Dave Winkler CSIRO Manufacturing, Parkville, VIC; Monash Institute of Pharmaceutical Science, Parkville, VIC; La Trobe Institute for Molecular Science, La Trobe University, Bundoora, VIC; Flinders University, Adelaide, SA, Australia

Robert A. Yokel College of Pharmacy, University of Kentucky, Lexington, KY, United States

Il Je Yu Institute of Nanoproduct Safety Research, Hoseo University, Asan, Korea

Tao Zhu University of Science and Technology of China, Hefei, Anhui, China

Yong Zhu University of Science and Technology of China, Hefei, Anhui, China

About the Editors

BENGT FADEEL, MD, PhD



Bengt Fadeel is Professor of Medical Inflammation Research at the Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden, and Adjunct Professor of Environmental and Occupational Health, University of Pittsburgh, Pittsburgh, Pennsylvania. He served as Vice Chairman of the Institute of Environmental Medicine from 2009 to 2013. He received his MD and PhD degrees from Karolinska Institutet and was elected as a Fellow of the Academy of Toxicological Sciences in 2012. He is a current or past member or coordinator of several EU-funded projects on nanosafety, including NANOMMUNE, MARINA, NANOREG, SUN, and NANOSOLUTIONS, and member of the EU-funded GRAPHENE Flagship Project, and of the national MISTRA Environmental Nanosafety consortium. Dr. Fadeel is also Chair of the Working Group on Systems Biology in the EU Nanosafety Cluster and a member of the WHO-IPCS Working Group on principles and methods to assess the immunotoxicity of nanomaterials. He is the Chair of the Scientific Panel of the National Platform for Nanosafety in Sweden. He is an author or coauthor of more than 200 original papers and review articles to date. Dr. Fadeel was awarded with the national Environmental Medicine Prize by the Cancer and Allergy Foundation in 2011 for his research on the opportunities and risks of the emerging nanotechnologies.

ANTONIO PIETROIUSTI, MD



Antonio Pietroiusti received his habilitation as Associate Professor in Occupational Medicine from the Italian Ministry of Education, University and Research in 2013 and he teaches Occupational Medicine at the Faculty of Medicine and Surgery of the University of Rome Tor Vergata (English course). He is also Professor of Occupational Medicine at the University "Nostra Signora del Buon Consiglio" of Tirana, Albania. He is/has been member or coordinator of several national projects funded by the Italian Ministry of Health and by the Italian Ministry of Education, University and Research. He is/has been member of two Seventh Framework Program projects on nanosafety including FP7-MARINA and member of one Seventh Framework Program project on the EU/LAC cooperation. He is an Editor of the

newsletter of the Toxicology Group of the Royal Chemical Society (United Kingdom), and served as coeditor of the special issue on Nanotoxicology published by the journal *Toxicology and Applied Pharmacology* in 2016. He was awarded by the Italian Society of Occupational Medicine in 2012 for his scientific activity in Occupational Medicine. He is an author or coauthor of 120 scientific articles.

ANNA A. SHVEDOVA, PhD, DSc



Anna A. Shvedova is Lead Research Physiologist at National Institute for Occupational and Safety Health (NIOSH) at the Center for Disease Control and Prevention (CDC) and Adjunct Professor of the Department of Physiology and Pharmacology, School of Medicine and Adjunct Professor of the Department of Pharmaceutical Sciences, School of Pharmacy of West Virginia University, West Virginia. Dr. Shvedova received her PhD and DSc degrees from Moscow University, Russia and is currently Principal Investigator of a number of NORA/National Occupational Research Agenda and NTRC/Nanotechnology Research Center projects at the Exposure Assessment Branch/NIOSH/CDC. Dr. Shvedova was Founder and first President of the Dermal Toxicology Specialty Section of the Society of Toxicology (SOT). She is member of advisory board committees for US Army, Air Force, and NASA projects and served as work package leader in the FP7-NANOMMUNE project (2008–11), and she is a partner of the FP7-NANOSOLUTIONS project (2011–17) and member of the ethical board review of the Graphene Flagship Project of the European Commission (2016–20). Dr. Shvedova has been honored with the SOT Public Communication Award in 2001; Alice Hamilton NIOSH Award for paper of the year in Occupational Safety and Health in 2006, 2009; Bullard–Sherwood Award: Research for Practice in 2011; and Women in Toxicology SOT Award in 2007. Dr. Shvedova is board member of the working group on Skin Notation at NIOSH/CDC, Associate Editor of *Toxicology and Applied Pharmacology*, and Editorial Board Member of *Regulatory Toxicology and Pharmacology*. Dr. Shvedova is an author or coauthor of more than 160 scientific articles and book chapters.

Preface

In the past decade, nanotoxicology has emerged as a specific domain within the toxicological sciences. In fact, there has been an exponential rise in the number of scientific articles on the subject; however, nanotoxicology as a discipline is still struggling with the fundamental question: are there specific environmental safety and health concerns associated with nanomaterials as a function of their particular or novel properties, and does this call for specific regulations to be applied for nanoenabled products and technologies? Furthermore, while considerable progress has been made during the last 10 years, and important lessons have been learned, such as the realization that a thorough physicochemical characterization is needed to properly evaluate the toxicological results, nanotoxicology still faces a number of challenges related to standardization and validation of test methods, and the implementation of reference materials, to allow for comparability of results across different studies; there is also a need for more realistic test scenarios, including low-dose, long-term exposures to nanomaterials. Notwithstanding, we have already made significant progress in terms of teasing out the properties that make some nanomaterials harmful and others essentially inert.

Indeed, in the 5 years that have passed since the first edition of the present volume, a vast number of new studies have been published that are of relevance for potential human health effects of nanomaterials. In this new and updated edition, several new chapters have been added, including a comprehensive chapter on risk assessment of nanomaterials, and all the chapters in the present volume have been updated to encompass new research findings regarding hazard, exposure, and risk of nanomaterials. The overall objective remains: to assess the potential of engineered nanomaterials to cause human disease, by systematically examining their possible effects on different organs and systems.

The first section of the book comprises eight chapters, aimed at providing sufficient background on nanomaterial exposure assessment and biomonitoring, as well as hazard assessment and risk assessment, to enable the reader to properly evaluate the subsequent chapters on potential human health effects. More specifically, the first chapter of the book gives an overview of the biological interactions of nanoparticles, focusing on the emerging concept of the biocorona, essentially the adsorption of proteins and other biomolecules onto the surface of nanoparticles, thus endowing the particles with a new, biological "identity." The following chapter provides a detailed description of the physicochemical characterization of the intrinsic properties of nanomaterials (or, the synthetic "identity"), with a discussion on the parameters measured as well as the methods used for these measurements. The authors also discuss the definition of a nanomaterial, which is important not least from a regulatory point of view. The third and fourth chapter deal with hazard assessment of nanomaterials and they describe two different, yet complementary approaches to the problem: on the one hand, the selection of the most appropriate in vitro (cell culture)

and/or in vivo (animal) tests, and on the other, the use of computational approaches and the development of predictive models. These chapters are followed by two chapters on exposure assessment and biomonitoring, respectively; focusing in particular on the occupational setting in which inadvertent exposure may occur (as opposed to the intentional administration of nanoparticles in the clinical setting). The chapter on exposure also discusses the most appropriate health surveillance programs, based on the current knowledge of the biological effects of nanomaterials. The chapter on biomonitoring includes a discussion on the emerging topic of systems toxicology and the application of global "omics" approaches with which to assess effect or exposure to nanomaterials. The first section of this volume closes with two chapters on regulation and legislation, and risk assessment and risk management, respectively. The former chapter is coauthored by experts from the European Union, United States, and Japan/Korea, and provides a unique view on international regulations applicable to nanomaterials. In the chapter on risk assessment, the authors discuss the concept of grouping or categorization of nanomaterials and how this may inform modern risk assessment approaches.

The second section of this volume includes 10 chapters addressing the pathogenic potential of engineered nanomaterials for specific organs and tissues. The first and second chapters in this section describe the possible adverse effects of nanomaterials on the respiratory system, one of the most important routes of exposure to engineered nanomaterials; the first one is focused on basic mechanisms of pulmonary effects of nanomaterials, with a special view to inflammation, fibrosis, and carcinogenicity, while the second one discusses allergic reactions and asthma, focusing on carbon nanotubes, which have been extensively studied from the point of view of pulmonary exposure (in animals). The subsequent chapters each deal with the effects of nanomaterials on specific organ systems, including the cardiovascular system, the neurological system/central nervous system, the immune system, the endocrine system, the gastrointestinal tract, and the skin. The body of evidence for the disease potential of nanomaterials is substantial: the possibility of pulmonary fibrosis, malignant mesothelioma, and atherosclerotic disease thus needs to be considered, at least for some nanomaterials. In other cases, the link with human diseases is much more tenuous, such as for neurodegenerative disorders, or inflammatory bowel disease, or for diseases or imbalances of the endocrine system. It should be considered, however, that the amount of research on the possible pulmonary and cardiovascular effects is much higher than for nanomaterial effects on neurological, gastrointestinal, or other organ systems. The skin seems the most impervious organ to the injurious action of nanomaterials, although, as pointed out by the authors, more long-term studies are needed to ascertain the potential for adverse effects. The skin is the principal, physical barrier protecting us from the outside world, while the immune system provides a first line of defense against foreign intrusion. Particular attention to the interactions of nanomaterials with the immune system are therefore warranted; indeed, assessment of immune effects may be viewed as a "sentinel" form of hazard assessment of nanomaterials. As noted by the authors, model systems that reflect vulnerable conditions, like chronic obstructive pulmonary disease, are needed; the majority of studies have been conducted using models of the healthy organism. The second section ends with a chapter on fertility and reproduction, and a chapter specifically devoted to genotoxicity (DNA damage) and cancer. Detrimental effects of

nanomaterials on embryonic development have been reported and this remains an area of great concern. Similarly, the potential for certain engineered nanomaterials to elicit genotoxicity is well documented, and recently, some nanomaterials were classified as being potentially carcinogenic in humans.

This book gives a state-of-the-art presentation of relevant research on adverse effects of engineered nanomaterials on human health. We are indebted to all the colleagues from around the globe who have contributed. We hope that this volume will serve as a useful guide for students and researchers in the field and for clinicians, policymakers, and regulators with an interest in nanosafety and human health.

Bengt Fadeel
Karolinska Institutet

Antonio Pietroiusti
University of Rome Tor Vergata

Anna A. Shvedova
National Institute for Occupational and Safety Health

CHAPTER

1

Bio–Nano Interactions

*Dana Westmeier**, *Shirley K. Knauer***, *Roland H. Stauber**, *Dominic Docter**

***University Medical Center of Mainz, Mainz, Germany; **Institute for Molecular Biology, CENIDE, Mainz Scientific Screening Center UG&Co. KG, University Duisburg-Essen, Essen, Germany**

OUTLINE

Introduction 3

Protein Corona on Nanoparticles 4

Development of the Protein Corona 4
Hard Corona Versus Soft Corona 4
The Biomolecule Corona Composition 5
Dynamic Formation of the Biocorona 6

Impact of the NP-Biomolecule Corona on Human Systems 7
In Vitro Studies 8
In Vivo Studies 8
Microbiome Interactions 10

Nanotoxicity and the Biomolecule Corona 10

Conclusions 11

Take-Home Messages 11

Acknowledgments 11

References 12

INTRODUCTION

The widespread utilization of engineered nanomaterials (NMs) in technical products, but also the application in biotechnology and biomedicine is steadily growing and caused by the exciting cross-disciplinary development of the intersection of nanotechnology and biomedicine (Docter et al., 2015a,b; Reese, 2013; Setyawati et al., 2015; Webster, 2013). Based on progresses in synthesis and design, the ability to create NMs with a wide variety of different features, for example, chemical properties, size, or surface functionalization, opens up new possibilities for the generation of NMs with optimally adapted characteristics for their intended technological or biomedical use (Docter et al., 2015a; Rauscher et al., 2013; Rizzo et al., 2013).

Adverse Effects of Engineered Nanomaterials. http://dx.doi.org/10.1016/B978-0-12-809199-9.00001-X
Copyright © 2017 Elsevier Inc. All rights reserved.

Whereas the design of NMs allows the control over the physicochemical properties in a stable environment, this is no longer the case in complex physiological or natural environments. Upon contact with any kind of biological environment, NMs adsorb (bio)molecules due to their high surface free energy, forming the so-called (bio)molecule corona. The physical and chemical interactions with biomolecules significantly alter the physicochemical properties of the NM and represent an important element of its biological identity (Kapralov et al., 2012; Monopoli et al., 2011a, 2012; Tenzer et al., 2013). This chapter gives (1) an overview of the concept of corona formation, (2) provides insight into the impact of the protein corona on cellular processes, the fate of NMs in vivo and the influence on NM–microbiome–host interactions, and (3) gives an introduction to the importance of the corona in the field of nanotoxicology.

PROTEIN CORONA ON NANOPARTICLES

Although the pioneering work about protein absorption by Vroman (1962) was published in 1962, the term "protein corona" was established much later by Cedervall et al. (2007b). Accordingly, these studies stimulated the whole "nanocommunity" and led to a large variety of new investigations dealing with the interface between NMs and biological components.

DEVELOPMENT OF THE PROTEIN CORONA

By using NMs, their exposition in complex physiological systems is unavoidable, for example, for biomedical application, NMs are intravenously injected into the patient (Webster, 2013). Here, NMs are subjected to many different, complex biomolecules like in blood, intestinal juice (Docter et al., 2015b; Monopoli et al., 2011b). Upon contact with these complex environments, NMs adsorb proteins and other (bio)molecules (e.g., phospholipids, sugars, nucleic acids, and so on) due to their high surface free energy, forming the so-called (bio)molecule corona (Monopoli et al., 2011b; Nel et al., 2009; Tenzer et al., 2013). Consequently, the transformation of the bare NM into a NM with a biological coating significantly affects the NMs fate and behavior (Cedervall et al., 2007b; Docter et al., 2015b; Monopoli et al., 2012; Westmeier et al., 2015a,b).

Furthermore, the surface of the NMs are clearly defined by the adsorbed biomolecules that represent an important element for the biological identity of NMs and are involved in mediating further interaction with the surrounded environment (Cedervall et al., 2007a; Klein 2007; Treuel and Malissek, 2013).

Hard Corona Versus Soft Corona

Nowadays, the term "hard protein corona" describes the tightly bound layer of biomolecules that represents the analytically accessible protein/biomolecule signature of NMs in a specific environment (Docter et al., 2015b; Monopoli et al., 2012; Tenzer et al., 2013). Additionally, some models suggest the existence of a "soft protein corona" on top of this "hard corona" and hereby not directly interacting with the NM (Fig. 1.1) (Monopoli et al., 2012; Walczyk et al., 2010; Walkey et al., 2014). Nevertheless, this "soft corona," that consists of

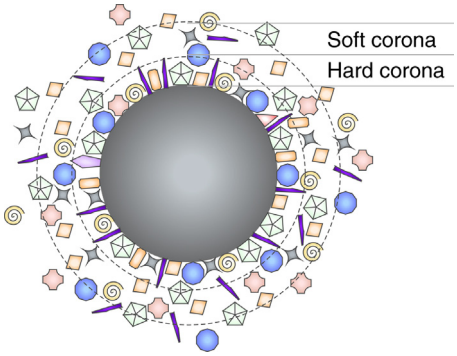


FIGURE 1.1 Protein corona models: hard versus soft corona. The hard corona is defined as the sum of the analytically accessible NP-protein complexes whereas the soft corona consists of loosely associated and rapidly exchanging proteins. The term "protein corona" or "biomolecule corona" is generally used for the analytically-accessible "hard corona."

more loosely associated and rapidly exchanging biomolecules, desorbs during current purification processes and its existence and relevance for effects at the nanobiointerface remains not fully confirmed. Thus, as the current literature shows up, more issues for the definition of the "hard" versus the "soft corona" then helps to describe and resolve scientific problems, we suggest to generally use the term "biomolecule corona" or just "corona" for the analytically-accessible "hard corona" (Docter et al., 2015b).

The Biomolecule Corona Composition

Typically, it is the biomolecule-corona that primarily interacts with the respective environment and hereby constitutes a major element of the biological identity of the NM (Lee et al., 2015; Monopoli et al., 2011a; Tenzer et al., 2013; Treuel et al., 2015; Walkey et al., 2014). Moreover, the biophysical properties of NMs significantly change by the biomolecule coating, which is why these NMs may be even considered as novel materials compared to the pristine NMs without biomolecule corona (Monopoli et al., 2011a; Nystrom and Fadeel, 2012; Tenzer et al., 2013; Wolfram et al., 2014). Furthermore, the corona profiles are extremely influenced by the composition of the physiological fluid in which they are investigated. Therefore, distinct proteins will be either enriched or display only weak affinity for the surface of the NM (Monopoli et al., 2012; Tenzer et al., 2011, 2013; Walkey et al., 2014). For example, although the blood plasma contains thousands of different proteins with different abundance, the most abundant ones are not always present in the protein corona or respectively, they are not also the most abundant in the protein corona (Martel et al., 2011; Tenzer et al., 2011; Zhang et al., 2011). Additionally, size, material, and surface properties of the NM as well as the exposure time and the relative ratio of the physiological fluid to the NM dispersion play an important role in the determination of the protein corona (Caracciolo et al., 2011; Dobrovolskaia et al., 2014; Gebauer et al., 2012; Monopoli et al., 2012; Tenzer et al., 2013; Walkey et al., 2012, 2014). Another question is, whether the corona changes its composition when NM moves through different physiological compartments, for example, from the blood system into phagocytes.

As the NM enters different environments, it is assumed that the final corona still contains some kind of a shaped fingerprint of its "travel history" (Lundqvist et al., 2011; Monopoli et al., 2012). However, even if all physiochemical properties of the NM and all other influencing factors are known, it still remains challenging to predict the composition of a biomolecule corona in a complex physiological environment (Westmeier et al., 2015b).

Dynamic Formation of the Biocorona

Typically, protein-adsorption takes place in a highly dynamic process with constantly adsorbing and desorbing proteins (Cedervall et al., 2007b; Dell'Orco et al., 2010; Natte et al., 2013; Vroman, 1962). As already published in 1962, the "Vroman-effect" postulated that the identities of adsorbed proteins are able to change over time but the total amount of adsorbed proteins is more or less stable (Vroman, 1962). Even though, Vroman demonstrated this binding kinetics only for a mixture of few proteins. Therefore, there is an urgent need for a time-dependent high-resolution knowledge of the biomolecule adsorption onto NMs in physiological fluids containing a complex, diverse mixture of thousands of proteins. Thus, snapshot kinetic proteomic profiling was performed and the existence of complex protein adsorption kinetics was shown for silica and polystyrene NMs, even in human plasma (Tenzer et al., 2013). According to the "Vroman-effect," increasing or reduced binding of proteins was also detected in human plasma whereas the new protein binding kinetics cannot solely be explained by the work of Vroman (Fig. 1.2). Moreover, some proteins groups showed an increased (rise) or decreased (fall) binding behavior over time. In addition, other protein groups were identified at low abundance at the beginning and at the end of plasma exposure but were present at very high abundance at intermediate exposure time points (peak), while conversely other proteins showed the exactly opposite binding character, which means a high abundancy at early and late exposure time points but not at the intermediate ones (cup).

Previous kinetic studies did not employ quantitative LC-MS-based proteomics, and this complex binding kinetics went unnoticed so far. In the study by Tenzer and colleagues, the authors could show that the binding patterns observed in the human blood plasma model cannot be explained by the current mathematical protein-corona-evolution models in simplified systems and a multiparameter classifier will likely be required to model nanoparticle–protein interaction profiles in biological relevant environments (Dell'Orco et al., 2010; Gebauer et al., 2012; Lundqvist et al., 2011; Tenzer et al., 2013). Additionally, in the same study, the authors could show that the protein coronas formed very rapidly and exhibited an unexpected complexity on all investigated nanoparticles (NPs). Previous studies suggested that the protein corona consists of only a few tens of proteins, even when NMs are introduced into a highly complex environment, such as the human blood plasma (Lesniak et al., 2012; Monopoli et al., 2011b). Tenzer et al. (2013) were able to detect and quantify as much as 166 different plasma corona proteins at the earliest exposure time point on all investigated NPs. By extending their analyses to prolonged plasma exposure time points and by employing a latest generation label-free quantitative liquid chromatography mass spectrometry instrument, they detected almost 300 different corona proteins for all exposure time points. Over the different investigated time-points, the corona composition changed only quantitatively and not qualitatively (Tenzer et al., 2013).

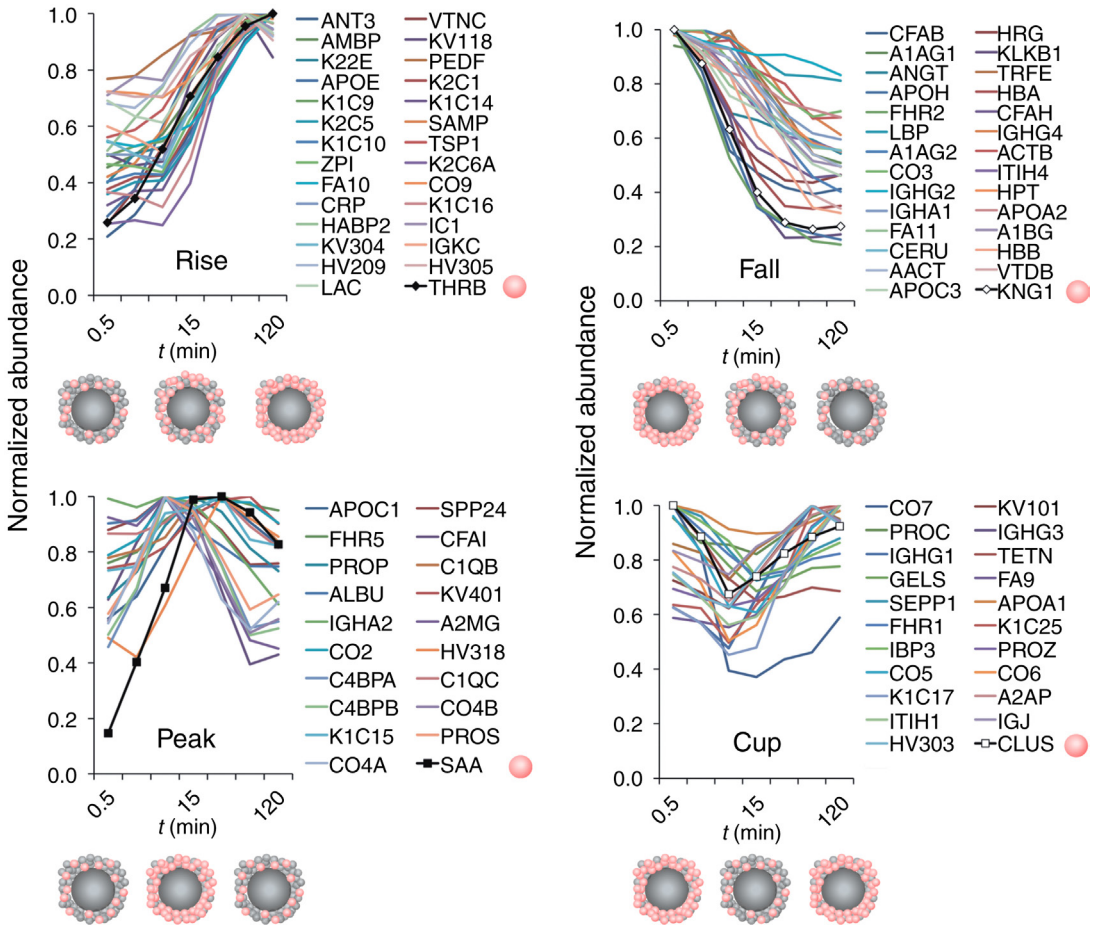


FIGURE 1.2 **Protein-binding kinetics during temporal evolution of the plasma corona.** Time-smoothed normalizes protein abundance profiles of negatively charged polystyrene NPs. NP coronae were divided into four groups by correlation analyses and relative values were normalized to the maximum amount over all time points for each protein. Protein groups displayed increasing (rise) or decreasing (fall) binding time over time. "Cup" proteins showed high abundance at the beginning of plasma exposure and at later time points, but not in the intermediate phase. Conversely, "peak" proteins display a high abundancy at the intermediate time points, but not at the beginning or at the end of the exposition.

IMPACT OF THE NP-BIOMOLECULE CORONA ON HUMAN SYSTEMS

As biomolecules rapidly adsorb onto NMs forming the biomolecule corona, thereby creating a material with novel characteristics, a mechanistic comprehension of the impact of this biomolecule corona is essential. In order to understand the impact of the corona, in vitro as well as in vivo analyses have to be conducted. Furthermore, as different types of microorganisms are present at all major NP entry routes, the impact of the corona in the human microbiome is also a mandatory topic.

In Vitro Studies

Upon exposition, NMs will interact with cells whereas almost all mammalian cell culture models are capable of an unspecific cellular uptake (Hillaireu and Couvreur, 2009). Several studies have already shown that the protein corona has an important impact on uptake and on other cellular mechanisms (Nazarenus et al., 2014), but studies on the specific role of the biomolecule corona in favoring toxicity have produced contrasting results. For example, in the earlier-mentioned study by Tenzer et al. (2013) the authors were able to show the (patho) biological relevance of the protein corona in in vitro studies of primary human cell models of the blood system and therefore established a direct link between the corona formation and their (patho)biological relevance. Pristine NPs existed only for a short period of time, but could immediately affect vitality of endothelial cells, trigger thrombocyte activation and aggregation, and result in hemolysis. Formation of the biomolecule corona rapidly modulated the NPs decoration with bioactive proteins, thus, protecting cells of the blood system against NP-induced (patho)biological processes, and also promoting cellular uptake (Tenzer et al., 2013). Also in the study by Wang et al. (2013) only the uncoated silica particles had a significant hemolytic effect on red blood cells, whereas the formation of a biomolecule corona on the particle surface protected the cells from this damage. As the protein corona plays a critical role in biodegradation, cellular uptake, and clearance of NPs (Pelaz et al., 2015; Saptarshi et al., 2013), researchers are now recognizing that the protein corona has also the ability to mediate or to alter immune responses. Furthermore, the immune system is expected to react to NPs, and thus, a better understanding of nano immunointeractions is critical for the safe application of engineered NPs in medicine (Farrera and Fadeel, 2015). In the work by Yan et al. (2013), the researchers showed the selective cell recognition for protein corona formation on poly-(methacrylic acid) nanoporous polymer particles. Compared to the uptake of bare NPs, the human monocytic cell line THP-1 internalized much fewer NPs in the presence of serum albumin. On the contrary, in the case of differentiated THP-1, that is, macrophage-like cells, the presence of albumin on the NP surface induced an increase in the internalization rate accompanied by the secretion of inflammatory cytokines as a proof of the activated phagocytosis (Lee et al., 2015; Yan et al., 2013). Attempts were also made to coat NPs by specific proteins. For example, transferrin, well known to be internalized via its cognate receptor, was used to create a protein corona and to study its effect on cellular uptake (Jiang et al., 2010; Salvati et al., 2013). Salvati et al. (2013) were able to demonstrate how transferrin-functionalized NPs can lose their targeting capabilities when a biomolecule corona adsorbs on their surface. They studied the uptake of transferrin-decorated silica NPs by A549 lung epithelial cells. After adding bovine serum to their experiments, proteins from the surrounding medium reportedly formed a corona around their prefunctionalized NPs shielding transferrin from binding to both its cognate receptors on cells and also to soluble transferrin receptors. While NPs were still taken up by the cells, the targeting specificity of transferrin was lost (Salvati et al., 2013). These findings underline very well, the complexity of the situation where even protein mediated cell-targeting suffers from corona formation under physiological conditions.

In Vivo Studies

NMs can enter the body in different ways (Fig. 1.3). As an intended NM administration is usually performed by intravenous injection (Riehemann et al., 2009; Webster, 2013), oral

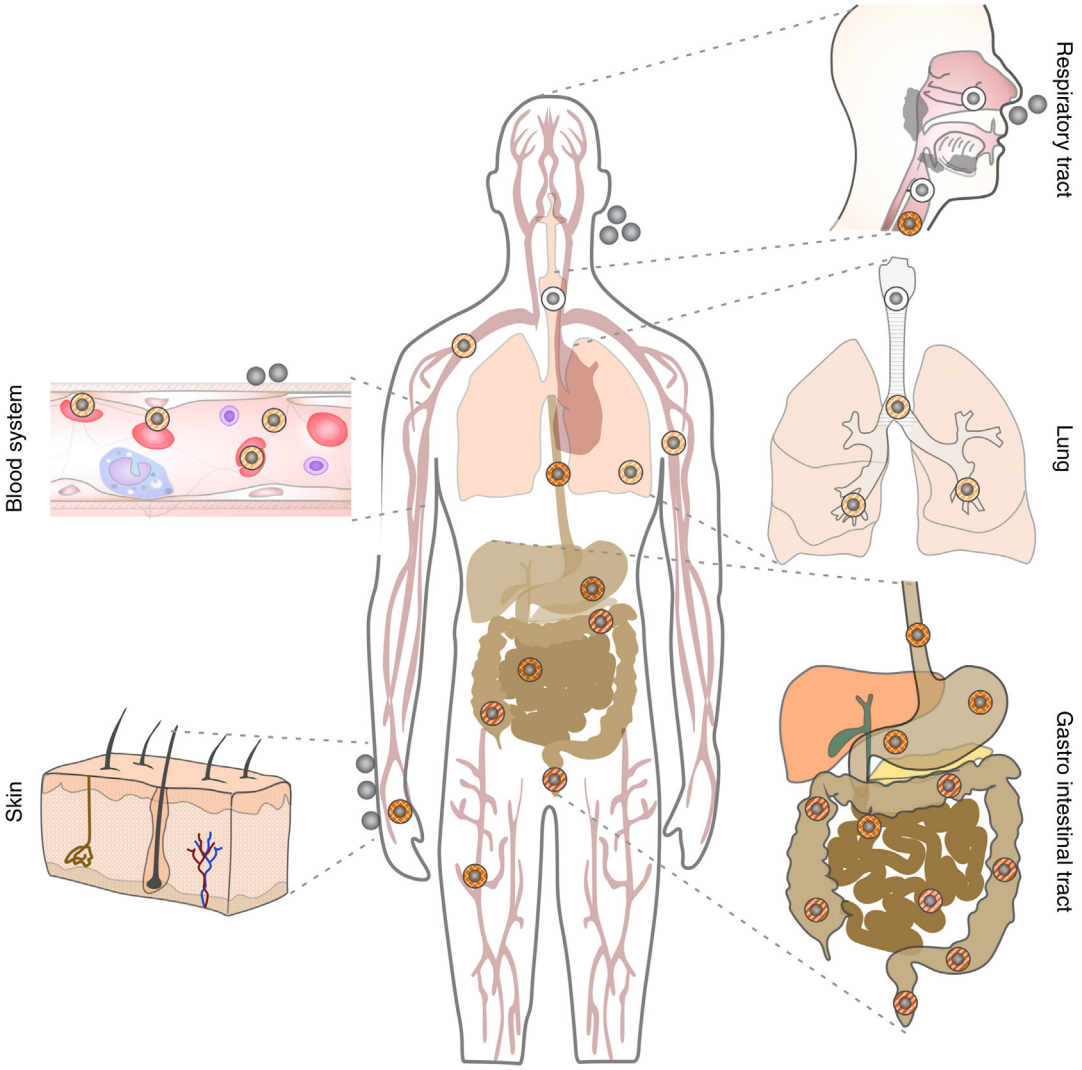


FIGURE 1.3 Possible entry routes for NPs. After accidental exposure or intended NP application, NPs enter the human body by intravenous injection or by oral, dermal, and pulmonic routes. Upon contact with physiological environments, biomolecules immediately absorb onto the NPs, forming the biomolecule-corona. Upon contact with other (micro)environments, the corona composition most likely changes, but still keeps a stable "fingerprint" from its initial entry site.

(Schleh et al., 2012), pulmonic (Lipka et al., 2010), and dermal (Lademann et al., 2011; Vogt et al., 2014) routes represent additional uptake options that are also involved in unintended NM exposure, for example, via air pollution. Regardless of whether NMs enter the human body by accidental exposure or by medical administration, in physiological environments, biomolecules immediately adsorb onto the NMs (Monopoli et al., 2011a; Tenzer et al., 2013).

During biodistribution in the body, the NMs' corona is expected to change due to the different physiological compartments. Nevertheless, the final corona most probably still remains a memory of the NMs travel route (Lundqvist et al., 2011; Monopoli et al., 2012). If NMs are able to overcome biological barriers, they will enter the blood stream which will spread the NMs to distinct organs. Here, it is mandatory to see the NM-biomolecule complexes as heterostructures, which means NMs in vivo should no longer be considered as a homogenous entity. Thus, these inorganic/organic/biological nanohybrids may have completely different distribution and degradation pathways for which the specific fate has to be analyzed individually (Feliu et al., 2016; Pelaz et al., 2013; Rivera-Gil et al., 2013). However, NMs are primarily cleared from the blood by the reticuloendothelial system of the liver and the spleen which are the major organs for NP excretion (Fabian et al., 2008; Kreyling et al., 2015; Li et al., 2010).

Microbiome Interactions

Interestingly, while we are facing many reports on the impact of the NM corona on human cells, it is more than surprising that no studies on the relevance of the NM corona on the microbiome as well as on its interaction with host cells have been reported so far (Docter et al., 2015b). The human microbiome is defined as the sum of all microorganisms and their respective genomes on and inside the human body (Docter et al., 2015b). Furthermore, the microbiome is involved in many physiological processes like digestion, but depending on the amount and type of microorganisms, the development of several diseases can also be promoted (Bergin and Witzmann, 2013; Brown et al., 2013; Goodrich et al., 2014; Human Microbiome Project, 2012; Merrifield et al., 2013; Williams et al., 2014). Notably, various types of microorganisms are present in all the main exposure and entry sites for NMs in the human body. Similar to previously described scenarios, microorganisms as well as the microorganisms-host environment probably face corona-covered NMs rather than pristine NMs. Although experimental data is still missing, the impact of NMs and their corona is expected to be extremely diverse. Therefore, the analysis of the influence of NMs and the corona on endogenous microbial communities and its subsequent relevance for nanotoxicology and organism health in mammalian certainly represents a major area that needs to be explored.

NANOTOXICITY AND THE BIOMOLECULE CORONA

As humans are exposed to increasing concentrations of NMs in the environment, "nanotoxicology" has developed to an independent research field and the nanotoxicological aspects regarding the NM characteristics present a highly important research area. Especially, the formation of the biomolecule corona influences NMs' toxicity and pathophysiology (Tenzer et al., 2013). For that reason, several studies on the role of the corona have been performed, albeit showing partially contrasting results. As an example, Tenzer et al. (2013) demonstrated the (patho)biological impact of the protein corona in in vitro studies using primary human cells of the blood system. Here, protein-coated NMs compared to pristine NMs showed less toxicity indicating that the corona protects the cells against

the NM-induced (patho)biological processes and furthermore, was also able to stimulate cellular uptake (Tenzer et al., 2013). In another study, the protein corona demonstrated an important role in cellular uptake and hereby again for toxicity (Docter et al., 2014). Here, in vitro studies showed that not only the size of NMs is involved in cellular toxicity, but also a reduced cellular uptake of NMs is caused by the corona-formation (Docter et al., 2014). Also, the ability of NMs to induce or alter immune responses and hereby activating the immune system is also dependent the NM-protein corona complex. Thus, it is important to understand nanoimmune interactions for the safe application of NMs in medicine (Farrera and Fadeel, 2015). As discussed in Chapter 4, there are also an increasing number of studies using computational (modeling) approaches to address the interactions between different NPs and (specific) proteins.

CONCLUSIONS

In this chapter, we briefly overviewed the most important aspects of the biomolecule corona, especially the protein coating including our current concept of the corona, the impact on cellular processes in vitro and in vivo as well as the influence on the novel field of nanotoxicology. Hereby, we provided multiple arguments to demonstrate that the corona is far from being an already resolved topic. Moreover, the understanding of the complexity of these "corona-nano-systems" is highly important for NM risk assessment and for minimizing nanotoxicity, but also to develop improved and safe applications of nanotechnology in medicine.

TAKE-HOME MESSAGES

- Besides the wide use of engineered NMs in technical products, their applications are not only increasing in biotechnology and biomedicine, but also in the environmental field.
- Upon contact with physiological environments, NMs rapidly adsorb biomolecules, forming the so-called biomolecule corona.
- Biological and environmental systems are mostly not facing pristine manufactured but rather corona-coated NPs.
- The composition of the biomolecule-corona is determined not only by physicochemical parameters of the NM itself, like size, charge, or surface functionalization, but also by the composition of the physiological medium and by the duration of the exposition.
- The biomolecule-corona represents an important element of the biological identity of the NM and has a major impact on many cellular processes.
- For the rational development of NMs for biological or biomedical application, it is the key to understand the formation and kinetic evolution of the biomolecule corona.

Acknowledgments

Supported by BMBF-MRCyte/NanoBEL/DENANA, Zeiss-ChemBioMed, Stiftung Rheinland-Pfalz (NanoScreen), DFGSPP1313, PTE-foundation, and *Fonds der chemischen Industrie*.

References

Bergin, I.L., Witzmann, F.A., 2013. Nanoparticle toxicity by the gastrointestinal route: evidence and knowledge gaps. Int. J. Biomed. Nanosci. Nanotechnol. 3 (1–2), 163–210.

Brown, J., de Vos, W.M., DiStefano, P.S., Dore, J., Huttenhower, C., Knight, R., Lawley, T.D., Raes, J., Turnbaugh, P., 2013. Translating the human microbiome. Nat. Biotechnol. 31 (4), 304–308.

Caracciolo, G., Pozzi, D., Capriotti, A.L., Cavaliere, C., Foglia, P., Amenitsch, H., Lagana, A., 2011. Evolution of the protein corona of lipid gene vectors as a function of plasma concentration. Langmuir 27 (24), 15048–15053.

Cedervall, T., Lynch, I., Foy, M., Berggard, T., Donnelly, S.C., Cagney, G., Linse, S., Dawson, K.A., 2007a. Detailed identification of plasma proteins adsorbed on copolymer nanoparticles. Angew. Chem. Int. Ed. Engl. 46 (30), 5754–5756.

Cedervall, T., Lynch, I., Lindman, S., Berggard, T., Thulin, E., Nilsson, H., Dawson, K.A., Linse, S., 2007b. Understanding the nanoparticle-protein corona using methods to quantify exchange rates and affinities of proteins for nanoparticles. Proc. Natl. Acad. Sci. USA 104 (7), 2050–2055.

Dell'Orco, D., Lundqvist, M., Oslakovic, C., Cedervall, T., Linse, S., 2010. Modeling the time evolution of the nanoparticle-protein corona in a body fluid. PLoS One 5 (6), e10949.

Dobrovolskaia, M.A., Neun, B.W., Man, S., Ye, X., Hansen, M., Patri, A.K., Crist, R.M., McNeil, S.E., 2014. Protein corona composition does not accurately predict hematocompatibility of colloidal gold nanoparticles. Nanomedicine 10 (7), 1453–1463.

Docter, D., Bantz, C., Westmeier, D., Galla, H., Wang, Q., Kirkpatrick, C.J., Maskos, M., Stauber, R.H., 2014. The protein-corona protects against size- and dose-dependent toxicity of amorphous silica nanoparticles. Beilstein J. Nanotechnol. 5, 1380–1392.

Docter, D., Strieth, S., Westmeier, D., Hayden, O., Gao, M., Knauer, S.K., Stauber, R.H., 2015a. No king without a crown - impact of the nanomaterial-protein corona on nanobiomedicine. Nanomedicine 10 (3), 503–519.

Docter, D., Westmeier, D., Markiewicz, M., Stolte, S., Knauer, S.K., Stauber, R.H., 2015b. The nanoparticle biomolecule corona: lessons learned—challenge accepted? Chem. Soc. Rev. 44 (17), 6094–6121.

Fabian, E., Landsiedel, R., Ma-Hock, L., Wiench, K., Wohlleben, W., van Ravenzwaay, B., 2008. Tissue distribution and toxicity of intravenously administered titanium dioxide nanoparticles in rats. Arch. Toxicol. 82 (3), 151–157.

Farrera, C., Fadeel, B., 2015. It takes two to tango: understanding the interactions between engineered nanomaterials and the immune system. Eur. J. Pharm. Biopharm. 95, 3–12.

Feliu, N., Docter, D., Heine, M., Del Pinto, P., Ashraf, S., Kolosnjaj-Tabi, J., Macchiarini, P., Nielsen, P., Alloyeau, D., Gazeau, F., Stauber, R.H., 2016. In vivo degeneration and the fate of inorganic nanoparticles. Chem. Soc. Rev. 45 (9), 2440–2457.

Gebauer, J.S., Malissek, M., Simon, S., Knauer, S.K., Maskos, M., Stauber, R.H., Peukert, W., Treuel, L., 2012. Impact of the nanoparticle-protein corona on colloidal stability and protein structure. Langmuir 28 (25), 9673–9679.

Goodrich, J.K., Waters, J.L., Poole, A.C., Sutter, J.L., Koren, O., Blekhman, R., Beaumont, M., Van Treuren, W., Knight, R., Bell, J.T., Spector, T.D., Clark, A.G., Ley, R.E., 2014. Human genetics shape the gut microbiome. Cell 159 (4), 789–799.

Hillaireu, H., Couvreur, P., 2009. Nanocarriers entry into the cell: relevance to drug delivery. Cell. Mol. Life Sci. 66, 2873–2896.

Human Microbiome Project, C, 2012. A framework for human microbiome research. Nature 486 (7402), 215–221.

Jiang, X., Weise, S., Hafner, M., Rocker, C., Zhang, F., Parak, W.J., Nienhaus, G.U., 2010. Quantitative analysis of the protein corona on FePt nanoparticles formed by transferrin binding. J. R. Soc. Interface 7 (Suppl. 1), S5–S13.

Kapralov, A.A., Feng, W.H., Amoscato, A.A., Yanamala, N., Balasubramanian, K., Winnica, D.E., Kisin, E.R., Kotchey, G.P., Gou, P., Sparvero, L.J., Ray, P., Mallampalli, R.K., Klein-Seetharaman, J., Fadeel, B., Star, A., Shvedova, A.A., Kagan, V.E., 2012. Adsorption of surfactant lipids by single-walled carbon nanotubes in mouse lung upon pharyngeal aspiration. ACS Nano 6 (5), 4147–4156.

Klein, J., 2007. Probing the interactions of proteins and nanoparticles. Proc. Natl. Acad. Sci. USA 104 (7), 2029–2030.

Kreyling, W.G., Abdelmonem, A.M., Ali, Z., Alves, F., Geiser, M., Haberl, N., Hartmann, R., Hirn, S., de Aberasturi, D.J., Kantner, K., Khadem-Saba, G., Montenegro, J.M., Rejman, J., Rojo, T., de Larramendi, I.R., Ufartes, R., Wenk, A., Parak, W.J., 2015. In vivo integrity of polymer-coated gold nanoparticles. Nat. Nanotechnol. 10 (7), 619–623.

Lademann, O., Richter, H., Kramer, A., Patzelt, A., Meinke, M.C., Graf, C., Gao, Q., Korotianskiy, E., Reuhl, E., Weltmann, K.D., Lademann, J., Koch, S., 2011. Stimulation of the penetration of particles into the skin by plasma tissue interaction. Laser Phys. Lett. 8 (10), 758–764.

Lee, Y.K., Choi, E.J., Webster, T.J., Kim, S.H., Khang, D., 2015. Effect of the protein corona on nanoparticles for modulating cytotoxicity and immunotoxicity. Int. J. Nanomedicine 10, 97–113.
Lesniak, A., Fenaroli, F., Monopoli, M.P., Aberg, C., Dawson, K.A., Salvati, A., 2012. Effects of the presence or absence of a protein corona on silica nanoparticle uptake and impact on cells. ACS Nano 6 (7), 5845–5857.
Li, M., Al-Jamal, K.T., Kostarelos, K., Reineke, J., 2010. Physiologically based pharmacokinetic modeling of nanoparticles. ACS Nano 4 (11), 6303–6317.
Lipka, M., Semmler-Behnke, M., Sperling, R.A., Wenk, A., Takenaka, S., Schleh, C., Kissel, T., Parak, W.J., Kreyling, W.G., 2010. Biodistribution of PEG-modified gold nanoparticles following intratracheal instillation and intravenous injection. Biomaterials 31 (25), 6574–6581.
Lundqvist, M., Stigler, J., Cedervall, T., Berggard, T., Flanagan, M.B., Lynch, I., Elia, G., Dawson, K., 2011. The evolution of the protein corona around nanoparticles: a test study. ACS Nano 5 (9), 7503–7509.
Martel, J., Young, D., Young, A., Wu, C.Y., Chen, C.D., Yu, J.S., Young, J.D., 2011. Comprehensive proteomic analysis of mineral nanoparticles derived from human body fluids and analyzed by liquid chromatography-tandem mass spectrometry. Anal. Biochem. 418 (1), 111–125.
Merrifield, D.L., Shaw, B.J., Harper, G.M., Saoud, I.P., Davies, S.J., Handy, R.D., Henry, T.B., 2013. Ingestion of metal-nanoparticle contaminated food disrupts endogenous microbiota in zebrafish (Danio rerio). Environ. Pollut. 174, 157–163.
Monopoli, M.P., Aberg, C., Salvati, A., Dawson, K.A., 2012. Biomolecular coronas provide the biological identity of nanosized materials. Nat. Nanotechnol. 7 (12), 779–786.
Monopoli, M.P., Bombelli, F.B., Dawson, K.A., 2011a. Nanobiotechnology: nanoparticle coronas take shape. Nat. Nanotechnol. 6 (1), 11–12.
Monopoli, M.P., Walczyk, D., Campbell, A., Elia, G., Lynch, I., Bombelli, F.B., Dawson, K.A., 2011b. Physical-chemical aspects of protein corona: relevance to in vitro and in vivo biological impacts of nanoparticles. J. Am. Chem. Soc. 133 (8), 2525–2534.
Natte, K., Friedrich, J.F., Wohlrab, S., Lutzki, J., von Klitzing, R., Osterle, W., Orts-Gil, G., 2013. Impact of polymer shell on the formation and time evolution of nanoparticle-protein corona. Colloids Surf. B Biointerfaces 104, 213–220.
Nazarenus, M., Zhang, Q., Soliman, M.G., del Pino, P., Pelaz, B., Carregal_Romero, S., Rejman, J., Rothen-Ruthishauser, B., Clift, M.J.D., Zellner, R., Nienhaus, G.U., Delehanty, J.B., Medintz, I.L., Parak, W.J., 2014. In vitro interaction of colloidal nanoparticles with mammalian cells: what have we learned thus far? Beilstein J. Nanotechnol. 5, 1477–1490.
Nel, A.E., Madler, L., Velegol, D., Xia, T., Hoek, E.M., Somasundaran, P., Klaessig, F., Castranova, V., Thompson, M., 2009. Understanding biophysicochemical interactions at the nano-bio interface. Nat. Mater. 8 (7), 543–557.
Nystrom, A.M., Fadeel, B., 2012. Safety assessment of nanomaterials: implications for nanomedicine. J Control Release 161 (2), 403–408.
Pelaz, B., Charron, G., Pfeiffer, C., Zhao, Y., de la Fuente, J.M., Liang, X.J., Parak, W.J., Del Pino, P., 2013. Interfacing engineered nanoparticles with biological systems: anticipating adverse nano-bio interactions. Small 9 (9-10), 1573–1584.
Pelaz, B., Del Pino, P., Maffre, P., Hartmann, R., Gallego, M., Rivera-Fernandez, S., de la Fuente, J.M., Nienhaus, G.U., Parak, W.J., 2015. Surface functionalization of nanoparticles with polyethylene glycol: effects on protein adsorption and cellular uptake. ACS Nano 9 (7), 6996–7008.
Rauscher, H., Sokull-Kluttgen, B., Stamm, H., 2013. The European Commission's recommendation on the definition of nanomaterial makes an impact. Nanotoxicology 7, 1195–1197.
Reese, M., 2013. Nanotechnology: using co-regulation to bring regulation of modern technologies into the 21st century. Health Matrix 23 (2), 537–572.
Riehemann, K., Schneider, S.W., Luger, T.A., Godin, B., Ferrari, M., Fuchs, H., 2009. Nanomedicine—challenge and perspectives. Angew. Chem. Int. Ed. Engl. 48 (5), 872–897.
Rivera-Gil, P., Jimenez de Aberasturi, D., Wulf, V., Pelaz, B., del Pino, P., Zhao, Y., de la Fuente, J.M., Ruiz de Larramendi, I., Rojo, T., Liang, X.J., Parak, W.J., 2013. The challenge to relate the physicochemical properties of colloidal nanoparticles to their cytotoxicity. Acc. Chem. Res. 46 (3), 743–749.
Rizzo, L.Y., Theek, B., Storm, G., Kiessling, F., Lammers, T., 2013. Recent progress in nanomedicine: therapeutic, diagnostic and theranostic applications. Curr. Opin. Biotechnol. 24 (6), 1159–1166.
Salvati, A., Pitek, A.S., Monopoli, M.P., Prapainop, K., Bombelli, F.B., Hristov, D.R., Kelly, P.M., Aberg, C., Mahon, E., Dawson, K.A., 2013. Transferrin-functionalized nanoparticles lose their targeting capabilities when a biomolecule corona adsorbs on the surface. Nat. Nanotechnol. 8 (2), 137–143.

Saptarshi, S.R., Duschl, A., Lopata, A.L., 2013. Interaction of nanoparticles with proteins: relation to bio-reactivity of the nanoparticle. J. Nanobiotechnology 11, 26.
Schleh, C., Semmler-Behnke, M., Lipka, J., Wenk, A., Hirn, S., Schaeffler, M., Schmid, G., Simon, U., Kreyling, W.G., 2012. Size and surface charge of gold nanoparticles determine absorption across intestinal barriers and accumulation in secondary target organs after oral administration. Nanotoxicology 6 (1), 36–46.
Setyawati, M.I., Tay, C.Y., Docter, D., Stauber, R.H., Leong, D.T., 2015. Understanding and exploiting nanoparticles' intimacy with the blood vessel and blood. Chem. Soc. Rev. 44, 8174–8199.
Tenzer, S., Docter, D., Kuharev, J., Musyanovych, A., Fetz, V., Hecht, R., Schlenk, F., Fischer, D., Kiouptsi, K., Reinhardt, C., Landfester, K., Schild, H., Maskos, M., Knauer, S.K., Stauber, R.H., 2013. Rapid formation of plasma protein corona critically affects nanoparticle pathophysiology. Nat. Nanotechnol. 8 (10), 772–781.
Tenzer, S., Docter, D., Rosfa, S., Wlodarski, A., Kuharev, J., Rekik, A., Knauer, S.K., Bantz, C., Nawroth, T., Bier, C., Sirirattanapan, J., Mann, W., Treuel, L., Zellner, R., Maskos, M., Schild, H., Stauber, R.H., 2011. Nanoparticle size is a critical physicochemical determinant of the human blood plasma corona: a comprehensive quantitative proteomic analysis. ACS Nano 5 (9), 7155–7167.
Treuel, L., Docter, D., Maskos, M., Stauber, R.H., 2015. Protein corona – from molecular adsorption to physiological complexity. Beilstein J. Nanotechnol. (6), 857–873.
Treuel, L., Malissek, M., 2013. Interactions of nanoparticles with proteins: determination of equilibrium constants. Methods Mol. Biol. 991, 225–235.
Vogt, A., Rancan, F., Ahlberg, S., Nazemi, B., Choe, C.S., Darvin, M.E., Hadam, S., Blume-Peytavi, U., Loza, K., Diendorf, J., Epple, M., Graf, C., Ruehl, E., Meinke, M.C., Lademann, J., 2014. Interaction of dermatologically relevant nanoparticles with skin cells and skin. Beilstein J. Nanotechnol. 5, 2363–2373.
Vroman, L., 1962. Effect of absorbed proteins on the wettability of hydrophilic and hydrophobic solids. Nature 196, 476–477.
Walczyk, D., Bombelli, F.B., Monopoli, M.P., Lynch, I., Dawson, K.A., 2010. What the cell "sees" in bionanoscience. J. Am. Chem. Soc. 132 (16), 5761–5768.
Walkey, C.D., Olsen, J.B., Guo, H., Emili, A., Chan, W.C., 2012. Nanoparticle size and surface chemistry determine serum protein adsorption and macrophage uptake. J. Am. Chem. Soc. 134 (4), 2139–2147.
Walkey, C.D., Olsen, J.B., Song, F., Liu, R., Guo, H., Olsen, D.W., Cohen, Y., Emili, A., Chan, W.C., 2014. Protein corona fingerprinting predicts the cellular interaction of gold and silver nanoparticles. ACS Nano 8 (3), 2439–2455.
Wang, F., Yu, L., Monopoli, M.P., Sandin, P., Mahon, E., Salvati, A., Dawson, K.A., 2013. The biomolecular corona is retained during nanoparticle uptake and protects the cells from the damage induced by cationic nanoparticles until degraded in the lysosomes. Nanomedicine 9 (8), 1159–1168.
Webster, T.J., 2013. Interview: nanomedicine: past, present and future. Nanomedicine 8 (4), 525–529.
Westmeier, D., Stauber, R.H., Docter, D., 2015a. The bio-corona and its impact on nanomaterial toxicity. Eur. J. Nanomed. 7 (3), 153–168.
Westmeier, D., Stauber, R.H., Docter, D., 2015b. The concept of bio-corona in modulating the toxicity of engineered nanomaterials (ENM). Toxicol. Appl. Pharmacol. 299, 53–57.
Williams, K., Milner, J., Boudreau, M.D., Gokulan, K., Cerniglia, C.E., Khare, S., 2015. Effects of subchronic exposure of silver nanoparticles on intestinal microbiota and gut-associated immune responses in the ileum of Sprague-Dawley rats. Nanotoxicology 9, 279–289.
Wolfram, J., Yang, Y., Shen, J., Moten, A., Chen, C., Shen, H., Ferrari, M., Zhao, Y., 2014. The nano-plasma interface: implications of the protein corona. Colloids Surf. B 124, 17–24.
Yan, Y., Gause, K.T., Kamphuis, M.M., Ang, C.S., O'Brien-Simpson, N.M., Lenzo, J.C., Reynolds, E.C., Nice, E.C., Caruso, F., 2013. Differential roles of the protein corona in the cellular uptake of nanoporous polymer particles by monocyte and macrophage cell lines. ACS Nano 7 (12), 10960–10970.
Zhang, H., Burnum, K.E., Luna, M.L., Petritis, B.O., Kim, J.S., Qian, W.J., Moore, R.J., Heredia-Langner, A., Webb-Robertson, B.J., Thrall, B.D., Camp, 2nd, D.G., Smith, R.D., Pounds, J.G., Liu, T., 2011. Quantitative proteomics analysis of adsorbed plasma proteins classifies nanoparticles with different surface properties and size. Proteomics 11 (23), 4569–4577.

CHAPTER

2

Physicochemical Characterization

Kirsten Rasmussen, Hubert Rauscher, Agnieszka Mech

Institute for Health and Consumer Protection, European Commission, Joint Research Centre, Ispra, Italy

OUTLINE

Introduction 15

Overview of Key Physicochemical Parameters 20

Modalities of Physicochemical Characterization 21

Particle Size, Size Distribution, Agglomeration, and Aggregation 21

Shape 26

Specific Surface Area and Porosity 26

Chemical Composition, Purity, and Crystal Structure (Crystallinity) 27

Surface Chemistry (and Contamination) 29

Surface Charge Density and Stability in Dispersion 30

Dispersibility and Solubility 31

Dustiness 32

Sample Preparation and Dispersion Media 32

Data Collection and Interpretation 41

Take-Home Messages 43

Acknowledgments 44

Disclaimer 44

References 44

INTRODUCTION

Physicochemical characterization of chemicals is important as it provides basic information on the exact nature of the chemical studied. Furthermore, physicochemical properties and (eco)toxicological effects of chemicals are often interlinked, for example, the solubility of a chemical in different media influences its bioavailability and thus also (eco)toxicological effects observed in tests performed in these media. Nanomaterials may be viewed as a particular subgroup of chemicals that are defined by their size. Hence, the term nanomaterial

Adverse Effects of Engineered Nanomaterials. http://dx.doi.org/10.1016/B978-0-12-809199-9.00002-1
Copyright © 2017 Elsevier Inc. All rights reserved.

derives from the fact that a nanomaterial has at least one (external) dimension in the nanoscale, and this is the only common feature among all nanomaterials, as highlighted in (Lövestam et al., 2010). A generally accepted definition of "nanomaterial" has not (yet) been agreed, and the most common understanding of that term implies that the material should have at least one (external) dimension smaller than 100 nm (Australia, 2010; EC, 2011; Health Canada, 2011; ISO, 2008a; Lövestam et al., 2010; Roco, 2001; Royal Society and Royal Academy of Engineering, 2004). Several definitions of "nanomaterial" have been proposed, both in scientific (Royal Society and Royal Academy of Engineering, 2004) and in regulatory contexts (Rauscher et al., 2014). The European Commission has proposed and published a recommendation for a regulatory definition of the term "nanomaterial" in 2011 (EC, 2011).

The physicochemical properties of nanomaterials can be different from those of materials with the same composition but with larger dimensions for two main reasons. First, nanomaterials have a relatively large specific surface area (SSA) and can have a different atomic surface structure compared to a macroscale material. The larger SSA can make materials chemically more reactive and in some cases materials that are inert in their larger form are reactive when produced in their nanoscale form, whereas a different surface structure can lead to changes in the surface reaction kinetics. Second, effects due to electron confinement, including quantum effects, can begin to dominate the behavior of matter at the nanoscale, an effect that becomes more pronounced as the particles get smaller. These electron confinement effects can affect the optical, electrical, and magnetic behavior of materials. Materials can be nanoscale in one dimension (e.g., very thin platelets), in two dimensions (e.g., nanowires and nanotubes), or in all three dimensions (e.g., nanoparticles). Even if a material does not exhibit "new" features at the nanoscale, it can have properties that are clearly different from those of the macroscale material just because of its reduced size. Besides being scientifically interesting this calls for a dedicated safety assessment in a regulatory context.

Depending on the context, it may be relevant to characterize different (sets of) physicochemical parameters. In a regulatory context, standard information requirements to assess the safety of chemicals include physicochemical characterization by the OECD screening information dataset (SIDS) (OECD, webpage). It was developed in 1991 for the assessment programme to systematically investigate existing industrial high production volume chemicals and includes data on chemical identity and physicochemical characterization (Table 2.1).

In 2006, the OECD launched a Working Party on Manufactured Nanomaterials (WPMN) to provide a global forum for discussion. The WPMN set up an exploratory test programme to examine the information needs and testing methods for manufactured nanomaterials, and a Guidance Manual for Sponsors (OECD, 2010) was drafted to help the sponsors. A list of nanomaterials, to test, was published in 2008, as well as a list of endpoints thought to be relevant for the safety assessment of a nanomaterial (OECD, 2010) including endpoints describing the identity and physicochemical characterization, see Table 2.1. These endpoints contain some of the endpoints investigated for normal chemicals, for example, SIDS, as well as new endpoints (Table 2.1). Later on, the OECD WPMN published an evaluation of the methods used for physicochemical characterization (OECD, 2016a,b). The Testing Programme was finalized in 2013 and the dossiers with the raw data from the testing were published in 2015 on the OECD website (OECD, 2016c). An overview of the WPMN work and outcomes of the testing programme are provided by Rasmussen et al. (2016). Analysis of methods (OECD, 2009) and initial considerations of test results (SCENIHR, 2009) concluded that not all proposed endpoints

TABLE 2.1 OECD WPMN Proposed Endpoints Regarding Chemical Identity and Physicochemical Properties for Nanomaterials Compared to SIDS Endpoints

Programme			
WPMN (OECD, 2010)	**SIDS (OECD, 2012a,b)**	**Preliminary review TG (OECD, 2009)**	**A. Nanomaterial information/ identification**
1	SIDS		Nanomaterial name[a]
2	SIDS		CAS number
3	SIDS		Structural formula/molecular structure
4	SIDS[b]	PR	Composition of NM being tested (incl. degree of purity, known impurities, or additives)
5	SIDS	PR (length and shape)	Basic morphology
6			Description of surface chemistry (e.g., coating or modification)
7			Major commercial uses
8		PR	Known catalytic activity
9			Method of production (e.g., precipitation, gas phase)
	SIDS		Quantity
			B. Physical–chemical properties and material characterization
10		PR	Agglomeration/aggregation
11	SIDS	PR	Water solubility
12		PR	Crystalline phase
13		PR	Dustiness
14		PR	Crystallite size
15			Representative TEM picture(s)
16		PR	Particle size distribution
17		PR	Specific surface area
18		PR	Zeta potential (surface charge)
19			Surface chemistry (where appropriate)
20			Photocatalytic activity
21			Pour density
22			Porosity
23			Octanol–water partition coefficient, where relevant
24			Redox potential
25			Radical formation
26			Other relevant information (where available)

(Continued)

TABLE 2.1 OECD WPMN Proposed Endpoints Regarding Chemical Identity and Physicochemical Properties for Nanomaterials Compared to SIDS Endpoints (*cont.*)

Programme			
WPMN (OECD, 2010)	**SIDS (OECD, 2012a,b)**	**Preliminary review TG (OECD, 2009)**	**B. Physical–chemical properties and material characterization**
		PR	Fat solubility/oleophilicity
	SIDS		Melting Point
	SIDS		Boiling Point
	SIDS		Relative density[c]
	SIDS		Vapor pressure
	SIDS		Dissociation constants[c]

[a]*Nanomaterial name and chemical name is suggested as an overlap as the information serves to identify, by a name, the substance of interest.*
[b]*For a single chemical: degree of purity, known impurities or additives, difference of impurities among products, details of stereoisomers if relevant. Additional descriptors are also listed for mixtures, for Class 21 compounds and for streams, but this information is not repeated here.*
[c]*Required if applicable.*

were relevant for all nanomaterials. For example, measurement of the water–octanol partition coefficient for nanomaterials turned out not to be meaningful as the coefficient relates to distribution of dissolved material between the two phases. Nanomaterials, however, are not dissolved, but dispersed as particles (if dissolved, they become normal chemicals without the need to apply nanospecific considerations).

Other sets of physicochemical endpoints for nanomaterials have been proposed by a number of sources. Stefaniak et al. (2013) provided an overview of physicochemical parameters suggested to be relevant for nanomaterials by 28 different sources, such as committees (e.g., European Commission's Scientific Committee for Emerging and Newly Identified Health Risks, SCENIHR), organizations (e.g., OECD), and researchers (e.g., Johnston et al., 2012). In the analysis by Stefaniak et al. only one of the 28 physicochemical parameters, "surface area (specific)," was found to be common among all references investigated, whereas several other parameters (defect density, hardness, magnetic properties, and optical properties) were listed only in a few sources. The list by Stefaniak et al. contains some parameters in addition to the WPMN parameters, such as biological solubility, stability, conductivity, defect density, hardness, magnetic, and optical properties. Thus, the needs for characterizing nanomaterials are variable, and the parameters considered relevant depend on the purpose of characterization, for example, research or addressing regulatory requirements.

In addition to the issues outlined earlier of deciding which properties should be investigated for nanomaterials, they pose, as a consequence of their size, unique measurement challenges for characterization (see, for instance, Linsinger et al., 2011; Roebben et al., 2011). An important gap to overcome here is the lack of harmonized, standardized, and (regulatory) recognized methods, including methods for physicochemical properties, to obtain quality data from testing nanomaterials. Globally recognized methods for regulatory testing of chemicals are developed and agreed in the OECD test guidelines programme and the data collected using the OECD test guidelines fall under OECD's system of Mutual Acceptance of Data (MAD). MAD is a legally binding instrument to facilitate the international acceptance of information for the regulatory safety assessment of chemicals. An initial analysis of the OECD test guidelines

(OECD, 2009) concluded that, for a majority of cases the physicochemical characterization methods described in the OECD test guidelines for conventional chemicals could not be considered as being directly applicable to nanomaterials in their current form. Furthermore, the analysis suggests 17 physicochemical properties (Table 2.1), to be necessary prerequisites (PR) of toxicological assessment of MNs, overlapping with the physicochemical endpoints for NMs proposed by the WPMN (OECD, 2010). Most of these WPMN endpoints are additional to the ones normally relevant to chemicals and therefore, at this time, there are no associated OECD test guidelines. Based on the outcomes from two expert meetings (OECD, 2014, 2016a), the WPMN suggested that the following test guidelines or guidance documents relevant to physicochemical characterization be revised or developed, see Table 2.2.

The standardization organizations are also working on method development for nanomaterials, with, for example, the International Standardization Organization (ISO) having established a technical committee, TC 229, that works on "Nanotechnologies," as does the European Committee for Standardization (CEN) through its technical committee 352 "Nanotechnologies." These organizations have published several documents describing methods for characterizing nanomaterials.

When developing test methods, validation and (for OECD test guidelines) regulatory acceptance are important aspects. Validation is supported by (certified) reference materials [(C)RMs], which are defined by ISO/REMCO (ISO, 2008b) as materials for which, as a minimum, homogeneity and stability are guaranteed for one or more relevant properties. The Certified RMs are supported by more data that ascertains one or several specific properties of the material. (C)RMs are commonly used and required by international standards to ensure the comparability of measurement data obtained in each step of method development and implementation cycle. To date only very few (C)RMs are available for nanomaterials. As an initial step to develop nano-(C)RMs, the European Commission's Joint Research Centre established the JRC Repository for Representative Nanomaterials. When using materials from this repository in tests, the same lot and batch of nanomaterial is tested thus eliminating one source of uncertainty when comparing data obtained, for example, from different laboratories. The

TABLE 2.2 Overview of OECD Test Guidelines or Guidance Documents Proposed to be Developed by the OECD WPMN for Physicochemical Methods

Title of OECD test guideline/guidance document to be revised or developed
Revision of TG 105 (water solubility) to reflect that it is not applicable to nanomaterials.
New test guideline or guidance document on determination of aggregation/agglomeration status of NMs.
New test guideline on determination of zeta potential.
New test guideline or guidance document on particle size and size distribution or update of the existing guidance.
New test guideline or guidance document on surface area (BET).
Review document describing what techniques/methods are available for measuring surface chemistry along with appropriate caveats, for example, which classes of nanomaterials are appropriate for the methods.
Update or add appendices to the guidance document on sample preparation and dosimetry.

materials from the Repository are industrially sourced Representative Test Materials from a single batch produced by industry. They are sufficiently homogeneous and stable with respect to one or more specified properties, and implicitly assumed to be fit for their intended use in the development of test methods which target properties other than the properties for which homogeneity and stability have been demonstrated (Roebben et al., 2013).

Based on the previously mentioned considerations, this chapter provides an overview of physicochemical characterization of nanomaterials in the context of their safety, including issues to consider specifically for nanomaterials. It is of particular relevance to identify the physicochemical properties appropriate for characterizing a nanomaterial for toxicological and environmental studies.

OVERVIEW OF KEY PHYSICOCHEMICAL PARAMETERS

The OECD WPMN proposed 26 physicochemical endpoints, and the analysis by Stefaniak et al. (2013) suggested that even more endpoints could be relevant for physicochemical characterization. Furthermore, ideally a nanomaterial should be characterized at different stages of its life cycle, for example, its pristine form (as received), as prepared for testing in vitro or in vivo (as administered), and under actual test conditions (at points of interaction with the test organism and histological examination of the exposed cells, tissue, and organs) (EFSA, 2011; Oberdörster et al., 2005). The nanomaterial physicochemical characteristics after administration (under actual test conditions) will also be related to the actual bioavailable dose (Naha et al., 2009; Rahman et al., 2013; Walczyk et al. 2010). However, only few methods are available for characterization of nanomaterial properties after administration, for example, in air or to cell culture media (in vitro), and technical limitations preclude measurement of many properties after administration to a living organism. Physicochemical characterization for toxicological testing has the additional challenge that some material properties depend on the environment (the medium) that surrounds the nanomaterial (refer to Chapter 1).

Stefaniak et al. (2013) collected recommendations for nanomaterial characterization during risk assessment from 28 scientific literature sources and found that they agree on a number of NM properties, which could be seen as an important step toward establishing a minimum list of physicochemical parameters for the risk assessment of NM. The NM properties that are listed in at least half of the 28 sources analyzed by Stefaniak et al. are:

(i) specific surface area,
(ii) elemental/molecular composition,
(iii) surface chemistry,
(iv) particle size,
(v) particle size distribution,
(vi) morphology/shape/form,
(vii) surface charge,
(viii) agglomeration/aggregation state,
(ix) crystal structure,
(x) surface reactivity,
(xi) solubility (water).

In the absence of a formal consensus on the minimum nanocharacteristics to be assessed, these parameters could be considered as starting point for such a list. All of these parameters are also listed by the OECD WPMN (Table 2.1). Stefaniak et al. furthermore analyzed the

18 sources that specifically focus on "priority" characterization for toxicological assessment. They arrived at the same endpoints as those listed previously (in a different order), except that surface reactivity and solubility (water) are no longer listed.

As for macroscale chemicals, the chemical composition, including the presence of impurities, is fundamental to understanding the nanomaterial. A description of the surface (treatment) of nanomaterials is particularly important for toxicology studies, as it may influence the transport of nanoparticles over barriers and into organisms. In addition, the surface chemistry affects the mechanism and the small particle size increases the probability of translocation of particles, which have entered into an organism. Furthermore, the nanoscale (approximately 1–100 nm) is a size range below cell size meaning that nanoparticles may enter cells, either passively or by active transport (Barua and Mitragotri, 2014; Juillerat-Jeanneret, 2008; Wohlfart et al., 2012; Zensi et al., 2009).

The purpose of the nanomaterial characterization will determine which physicochemical data are deemed relevant. Characterization data for a nanomaterial, Material Safety Data Sheet (MSDS), which is prepared to inform actors in the supply chain and at the workplace about hazards, may share common requirements with the characterization suggested when performing toxicological assessment of a nanomaterial. There are also some specific elements: for the workplace, information on dustiness is particularly relevant as it informs about the propensity of a material to become airborne leading to exposure via air; this is of less interest for toxicological assessment, thus it is listed for the MSDS. Airborne particles with high aspect ratio can be asbestos-like and thus the aspect ratio is specifically mentioned for MSDS.

MODALITIES OF PHYSICOCHEMICAL CHARACTERIZATION

The interest in nanomaterials has sparked development of methods to detect and characterize them. This section will provide an overview of the most common experimental methods for characterizing the OECD WPMN endpoints (Table 2.1), except the water–octanol coefficient, pour density, and photocatalytic activity. Since the field of nanomaterial characterization is progressing fast, more methods or combinations of methods in addition to those described in this chapter are under development or already used for specific purposes. As mentioned earlier, the characterization and (eco)toxicological testing should be performed on a representative sample of the nanomaterial, possibly at several different points in the material's life cycle. For nanomaterials in complex media there are a number of requirements, challenges, and solutions for characterization that need to be carefully thought through when performing the testing and analyzing the data. The methods described later in the chapter are for the pristine material; some may also be used at later points in the life cycle.

Particle Size, Size Distribution, Agglomeration, and Aggregation

A first step in physicochemical characterization of nanomaterials is to determine the particle size and size distribution. There are several methods and method combinations, based on different physical phenomena, that can be applied in the determination of size and size distributions (e.g., based on particle imaging, sedimentation phenomena, or light scattering) (Gilliland et al., 2014; NanoDefine, 2016). Methods to determine the particle size distribution

in a sample can also be grouped according to whether particles are measured individually or as ensemble (Stintz et al., 2010):

1. Ensemble techniques (many particles in the sample are measured at the same time and the size distribution is extracted from the combined signal of the measured particles). Ensemble techniques in general give statistically relevant results as they refer to many particles, but may have an insufficient resolution and a size-dependent sensitivity.
2. Fractionation techniques (an outside force/process is applied to separate particles according to their size. Subsequently a detection technique is applied to each size fraction to determine the particle amount, which may, for example, be the number or the weight of the particles, in each different size fraction to construct a size distribution). The fractionation techniques can, in combination with ensemble methods, overcome problems of resolution while retaining statistical relevance.
3. Counting techniques (the size of individual particles is measured and counts of similar-size particles are placed into "size bins" to construct a size distribution). The counting methods overcome the problems of resolution, but require much effort to have data, which are statistically relevant.

To obtain reliable particle size distribution measurements, some techniques require that the particles of the sample, which ideally should be representative of the material investigated, are well dispersed in a medium. For monodisperse nanomaterials it is relatively straightforward to obtain and measure a representative sample, whereas polydisperse materials require measuring of a larger number of particles to determine the true particle size distribution. Generally, ensemble methods require less time and effort, but may give results whose informative value needs to be carefully scrutinized with respect to their range of applicability. Counting methods give more direct results but usually are more time-consuming and expensive.

Particulate nanomaterials are available in two main states: as dry powders or in suspension. The various measurement techniques require that the nanoparticles (NPs) are in a certain state, and the choice of characterization techniques is partly determined by the state in which the nanomaterial is, or can be made, available. Furthermore, as nanoparticles in general are prone to aggregate and/or agglomerate, the selected measurement method has to have a reliable measurement range far beyond 100 nm to assure reliability and accuracy of the performed analysis. An additional factor in size measurements is the particle shape: for perfectly spherical particles, the actual particle diameter is the quantity to be determined. However, many nanoparticles are not spherical and may have (very) irregular shapes. It is therefore necessary to explain which quantity is reported in a size measurement (Fig. 2.1). The output reported from the measurement techniques therefore often corresponds to a characteristic quantity [e.g., Feret (min, mean, or max) diameter] or an equivalent size, for example, equivalent spherical diameter. For size measurement results it is thus extremely important to associate the result with the measurement techniques applied, provide the standard operating procedures (SOPs) for sample preparation and measurement, and describe precisely what is reported.

Each method used to determine particle size and size distribution has its particular advantages and limitations. DLS, for example, measures the hydrodynamic diameter and is much more sensitive for large particles than for small ones. The signal for large particles can therefore easily cover the signal from small particles. TEM gives 2D information on 3D particles. Thus, multiple techniques should be used whenever possible in order to develop a complete understanding of the material (Linsinger et al., 2012).

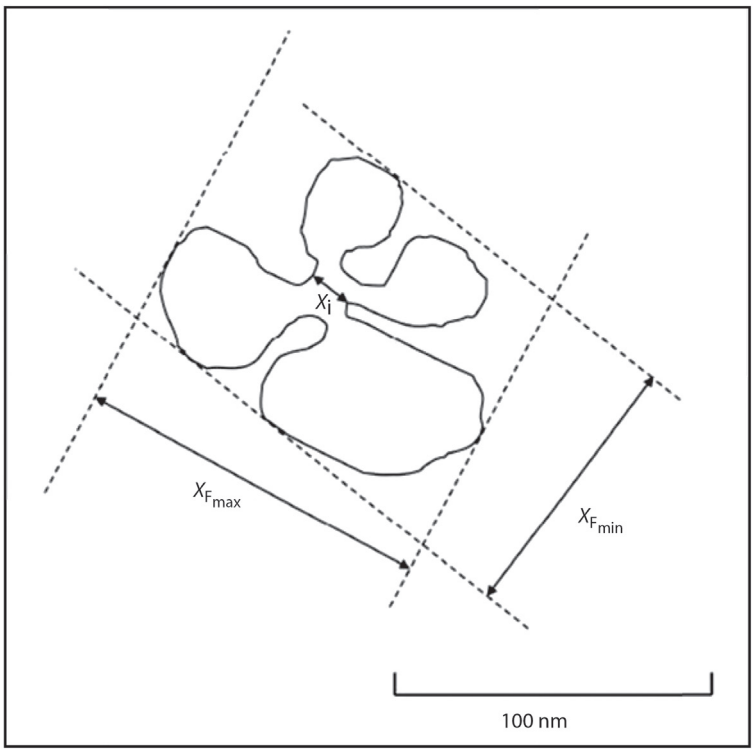


FIGURE 2.1 Illustration of a number of possible external dimensions of an irregular particle as apparent from a 2D image of the particle. The following dimensions are indicated: X_i, the diameter of the central part of the particle; $X_{F_{max}}$, the maximum Feret diameter; and $X_{F_{min}}$, the minimum Feret diameter. *Source: Linsinger, T.P.J., Roebben, G., Gilliland, D., Calzolai, L., Rossi, F., Gibson, N., Klein, C., 2012. Requirements on Measurements for the Implementation of the European Commission Definition of the Term 'Nanomaterial'. European Union, Luxembourg, 52 p., with permission from the JRC.*

One of the most commonly used techniques for determination of size, shape, and agglomeration and aggregation state is electron microscopy (EM) which includes many qualitative and semiquantitative variations of the technique (de Temmerman et al., 2012; NanoDefine, 2016; Smith, 2015). Scanning electron microscopy (SEM) operates with spatial resolution below 5 nm and in transmission electron microscopy (TEM) the resolution can be in the subnanometer region. Certain variants of EM, for example, high-resolution transmission electron microscopy (HRTEM), operate with resolution nominally below 1 nm (Ramallo-Lopez et al., 2007; Sales et al. 2012) In general despite its limitations, EM is considered to be the most useful and appropriate technique for direct investigation of NMs. The main disadvantage of traditional EM methods is the requirement to operate in high-vacuum and thus for many commercial instruments EM is applicable only to solid samples. Recently, specialized procedures and equipment have been developed which can, to a certain extent, overcome some of the limitations of working under vacuum conditions. However, they are not routinely used or universally applicable. Accessories for the investigation of liquid dispersions with SEM have been commercialized (cryo-SEM) (Callaway, 2015). An additional limitation of SEM is that

it provides information on conductive materials, which can potentially be overcome by, for example, sputtering a thin layer of conductive material, such as silver or gold, on the sample. However, this may lead to changes of the material and the added layer increases the size of particles. Another way to resolve the conductivity issue for SEM is to operate under low voltage/current conditions, but this leads to a significant reduction of image resolution. The particle size distribution may be obtained by EM techniques by measuring the size of particles and counting them, but the number of particles that need to be sized to ensure statistical reliability is high. A semiautomated procedure for TEM that would significantly reduce the time and cost of the analysis is currently under development (de Temmerman et al., 2014). TEM can provide some additional crystallographic data, such as surface atom arrangements or defects and when coupled with energy-dispersive X-ray spectroscopy (EDS) can reveal additional information on the chemical nature of the analyzed material.

Another microscopy technique that can be applied to size (and shape) measurements is scanning probe microscopy (Samori, 2006), which includes atomic force microscopy (AFM) (Grobelny et al., 2011) and scanning tunnelling microscopy (STM) (Marchini et al., 2007) and variations thereof. Both AFM and STM use a scanning probe (the tip), which is moved along the grid where sample has been deposited and senses the differences in height due to the presence of particles. In contrast to SEM and TEM, in scanning probe microscopy nanoparticles and aggregates/agglomerates can in principle be measured along all three dimensions (maximum length, width, and height), albeit with different resolution parallel and perpendicular to the substrate. In fact, STM images may directly provide the 3D morphology of certain complex samples (e.g., CNT) (Terrones et al., 2004) and can resolve simultaneously both their electronic density and atomic structure under appropriate working conditions.

Dynamic light scattering (DLS), quasi-elastic light scattering (QELS), photon correlation spectroscopy (PCS), and multiangle light scattering (MALS) are commonly used nondestructive techniques that measure particle size in liquid dispersions (Lee et al., 2005; Xu, 2000). These techniques provide information on the equivalent hydrodynamic diameter, that is, the diameter of a spherical particle moving as an individual unit in a liquid surrounded by a solvation layer. DLS measures the intensity of light scattered from the sample and gives a backscattered light intensity based size distribution. A mathematical transformation of the intensity-based distribution is applied to calculate the particle number size distribution (i.e., number of particles and agglomerates/aggregates) and particle volume size distribution (i.e., volume of particles and agglomerates/aggregates). The scattered light intensity is proportional to the sixth power of the size of the scatterers (the particles), and therefore the signal of small particles can easily be covered by that of large particles. If this is the case, the transformation from an intensity to a number or volume-based distribution does not give true values. However, if the size distribution is narrow or quasi-monodisperse, the light-scattering techniques can render reasonable results. The instrument's software evaluates the scattered intensity usually assuming spherical particles. This means that even for nonspherical particles a spherical equivalent hydrodynamic diameter is reported, which must be kept in mind when drawing conclusions from such results. Additionally, this technique requires information about viscosity of the medium and refractive index of the test material, but for some samples this can be difficult to obtain. Multiangle light scattering (MALS) is a static light-scattering technique that complements dynamic light-scattering methods. MALS measures the intensity of the scattered light at various angles at the same time. The intensity of the scattered light at each

angle is proportional to the molar mass and the concentration of the molecules/particles under investigation, and it is used to calculate both particle mass and size for nanoparticles. MALS is a commonly used technique for the characterization of proteins and polymers and it can be applied to nonspherical nanoparticles. Other techniques that employ light scattering and can be used for size distribution analysis are small-angle X-ray scattering (SAXS) (Glatter and Kratky, 1982) or wide-angle X-ray scattering (WAXS) (Graewert and Svergun, 2013) which are further described in the section dedicated to SSA characterization.

Scanning mobility particle size (SMPS) spectrometry provides information on the size of particles, agglomerates, and aggregates. SMPS is a nondestructive method which requires that samples are aerosolized, and it separates the particles according to their electrical mobility, that is, their charge. It has relatively high resolution ~2 nm and gives reliable results in the size range 2.5–1000 nm. The main limitation of this method is the stability of the aerosol, as SMPS results are reliable only for aerosols which are stable while scanning. The model associated with SMPS is based on the assumption that the particles are spherical and thus nonspherical particles or aggregates are expressed as their equivalent sphere (Wiedensohler et al., 2012).

Recently, fractionation techniques have been combined with detection techniques to determine the size distribution of particles (NanoDefine, 2016). Field flow fractionation (FFF) techniques are very powerful chromatographic methods and can, in principle, separate particles from 1 nm to several microns, regardless of their chemical composition. FFF separates the particles according to differences in mobility induced by a force (cross-flow, thermal, electric, gravitational, etc.) perpendicular to the main laminar flow direction of the injected dispersion of particles. Different FFF techniques separate analytes according to different properties depending on the type of field applied. The selectivity of the separation depends on the relationship between retention time and the specific sample property investigated. The techniques most used are AF4 (asymmetric flow FFF) and FIFF (crossflow FFF) due to their very high sensitivity (Williams et al., 2011). The nondestructive FFF techniques can be coupled with different detectors, such as DLS, MALS, PCS (Bandyopadhyay et al., 2013), or ICP-MS. FFF separation techniques provide a means to analyze the particle size distribution even in very complex mixtures and also allow to collect the separated fractions of the injected dispersion for further physicochemical analysis (Dubascoux et al., 2010). Another widely used chromatographic technique is capillary hydrodynamic fractionation (CHDF). It operates similarly to FFF methods; however, the principle of particle separation is different based on differences in transport rates in a capillary, related to their location in the eluent. It can separate particles in the range 15–1000 nm and, as FFF techniques, it can be coupled with different detectors (Philippe et al., 2014).

Also single particle (sp) ICP-MS can provide information on size and size distribution of nanoparticles. It is based on the measurement of highly diluted nanoparticle dispersions by ICP-MS operated in time-resolved mode for a preselected mass-to-charge ratio (Degueldre and Favarger, 2003; Laborda et al., 2011, 2014; Linsinger et al., 2014; Pace et al., 2012). The diameter of spherical particles can then be calculated from the measured mass based on the known or assumed stoichiometry and density of the target material, though mathematical conversion may in certain cases lead to less reliable results. In general sp-ICP-MS is a relatively robust technique and can be run on modified conventional ICP-MS instruments, which are, however, limited in terms of time resolution and detection efficiency; sample preparation

is simple (often only dilution) and the measurement time per sample relatively short (1 min) which allows high throughput analysis. Furthermore, it is specific to chemistry and provides number-based size distributions directly (Linsinger et al., 2014).

Thus, different sizing techniques provide information, which also depends on the media, on different measurands. Most of the suspension-based techniques (ensemble techniques), such as DLS or CLS provide information about size in terms of equivalent hydrodynamic diameter, whereas in other techniques, such as SAXS, the size is the equivalent geometric diameter. EM based techniques provide a 2D projection of a 3D particle yielding the number weighted geometric size (and shape) distributions. Therefore, as already pointed out, it is very important to understand and report exactly which of these sizes has been measured, also in the context of later use in assessing results of toxicological tests.

In (eco)toxicology testing and for safety assessment the traditional measurand is milligram chemical per kilogram test organism. Regarding nanoparticles, the European Commission's Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) noted that "For the determination of dose–response relationships, special attention should be given to the expression of the metrics of the nanoparticle dose since mass concentration is not necessarily the best description of dose for these materials and number concentration and surface area are likely to be more appropriate." (SCENIHR, 2007). Though stated about 10 years ago, this observation is still valid and the (USA) National Nanotechnology Initiative strategic plan lists dose metrics as a research area for increased emphasis (National Science and Technology Council, 2014).

Shape

The concept of shape appears intuitive, but it is not easily quantifiable, making it a very challenging issue. Particulate materials rarely have well-defined shapes, such as spheres or rods, and for most materials the shape is much more variable. Many shape descriptors have been devised in attempts to quantify shape (de Temmerman et al., 2012), including sphericity, convexity, aspect ratio, and fractal dimension (Klobes et al., 2006). ISO has published a standard on image analysis of shape, which includes standardized shape factors (ISO, 2008c). This and other national standards should be considered when defining the particle shape (ISO, 2013).

Techniques based on EM are often applied to obtain information on the shape of particles, and especially for nanomaterials only EM techniques have a sufficient resolution to provide reliable images and thus information regarding the shape of nanoparticles. As already mentioned, EM provides a 2D projection of a 3D particle and thus care should be taken to avoid bias due to orientation effects. It is especially important when analyzing platelet-like particles as their smallest dimension is often not well accessible for EM and hence can remain undetected. Though there are approaches to standardize reporting of shape data (ISO, 2008c), a number of different protocols, that is, ways of expressing the relevant dimension, are still commonly used.

Specific Surface Area and Porosity

The SSA of a particle is closely related to its size, shape, and porosity. SSA may influence the reactivity of the particle, as well as its dissolution or adsorption properties. Hence it is also relevant for toxicity testing.

Porosity measurements can provide information on the SSA of an NM and on the presence, size, and shape of the pores (Klobes et al., 2006). Information on the SSA of a nanomaterial can be obtained using the Brunauer-Emmet-Teller (BET) method while additional information on the porosity can be obtained by extending the measured pressure range to very low values (ISO, 2006). The BET technique is based on the measurement of adsorption isotherms of an inert gas (typically N_2) on a dry solid material (Brunauer et al., 1938). BET is a standard measurement for solid nanomaterial characterization (ISO, 2010), but has its limitations. It is applicable only to dry solid samples, and its lack of applicability to liquid suspensions is a major limitation for toxicological testing. Nuclear magnetic resonance (NMR) methods may also be applied for measuring surface area for materials in suspension, but NMR-based methods have a limited sensitivity and need a relatively high, mass concentration, around 1 wt.% (Tantra, 2016). Recently, Transmission Electron Tomography was reported to be a useful tool for determination of the SSA for NMs in frozen suspension (van Doren et al., 2011).

The SSA can also be measured utilizing SAXS by relating the tail-end constant of the small-angle curve to the integral scattering of the material. This technique can also provide information on porosity and pore size distribution as well on size, shape, interparticle correlations, and density fluctuations of nanostructures (Saw, 2005). As SAXS is suitable for testing of biological samples and complex matrix materials, it is commonly used to investigate biological material, for example, cells and tissues, proteins and protein folding, colloids, micelles, bacteria, and viruses, which is important when considering toxicity testing of nanomaterials (Saw, 2005).

The porosity of a nanomaterial can be characterized by using Mercury Intrusion Porosimetry (Powers et al., 2006), which is based on the nonwetting mercury properties that, combined with the high surface tension of mercury, allow intrusion of mercury into the pores of a material at high pressure. The pore size can be determined based on the external pressure needed to force the mercury into the pores against the opposing force of the liquid's surface tension. However, the high pressure needed for the measurement may deform the material tested, and when applied to metals, amalgams may form.

Chemical Composition, Purity, and Crystal Structure (Crystallinity)

The determination of an NM's chemical composition may be challenging and often requires the application of several techniques to assure the accuracy of the result. Typically, the analysis includes methods, such as inductively coupled plasma (ICP), X-ray diffraction (XRD), NMR, UV-VIS (Okitsu, 2013), and fluorescent spectroscopy, which are used to measure the atomic structure and composition of NMs. When carrying out chemical composition analysis, the methods should be carefully selected based on the chemistry of the NM, as some techniques are relevant only for one type of material, for example, organic or inorganic materials.

One of the best suited methods for detailed chemical analysis is ICP combined with suitable detection methods (Tantra, 2016). It is a destructive method that simultaneously produces ions of any element present. The method can be combined with mass spectrometry (MS) and optical emission spectrophotometry (OES), leading to qualitative (ICP-MS) and quantitative identification (ICP-OES) of the components and impurities of almost all nanomaterials. ICP-MS and ICP-OES do not provide information on the structure of the investigated material

and, furthermore, ICP-MS has a low sensitivity toward some of the lighter elements, such as H, I, O, N, C, S. ICP-OES can, in principle detect any element and thus it can be used to characterize both organic and inorganic nanomaterials.

A technique that is often used for the analysis of organic nanomaterials is common elemental analysis (CHN combustion analysis) in which a sample is burned in an excess of oxygen, and various traps collect the combustion products (carbon dioxide, water, and nitrogen dioxide). The masses of these combustion products can be used to calculate the elemental composition of the unknown sample. This technique can successfully be employed for analysis of certain inorganic materials with high carbon content (e.g., Teflon) although some materials with high thermal stability, for example, fullerenes and CNTs, could pose difficulties in the combustion process.

Various time-of-flight mass spectrometry (TOF-MS) (Tiede et al., 2008) methods can be used to analyze the composition of nanomaterials and, when coupled with separation methods, for example, FFF, they can provide information on the size of analyzed particles. Recently, Aerosol time of flight-mass spectrometry (ATOF-MS) (TSI, 2004) was used to analyze the chemical composition of nanoparticles with simultaneous determination of their size.

For inorganic NMs a number of methods for characterization of their composition are available. Organic materials and the presence of organic molecules in NM are much less investigated. Certain methods commonly used to characterize conventional organic substances can also be employed for NM characterization. Fourier transformed infrared spectroscopy (FTIR) and Raman spectroscopy (RS) (Carey, 2006) are two nondestructive, qualitative, and in some cases, quantitative methods that can provide information about organic and inorganic structures by analyzing their vibrational modes. FTIR and especially Raman analysis are commonly used to characterize carbon-based NM, such as CNT (Branca et al., 2004) and graphene (Dresselhaus et al., 2010). FTIR was also applied for characterization of functionalized nanoparticles of metals (e.g., gold), metal oxides (e.g., zinc oxide), and quantum dots (CdS) (Dablemont et al., 2008). Raman spectroscopy can also provide information on phase changes (both amorphous and crystalline) or lattice stress in NM (Challa and Kumar, 2012). Furthermore, Differential Scanning Calorimetry (DSC) and Thermogravimetric Analysis (TGA) are destructive methods that also can provide information on phase transitions (see section on surface characterization).

Another nondestructive method that is relevant for chemical and structural analysis of organic NMs is NMR. NMR can detect the presence of contaminants and monomers in the case of organic polymeric nanoparticles. In addition, in some cases it can also provide information on the surface functionalization and on the kinetics of functional group release (Wilson et al., 2015). It allows an accurate identification of the position of individual atoms in a given chemical structure eliciting the chemical environment and the surrounding elements. This technique can be used both with organic and inorganic NMs in solid state or in dispersion (Roming et al., 2008; Torres and Bottari, 2013).

X-ray diffraction is one of the most commonly used techniques to resolve the crystal structure of a material. Atoms arranged in a crystalline structure will cause an X-ray beam to diffract in well-defined directions. By measuring the angles and intensities of these diffracted beams, a three-dimensional picture of the density of electrons within the crystal is generated. From this electron density map, the positions of the atoms in the crystal are determined. SAXS and WAXS, two methods based on elastic scattering of X-rays of wavelength 0.1–0.2 nm, can provide information about the shape and size of nanoparticles, characteristic distances of

partially ordered materials, pore sizes, and many other data. SAXS is capable of delivering structural information of nanoparticles between 5 and 25 nm. WAXS can resolve even smaller dimensions and is used for analyzing highly ordered or crystalline structures, while SAXS is more common to confirm that materials are amorphous or nonhomogeneous.

For crystallinity characterization, electron- and neutron-scattering techniques can also be employed; but due to their higher cost they are less commonly chosen. Electron diffraction is used to study the short-range atomic order in amorphous nanoparticles. It can complement X-ray diffraction, which is based on electron scattering, for studies of very small crystals (less than 0.1 μm) of inorganic, organic, and biological materials, such as proteins that cannot easily form the larger 3D crystals required for XRD. Neutron crystallography involves the application of neutron scattering to determine the atomic and crystallographic structure of a nanomaterial. The technique is similar to X-ray diffraction but due to their different scattering properties (neutrons interact primarily with the nuclei of atoms), neutrons and X-rays provide complementary information. The technique is most commonly performed as powder diffraction on polycrystalline nanopowders. It is mostly used to characterize and identify phases and to refine details of an already known structure, rather than solving unknown structures (Blakeley et al., 2008).

Surface Chemistry (and Contamination)

X-ray photon spectroscopy (XPS) is a nondestructive technique commonly used for the determination of surface elemental composition of NMs (Grant and Briggs, 2003). It provides detailed, qualitative information on chemical elements present on the surface and their chemical environment, for example, coating, binding states, oxidation states, etc. (Ray and Shard, 2011). XPS can detect elements from lithium to uranium with an abundance of 0.1 at.% in a layer as thick as 5 nm. Until recently, the use of this technique was limited to solid samples only, as it required the use of ultra-high vacuum to allow the photoelectrons to reach the detector and to maintain the analyzed sample surface free from contamination. Recently, the use of special sample cells permits the use of liquid samples, and this development may significantly broaden the application of XPS (Beloqui Redondo et al., 2015). It is used to characterize surfaces of any nanomaterial, for example, gold, aluminum, CNTs (Rong et al., 2004) and core–shell particles (Hota et al., 2007).

The surface chemistry of nanoparticles can also be analyzed by means of Auger electron spectroscopy (AES) (Briggs and Seah, 1983; Grant and Briggs, 2003), which can provide information on elemental composition of a sample in 2–20-nm depth range. Its major limitation is the requirement of ultra-high vacuum, which restricts its applicability only to very stable materials, such as metals, metal oxides, and CNTs (Ohdaira et al., 2002).

Electron energy loss spectroscopy (EELS) can also provide valuable information on NM surface chemistry (Egerton, 2011). In this technique, the material is exposed to a beam of electrons with a known kinetic energy, where some of the electrons will undergo inelastic scattering. The amount of energy lost can then be measured via an electron spectrometer and interpreted in terms of what caused the energy loss. This technique usually operates in a Transmission Electron Microscope and can be considered as complementary to, yet 10 times more sensitive, energy dispersive X-ray spectroscopy (EDS) which provides better results for higher atomic number elements (Oomen et al., 2011).

Identification of trace elements, organic molecules, or polymers on the surface of NMs with sensitivity higher than 1 ppm can be obtained by means of secondary ion mass spectroscopy (SIMS). In this technique the elemental, isotopic, or molecular composition of the surface to a depth of 1–2 nm can be analyzed with very high sensitivity. Despite the fact that this method has c. 200 nm of spatial resolution it can be also applied to analyze the chemical composition of both nanomaterials and materials that contain nanoparticles. Similarly, X-ray fluorescence (XRF) spectroscopy can be applied for chemical composition analysis and can identify a wide range of elements from sodium to uranium with detection limits (LOD) in the ppm range; the LOD for XRF is, however, orders of magnitude higher than, for example, ICP-MS. Analyzed samples do not need any particular treatment before analysis, except removal of surface contaminations. The analysis is performed under vacuum conditions to allow detection of light elements. The resolution of the method depends on how the effect is induced. Several methods exist for inducing X-ray fluorescence, such as irradiation of the sample by electrons or X-rays. A modification of this technique, Total Reflection X-ray Fluorescence (TRXF) spectroscopy, is currently widely used in the electronic industry for quality control and ISO standards are also available (ISO, 2015).

Some of the techniques used for chemical composition analysis, such as UV-VIS or FTIR can also be applied to characterize the surface chemistry.

Surface Charge Density and Stability in Dispersion

When dispersed in liquid media, nanoparticles may carry an electrical charge on their surface which depends on the particle itself and the surrounding medium. Surface charge, as well as particle size and surface chemistry are major factors affecting the stability of NM dispersions. Stability in dispersion here is considered in terms of changes in the particle size distribution by aggregation/agglomeration and subsequent sedimentation. If sedimentation occurs because smaller particles start to agglomerate irreversibly then this is an indicator of true instability, but often sedimentation occurs because the primary particles or primary agglomerates are sufficiently large and/or heavy that the force of gravity can overcome the back-diffusion due to Brownian motion. Gentle mixing of such a solution will return it to its starting state. The determination of the dispersion stability is fundamental prior to any toxicity testing, especially if the nanoparticles have to be dispersed in a medium before administration. The stability of the dispersion can be estimated by measuring its zeta potential which is basically the potential difference between the dispersion medium and a stationary layer of fluid attached to the dispersed particles. Often a DLS apparatus with zeta potential option is used for the measurement. If the zeta potential is sufficiently positive or negative, the suspended particles repel each other to a degree resulting in a stable dispersion, and generally, a zeta potential larger than +30 mV or less than −30 mV is considered to indicate a stable dispersion. Zeta potential values in the range between ±30 mV imply that the repulsive interparticle forces are most likely not strong enough to prevent aggregation/agglomeration (and possibly sedimentation) with time. Even small changes in the environment, such as a slight pH change or concentration of ligands, can considerably influence the aggregation rate of dispersed particles, and therefore it is common practice to measure the zeta potential as a function of pH, as well as to determine the isoelectric point, that is, the pH value at which the zeta potential is equal to zero. Electrophoretic light scattering (ELS) and electroacoustic phenomena measurements can also be used to determine the zeta potential of dispersed particles.

Dispersibility and Solubility

The state of dispersion of particulate systems indicates the degree to which particles are agglomerated. Forces that hold together particles in groups or clusters may have different sources, of which the most fundamental ones are attractive van der Waals forces and Coulomb forces. The strength of the van der Waals forces is related to the atomic properties of the surface atoms, shape (geometry) and distance of these surfaces to each other. The state of dispersion influences the result(s) of any analysis that requires the sample to be in liquid dispersion, as the particles, due to the attractive forces, tend to agglomerate and aggregate in suspensions. Steric effects may prevent this, and furthermore, the particles may be stabilized by surface charge or added stabilizers. For toxicological testing it is thus important to understand the degree of aggregation/agglomeration relative to the presence of individual nanoparticles to know what is actually tested. Particle size affects the dispersion state in two ways: for larger particles (including agglomerates and aggregates) sedimentation may occur, and for smaller particles the attractive van der Waals forces per particle are relatively stronger than for large ones, and thus with decreasing size it becomes more difficult to redisperse agglomerated particles. Particle aggregates/agglomerates can, to a certain extent, be redispersed; however, for strong aggregation gentle stirring is insufficient. In this case, the sample requires controlled energy input, such as grinding or sonication. However, these are high-energy input processes that can easily affect the properties of the material.

Many of the techniques, which are used to determine size and size distribution, are also suitable for measuring the state of dispersion of a system, which implies quantification of both primary particle size distribution (unagglomerated) and the agglomerate/aggregate size distribution of the system. Agglomeration can be a slow process, depending on the environment, and can be affected by many factors (including sonication, etc.). Thus it is advisable to determine the state of dispersion as often as possible (Jarvis et al., 2005). Despite these limitations some assessment can often be made regarding dispersed versus agglomerated size distributions and in addition qualitative observations can be made based on imaging techniques (Tantra, 2016).

Solubility refers to the maximum mass of a nanomaterial which can be dissolved in a solvent so that a single, homogenous, and (temporarily) stable phase is obtained. It is important to not confuse solubility, which occurs at molecular levels, with dispersibility that occurs at particle levels; the distinction between the two can be difficult in case of colloidal suspension of nanomaterials. Currently used methods for solubility assessment of a bulk material could in principle be used for nanomaterials; specific nanotailored protocols and guidelines are under development (Hartmann et al., 2015; Tantra, 2016). Solubility of nanomaterials can also, to a certain extent, be determined by means of light-scattering techniques (e.g., DLS) or by other sizing techniques that can determine the presence of nanoparticles in a liquid. Due to higher relative surface area nanomaterials may have different (usually higher) reactivity, including higher dissolution rate, than their bulk counterparts. As for chemicals in general, the solubility of nanomaterials is solvent dependent and has to be measured for each solvent–solute combination. Many other factors may affect solubility, such as pressure, temperature, impurity concentration, or forces between particles. It could actually be of high importance for toxicity studies to have information on the kinetics of dissolution of nanomaterial as it may change with time and therefore it may modify the toxicity profile of a nanomaterial with time. This could, for instance, be assessed by measuring the time-dependent particle size

distribution of the solution, which may allow monitoring changes in the equilibrium between dissolved solute and particulate fraction.

Dustiness

Dustiness is defined as "the propensity of a material to generate airborne dust during its handling" (ISO, 2012b) and is especially important when analyzing inhalation toxicity of a powder. Dustiness is not an intrinsic physicochemical property of a powder because besides the physicochemical properties of the particles of the powder (size, shape, surface area, coating, hydrophilicity/phobicity, aggregation, and so on) there are many other, external factors which may influence the dustiness of a material, for example, its moisture content, as well as the environment (temperature, pressure, humidity). The results obtained for dustiness are linked to the test method used and are measured in arbitrary units, and do not provide information on the number of particles and their size distribution nor on the particle shape.

Currently, there are methods available to test the dustiness of bulk materials: the rotating drum method, the continuous drop method, and the vortex shaker (VS) method. A European standard (EN15051) (British Standards, 2013) has been published describing two methods for determining dustiness (the rotating drum and continuous drop methods), but their suitability for nanomaterials is limited. Measurements according to EN15051 are done using mass-based protocols, that is, they do not distinguish between different particle sizes and do not give a clear indication on the presence or absence of particles smaller than 100 nm, or on submicron particles. Furthermore, the test requires a large amount of material, typically more than 500 g, and nanomaterials are often not available in such large quantities.

Both the rotating drum method and the VS method have been modified to attempt to extend their applicability to nanomaterials (Nanogenotox, 2012); the standard drum was, for example, substituted with a small drum (SD). A useful feature of the SD and the VS methods which improve their suitability for nanomaterials is that only a small quantity of material (between less than 0.1 and 6 g) is needed for a test. In addition, the smaller-size equipment is easier to place in an approved fume cupboard or safety cabinet, greatly improving the safety of the experimenters. However, the results of the two methods did not agree in tests (Rasmussen et al., 2013) and work is still on-going to improve their applicability to nanomaterials.

In conclusion, Tables 2.3 and 2.4 provide an overview of the methods described earlier. Table 2.3 indicates the suitability of the methods for characterizing the properties of nanomaterials, and Table 2.4 lists the propert(ies) and type of material that the methods can analyze, as well as strengths and limitations of each method.

SAMPLE PREPARATION AND DISPERSION MEDIA

An issue raised consistently when testing nanomaterials, both within the WPMN and in the scientific literature, is the sample (test item) preparation in appropriate media. It is recognized that possible hazards associated with nanomaterials may be affected by, for example, shape, size, and surface area, because these parameters affect both the mode of action and toxicokinetics of particles (absorption, distribution, and excretion).

TABLE 2.3 Applicability of Analytical Methods for Characterization of Physicochemical Properties of Nanomaterials

	Technique	Composition	Size/size distribution	Agglomeration/aggregation	Shape	Surface chemistry	Specific surface area	Crystalline phase	Surface charge/zeta potential	Dustiness	Solubility	Dispersibility	Stability	Porosity
Microscopy	SEM		++	++	++			+						
	TEM		++	++	++		+[a]	+						
	SPM (AFM, STM)		++	++	++				+/−					
Spectroscopy	DLS, PCS, QELS		++						+		++	++	++	
	MALS		++	+							++	++	++	
	SAXS		+	+/−			+/−							
	Neutron/electron scattering	+						++						
	XRD	+	+[b]					++						
	ICP-MS/OES	++				+/−								
	SP ICP-MS	++	++											
	EDX	+				+								
	NMR	++				+								
	XPS					++								
	XRF	++												
	SIMS	+				++								
	EELS	+				++								
	ELS								++				++	

(*Continued*)

TABLE 2.3 Applicability of Analytical Methods for Characterization of Physicochemical Properties of Nanomaterials (*cont.*)

	Technique	Composition	Size/size distribution	Agglomeration/aggregation	Shape	Surface chemistry	Specific surface area	Crystalline phase	Surface charge/zeta potential	Dustiness	Solubility	Dispersibility	Stability	Porosity
	TOF-MS/ATOF-MS	++	+[c]			+								
	FTIR/RS	++	+[d]			+		+[e]						
	UV-VIS	+	+[f]											
	AES	+/−				++								
Chromatography	CHDF		++											
	FFF/A4F/FIFFF		++											
Other	BET		+/−				++							++
	Mercury Intrusion													++
	CLS		++											
	AC		++											
	TGA/DSC					++		+					++	
	SMPS		++	++										
	SD, VS									+				

The (material dependent) suitability of the methods is indicated as follows: ++, very good; +, good; +/−, fair.

[a] *Electron tomography.*

[b] *Volume weighted mean diameter of primary crystalline domains.*

[c] *When coupled with, for example, FFF.*

[d] *Raman for CNT or layer thickness of graphene.*

[e] *Raman.*

[f] *For plasmonic materials (e.g., gold, silver, copper).*

TABLE 2.4 Overview of Commonly Used Analytical Methods for Characterization of Physicochemical Properties of Nanomaterials

Method	Physicochemical characteristics analyzed/ parameters	Type of material	Strengths	Limitations
SEM	Size and size distribution Shape Aggregation Dispersion (cryo-SEM) Crystal structure (lattice system, electron backscattering detection SEM: crystallinity)	Inorganic, organic, carbon based, biological, complex materials (coated, core–shell). Spherical, equiaxial particles, tubes, flakes, rods, fibers.	High resolution, down to sub-nanometer).	Conductive materials (sample or coating) required for high resolution. Dry samples required Sample analysis in nonphysiological conditions. Expensive equipment. Cryogenic method required for most NP-biomaterials.
TEM	Size and size distribution Shape Aggregation Dispersion (environmental TEM) Crystal structure	Inorganic, organic, carbon based, biological, complex materials (coated, core–shell). Spherical, equiaxial particles, tubes, flakes, rods, fibers.	Direct measurement of the size/ size distribution and shape of nanomaterials (higher resolution than SEM, down to sub-nanometer). Several analytical methods can be coupled with TEM for analysis of chemical composition and electronic structure.	Very thin samples are required. To achieve statistic reliability several scans of one sample which means time-consuming and expensive measurements. Samples measured in nonphysiological conditions. Damage/change of sample. Expensive equipment.
STM	Size and size distribution Shape Aggregation Structure Surface charge density (mapping)	Inorganic, organic, carbon based, biological, complex materials (coated, core–shell). Spherical, equiaxial particles, tubes, flakes, rods, fibers	Direct measurement. High spatial resolution down to atomic scale.	Conductive samples only.
AFM	Size and size distribution Shape Structure Surface properties (modified AFM) Surface charge density (mapping)	Inorganic, organic, carbon based, biological, complex materials (coated, core–shell). Spherical, equiaxial particles, tubes, flakes, rods, fibers.	3D sample surface topography. Sub-nanoscale topographic. Resolution. Direct measurement of samples in dry, aqueous or ambient environment.	Difficult and time consuming sample preparation. Tip effects often disturb measurements. Analysis in general limited to the external of nanomaterials.

(*Continued*)

TABLE 2.4 Overview of Commonly Used Analytical Methods for Characterization of Physicochemical Properties of Nanomaterials (*cont.*)

Method	Physicochemical characteristics analyzed/ parameters	Type of material	Strengths	Limitations
DLS in combination with electrophoretic light scattering	Hydrodynamic size Distribution/particle size distribution Zeta potential	Inorganic, organic, carbon based, biological, complex materials (coated, core–shell). Spherical and equiaxial particles.	Nondestructive/ invasive method. Measures in many liquid media (solvent of interest). Hydrodynamic sizes accurately determined for monodisperse samples. Modest cost of apparatus. Rapid technique.	A small number of large particles may shield small particles. Not suitable for polydisperse sample measurements. Limited size resolution. Model for data evaluation assumes spherical particles.
MALS	Particle size distribution Mass distribution	Inorganic, organic, carbon based, biological, complex materials (coated, core–shell). Spherical, equiaxial particles, tubes, flakes, rods, fibers.	Good resolution. Can correctly characterize nonspherical particles. Can be coupled with separating methods, for example, FFF.	Sample dispersion should be transparent and do not absorb the light wavelength. Typically large samples volume (1 mL).
SAXS	Size/size distribution SSA Shape Crystal structure	Inorganic, organic, carbon based, biological, complex materials (coated, core–shell). Spherical, equiaxial particles to a certain extend also: tubes, flakes, rods, fibers	Nondestructive method. Simple sample preparation. Possible for amorphous materials and sample in solution.	Low resolution. Not suitable for polydisperse samples.
TOF-MS	Particle size distribution	Inorganic, Organic, Carbon based, Complex materials (coated, core–shell). Spherical, equiaxial particles. Nonspherical particles.	Fast analysis. Broad total range of measurements (0.1–7 μm).	The size of nonspherical particles is underestimated. Suspension of particles may be difficult or impossible to measure. High resolution analysis is limited.

TABLE 2.4 Overview of Commonly Used Analytical Methods for Characterization of Physicochemical Properties of Nanomaterials (*cont.*)

Method	Physicochemical characteristics analyzed/ parameters	Type of material	Strengths	Limitations
SIMS	Composition Surface chemistry	Inorganic, organic, carbon based, biological, complex materials (coated, core–shell). Spherical, equiaxial particles to a certain extend also: tubes, flakes, rods, fibers.	Despite the fact that it has 200-nm spatial resolution it can used for nanomaterials chemical identification. Depth resolution up to 1 nm for inorganic and up to 10-nm for organic materials.	Samples analyzed are in vacuum. Quantification may be challenging.
XRD	Size, shape, and crystal structure for crystalline materials	Inorganic, carbon based, complex materials (coated, core–shell not organic).	Well-established technique. High spatial resolution at atomic scale.	Limited to crystalline materials. Low intensity compared to electron diffraction.
CHDF	Particle size distribution	Inorganic, organic (particulate and nonparticulate), carbon based, complex materials (coated, core–shell). Spherical, equiaxial particles	Relatively fast analysis time (7–10 min).	Aqueous emulsifier medium only. Poor resolution (40% difference between sizes). Capillary plugging. Nonspherical particles may not be correctly measured.
FFF	Particle size distribution (in combination with a detection method)	Inorganic, organic, carbon based, complex materials (coated, core–shell). Spherical, equiaxial particles.	Very good resolution with small particles, narrow peaks as little as 5%–6% different in size. Certified reference materials are available.	Relatively complex algorithm for size separation. Relatively long run times (>1 h) Assumption of spherical particles. It does not determine particles in aggregates and agglomerates.
SMPS	Particle size Particle size distribution	Inorganic, organic, carbon based, complex materials (coated, core–shell). Spherical, equiaxial particles.	Most widely used technique for aerosols. Can also be used on site.	Only aerosol samples in size range 2.5–1000 nm with resolution 2 nm Particles needs to be charged.

(*Continued*)

TABLE 2.4 Overview of Commonly Used Analytical Methods for Characterization of Physicochemical Properties of Nanomaterials (*cont.*)

Method	Physicochemical characteristics analyzed/ parameters	Type of material	Strengths	Limitations
Electrophoretic mobility	Zeta potential	Inorganic, organic, carbon based, biological, complex materials (coated, core–shell). Spherical, equiaxial particles, tubes, flakes, rods, fibers.	Simultaneous measurement of many particles (using ELS).	Not very precise method. Very diluted sample required.
BET	SSA Porosity (if extended pressure range and modified data analysis is applied)	Inorganic, carbon based, complex materials (coated, core–shell). Spherical, equiaxial particles.	Certified reference materials are available. Fast and relatively cheap method.	Particles and nonparticulate porous materials cannot be distinguished. Materials must be free of any volatile compounds. Long measurement time.
sp-ICP-MS	Particle size distribution Surface chemistry	Inorganic. complex materials (coated, core–shell if contains detectable element). Spherical, equiaxial particles.	Measures individual particles. Method is chemically specific, rapid, cost-efficient.	Can detect only one element at a time. Accurate size determination limited to spherical particles only. The method does not resolve particles in aggregates and agglomerates.
IR/FTIR	Structure Molecular composition Surface properties (FTIR)	Inorganic, organic, carbon based, biological, complex materials (coated, core–shell). Spherical, equiaxial particles, tubes, flakes, rods, fibers.	Fast and inexpensive measurement. Minimal or no sample preparation requirement.	Interference and strong absorbance of H_2O (IR). Relatively low sensitivity in nanoscale analysis. In complex samples analysis may be very challenging. FTIR mostly for dry powder samples. Molecules must be active in IR region. Detection limits routine: 2%, Special techniques: 0.01%.

TABLE 2.4 Overview of Commonly Used Analytical Methods for Characterization of Physicochemical Properties of Nanomaterials (*cont.*)

Method	Physicochemical characteristics analyzed/ parameters	Type of material	Strengths	Limitations
Raman Spectroscopy	Composition Surface chemistry Particle size (for certain materials)	Inorganic, organic, carbon based, biological, complex materials (coated, core–shell). Spherical, equiaxial particles, tubes, flakes, rods, fibers.	Commonly available, easy and cheap technique.	It can determine size for certain materials only, for example: CNT, thickness of graphene flakes. It cannot be used for metals and alloys. Very sensitive to impurities. Samples heating through intense laser radiation can lead to sample decomposition.
UV-VIS	Composition Surface chemistry Optical properties Particle size (for certain materials)	Inorganic, organic, carbon based, biological, complex materials (coated, core–shell). Only certain elements.	Easy and cheap technique. Both solid and liquid samples can be analyzed.	Very material dependent. Size determination possible only for plasmonic samples. Only for diluted samples (not in powders), for example, thin films, dispersions, and so on. Samples in powders require reflectance accessory.
NMR	Size (indirect analysis) Structure Composition Purity Conformational change	Inorganic, organic, carbon based, biological, complex materials (coated, core–shell). Only for certain elements.	Nondestructive/ noninvasive method. Sample preparation easy.	Low sensitivity. Time consuming. Relatively large amount of sample required. Only for certain elements.
EELS	Composition Surface chemistry Electronic properties	Inorganic, organic, carbon based, complex materials (coated, core–shell). Spherical, equiaxial particles, tubes, flakes, rods, fibers.	Depth resolution up to 10 nm. Can be used in combination with TEM.	Tends to work best at relatively low atomic number. Need very thin specimens <30 nm. Intensity weak for energy losses >300 eV.
XRF	Composition	Inorganic, organic, carbon based, complex materials (coated, core–shell). Spherical, equiaxial particles, tubes, flakes, rods, fibers	Despite the fact that it has 100-nm spatial resolution it can be used for nanomaterials chemical identification.	Fairly high limits of detection. Possibility of matrix effects.

(*Continued*)

TABLE 2.4 Overview of Commonly Used Analytical Methods for Characterization of Physicochemical Properties of Nanomaterials (*cont.*)

Method	Physicochemical characteristics analyzed/ parameters	Type of material	Strengths	Limitations
XPS	Surface chemistry Composition	Inorganic, organic, carbon based, complex materials (coated, core–shell). Spherical, equiaxial particles, tubes, flakes, rods, fibers.	Very sensitive for surface chemistry.	Only detects the elements in a very thin layer close to surface (about 10 nm). Very sensitive, requires careful sample preparation. Ultra high vacuum.
EDX	Composition Surface chemistry	Inorganic, organic, carbon based, complex materials (coated, core–shell). Spherical, equiaxial particles, tubes, flakes, rods, fibers.	Gives information on elements from beryllium to einsteinium. Detection limit 0.1 at.%.	Used in conjunction with TEM or SEM. Not real surface science technique. Not suitable for nonconductive samples. Elements of low atomic number can be difficult to detect (overlap with X-ray peaks).
TGA/DCS	Composition Thermal properties Crystalline phase	Inorganic, organic, carbon based, complex materials (coated, core–shell). Spherical, equiaxial particles, tubes, flakes, rods, fibers.	DSC can operate from −180 to 1750°C.	Large samples required: 1–50 mg. Destructive method.
ICP-MS	Composition Surface chemistry	Inorganic, organic, carbon based, complex materials (coated, core–shell). Spherical, equiaxial particles, tubes, flakes, rods, fibers.	Very sensitive: limits of detection 1 ng/L.	Lower sensitivity toward some of the lighter elements, such as H, I, O, N, C, S.
ICP-OEC	Composition Surface chemistry	Inorganic, organic, carbon based, complex materials (coated, core–shell). Spherical, equiaxial particles, tubes, flakes, rods, fibers.	Can basically characterize all types of material.	2–3 orders of magnitude lower sensitivity than ICP-MS.

Test item preparation and dispersion (including stability) in appropriate media is a critical issue, since many of the nanomaterials are insoluble or sparingly soluble in water and other media used in (eco)toxicological tests. Moreover, their physicochemical properties, as well as (eco)toxicological effects can be affected by the interactions with the bio-physicochemical surroundings in these media. Thus, sample characterization should be performed for a number of different stages of testing nanomaterials. The testing stages may include powder and/or dispersion forms depending on the endpoint and/or the stage tested, and the actual material tested (the test item) has undergone a critical process of preparation that needs to be controlled, consistent, relevant, reliable, and robust.

The OECD Guidance Document on Sample Preparation and Dosimetry (OECD, 2012b) can be considered an important step forward in the development of guidance for adequately addressing hazard testing of nanomaterials, as indicated for instance in the OECD Council Recommendation (OECD, 2013). This document refers and applies to water-insoluble manufactured nanomaterials, as it was considered unlikely that soluble nanomaterials would need a sample preparation different from what is applied to chemicals in general. It should be noted that the classical dose metrics, milligram test substance per kilogram test organism, may not be the most suitable one for nanomaterials (SCENIHR, 2007). Since publication of the Guidance Document on Sample Preparation and Dosimetry (OECD, 2012b) a number of protocols have emerged [NIST, http://www.nist.gov/mml/np-measurement-protocols.cfm; PROSPEcT (2010), Nanogenotox (2011), and ENPRA (Jacobsen et al., 2010), and recommendations (Tantra et al., 2014)] aiming to become reference for future studies. Also the dispersion of nanomaterials for testing has been further studied, and several aqueous dispersion protocols for nanomaterials in aqueous media (Hartmann et al., 2015) were reviewed, analyzing which aspects of dispersion procedures could be harmonized at this point in time. The "perfect" dispersion protocol is yet to emerge, if at all existing, taking into consideration the specificities of nanomaterials and test method requirements.

DATA COLLECTION AND INTERPRETATION

Interlaboratory and even intralaboratory reproducibility of physicochemical data on nanomaterials has turned out to be problematic in many cases. In order to improve the reliability, comparability, and usefulness of specific physicochemical information, it is worthwhile to consider some general elements that affect the usefulness of data on nanomaterials, including generation, quality, completeness, and interpretation of data. These factors are discussed in the context of nanomaterials, but in principle they are also valid for chemicals in general.

Data generation is understood as direct measurement or derivation of a quantity from measured data. As far as possible, it should be done following published standards (e.g., by ISO) or guidelines (e.g., by OECD), if available. The problem with nanomaterials is that guidelines and standards on how to determine certain physicochemical properties of substances were developed for standard chemicals which are usually not nanomaterials. Therefore, one has to be aware that in many cases they are not directly applicable to NMs and need to be adapted to NMs, or new ones need to be developed (OECD, 2009). Currently, apart from size almost no standards and reference materials are available for physicochemical properties

of nanomaterials. Such standards and reference materials are urgently needed to improve the comparability of data obtained in different laboratories.

If generally agreed standards or guidelines are not available, the next best choice is to look for SOPs for sample preparation and measurements published in the scientific literature (e.g., Hartmann et al. 2015; MARINA, webpage; Nanogenotox 2011). Such SOPs are usually developed for specific combinations of materials and methods, and most likely they cannot be directly applied to other systems. But taking advantage from extended experience made in such research projects, it may be possible to adapt them to similar systems without developing them from the start. In order to achieve interlaboratory reproducibility and uniformity of results on physicochemical properties of nanomaterials, for which validated methods are not yet available, it is crucial to disclose all details of a measurement procedure, from sample preparation to final data evaluation and to work toward a common understanding of the measured data. Such an approach was followed, for example, to obtain a basic comparison of particle size distribution measurements of pigments and fillers using commonly available industrial methods (Gilliland et al., 2014).

Regardless whether data generation is made according to fully developed standards, general guidelines, or case-specific protocols, it is essential in all cases that the data collection from sample preparation to measurement is documented in as much detail as possible. In that context it is also helpful to use or develop standardized templates for data collection, sharing, and reporting of nanomaterial research data (OECD website; Thomas et al., 2013).

A crucial problem to which a common solution has not yet been found, is the decision on which data should be collected and the assessment of the quality of the data (Marchese Robinson et al., 2016). This problem exists not only for pure physicochemical characterization of NMs, but for many other aspects of nanomaterial research, specifically concerning the safety of NMs.

To answer a specific question, for example, in the context of the safety of nanomaterials, the data should be complete, that is, the necessary experimental details should be described and associated experimental results reported. Data completeness assesses the extent to which such data, including related metadata, are available. Key international organizations, such as the Organisation for Economic Co-operation and Development (OECD) and the International Standards Organisation (ISO), have made recommendations on which physicochemical parameters and other experimental variables should be reported for various kinds of experimental studies of nanomaterials (ISO 2012a; OECD, 2012b). The data necessary to answer a specific question can be seen as a subset of a complete physicochemical nanomaterial characterization (Stefaniak et al., 2013) that encompasses all available information on the properties of a nanomaterial. Data completeness can therefore be regarded as the extent of nanomaterial characterization under a specified set of experimental conditions and time points, the degree to which experimental details are described, as well as the availability of raw and processed data (OECD website). It can be seen as a measure of the extent to which the data and metadata which serve to address a specific need are available.

Data quality according to a recent proposal is a measure of the potential usefulness, clarity, correctness, and trustworthiness of data and datasets (OECD website). However, as with data completeness, there is no definitive consensus regarding exactly how data quality should be defined in the nanoscience community. It may include a variety of issues, such as uncertainty in the data and also aspects of data completeness. For example, data quality may be

considered to be dependent upon the reproducibility of the data, and this in turn will depend upon the degree of data completeness.

In order to achieve high data quality, that is, a high degree to which data are clear and the extent to which they can be considered correct, it is useful not only to consider the measured data themselves, but to assess whether experimental protocols were carried out appropriately. For example, Lubinski et al. (2013) proposed an extension of the Klimisch framework (Klimisch et al., 1997) for evaluating the reliability of physicochemical data on nanomaterials (and nanotoxicology data). This was considered to depend upon compliance with Good Laboratory Practice (GLP) (OECD website) and standardized test guidelines and protocols.

It is also important that sufficient metadata are provided to precisely identify any measured data. For example, if the size distribution of nanoparticles is reported, this should also be accompanied by the used sample preparation protocols, methods, source, and batch number of the sample including its history (producer, age, storage, and handling conditions, any preprocessing, etc.) and everything that is needed to assess the data, experimental errors, uncertainty, and possible artefacts. Nanomaterial identification and characterization based on physicochemical measurements is not meaningful unless the corresponding experimental protocols are adequately described, because many of the nanomaterials' physicochemical properties are context-dependent. For many physicochemical properties it may even be safer to determine them in-house, given the often unknown quality of such data provided externally. In-house measurement of physicochemical data allows a much better assessment of their quality and moreover gives the possibility to determine them under conditions relevant for subsequent in-house toxicological studies.

In relation to the safety of nanomaterials it appears that at present there are the following main challenges: there is an incomplete understanding of which physicochemical properties most significantly influence biological effects and are therefore most relevant for the safety of nanomaterials. Many physicochemical properties depend on the experimental conditions, including but not limited to zeta potential and state of agglomeration. Those properties potentially, also, are time-dependent and can change with the aging, as well as with the duration of contact with the media. Often there is a batch-to-batch variability of nanomaterials, and, as highlighted by OECD and other organizations, there is a lack of guidelines specifically suitable for physicochemical characterization of nanomaterials.

TAKE-HOME MESSAGES

- Safety evaluation and research on the safety of nanomaterials is meaningful only if the nanomaterials to be studied are well characterized.
- Physicochemical characterization of nanomaterials is a fundamental prerequisite for any analysis of their toxicological effects.
- For safety assessment of nanomaterials, it is essential to be able to link measured properties unequivocally with a specific nanomaterial. This includes complete purpose-specific physicochemical characterization of the material.
- There are several suggestions on which physicochemical properties are relevant for the toxicological assessment of nanomaterials which agree to some extent. A definitive list of relevant physicochemical properties is, however, not (yet) available.

- Research and development of methods that can characterize nanomaterials at points of interaction with organisms is still needed.
- The selected set of physicochemical parameters should be fit-for-purpose of the characterization, for example, research, one particular toxicity test or regulatory requirements.
- There is no single characterization technique that can be used to characterize one property for all nanomaterials. Furthermore, different methods can yield different results for the same nanomaterial. Thus, where possible, measurements of a specific property should be made using several methods to obtain a comprehensive characterization and to compensate for the weaknesses of individual methods.
- Sample preparation and dispersion in test media is a critical issue for reliable physicochemical characterization of nanomaterials, as well as for interpretation of toxicity tests results. No universal dispersion protocol, applicable to all types of nanomaterials in all types of media, exists.
- Appropriate data collection from sample preparation to measurement should be documented in as much detail as possible to assure correct data interpretation and repeatability of the results. Data completeness check is highly recommended and should be regarded as an extension of nanomaterial characterization.
- There is still a lack of test guidelines validated for physicochemical characterization of nanomaterials thus each nanomaterial should be tested on a case-by-case basis.

Acknowledgments

The authors wish to thank Dr. Douglas Gilliland from the European Commission's Joint Research Centre, Ispra site, for his comments and observations on an earlier version of the manuscript.

Disclaimer

The views expressed in this chapter are those of the authors and do not represent the views and/or policies of the European Commission or European Agency. The authors are not responsible for any subsequent use which might be made of this text.

References

Australia, 2010. Australia Announces Adjustments to NICNAS New Chemicals Processes for Industrial Nanomaterials. Available from: http://nanotech.lawbc.com/2010/10/australia-announces-adjustments-to-nicnas-new-chemicals-processes-for-industrial-nanomaterials/

Bandyopadhyay, S., Peralta-Videa, J.R., Gardea-Torresdey, J.L., 2013. Advanced analytical techniques for the measurement of nanomaterials in food and agricultural samples: a review. Environ. Eng. Sci. 30 (3), 118–125.

Barua, S., Mitragotri, S., 2014. Challenges associated with penetration of nanoparticles across cell and tissue barriers: a review of current status and future prospects. Nano Today 9 (2), 223–243.

Beloqui Redondo, A., Jordan, I., Ziazadeh, I., Kleibert, A., Giorgi, J.B., Wörner, H.J., May, S., Abbas, Z., Brown, M.A., 2015. Nanoparticle-induced charge redistribution of the air-water interface. J. Phys. Chem. C 119, 2661–2668.

Blakeley, M.P., Langan, P., Niimura, N., Podjarny, A., 2008. Neutron crystallography: opportunities, challenges, and limitations. Curr. Opin. Struct. Biol. 18 (5), 593–600.

Branca, C., Frusteri, F., Magazù, V., Mangione, A., 2004. Characterization of carbon nanotubes by TEM and infrared spectroscopy. J. Phys. Chem. B 108 (11), 3469–3473.

Briggs, D., Seah, M.P., 1983. Practical Surface Analysis by Auger and X-Ray Photoelectron Spectroscopy. John Wiley & Sons, Chichester.
British Standards, 2013. Workplace exposure. Measurement of the Dustiness of Bulk Materials. Requirements and Choice of Test Methods. BS EN 15051-1:2013, 2013, ISBN 978 0 580 76532 2.
Brunauer, S., Emmett, P.H., Teller, E., 1938. Adsorption of gases in multimolecular layers. J. Am. Chem. Soc. 60, 309–319.
Callaway, E., 2015. The revolution will not be crystallized: a new method sweeps through structural biology. Nature 525, 172–174.
Carey, P.R., 2006. Raman crystallography and other biochemical applications of Raman microscopy. Annu. Rev. Phys. Chem. 57, 527–554.
Challa, S.S., Kumar, R. (Eds.), 2012. Raman Spectroscopy for Nanomaterials Characterization. Springer, ISBN 978-3-642-20620-7.
Dablemont, C., Lang, P., Mangeney, C., Piquemal, J.-Y., Petkov, V., Herbst, F., Viau, G., 2008. FTIR and XPS study of Pt nanoparticle functionalization and interaction with alumina. Langmuir 24 (11), 5832–5841.
Degueldre, C., Favarger, P.Y., 2003. Colloid analysis by single particle inductively coupled plasma-mass spectroscopy: a feasibility study. Coll. Surf. A 217, 137–142.
de Temmerman, P.-J., van Doren, E., Verleysen, E., van der Stede, Y., Abi, D.F.M., Mast, J., 2012. Quantitative characterization of agglomerates and aggregates of pyrogenic and precipitated amorphous silica nanomaterials by transmission electron microscopy. J. Nanobiotechnol. 10, 24.
de Temmerman, P.-J., Verleysen, E., Lammertyn, J., Mast, J., 2014. Semi-automatic size measurement of primary particles in aggregated nanomaterials by transmission electron microscopy. Powder Technol. 261 (1), 191–200.
Dresselhaus, M.S., Jorio, A., Hofmann, M., Dresselhaus, G., Saito, R., 2010. Perspectives on carbon nanotubes and graphene Raman spectroscopy. Nano Lett. 10 (3), 751–758.
Dubascoux, S., Hècho, I., Hassellöv, M., von der Kammer, F., Gautier, M., Lespes, G., 2010. Field-flow fractionation and inductively coupled plasma mass spectrometer coupling: history, development and applications. J. Anal. Atom. Spectrom. 25, 613–623.
EC, 2011. European Commission Recommendation of 18 October 2011 on the definition of nanomaterial. Off. J. Eur. Union 2011/696/EU, 38–40.
European Food Safety Authority (EFSA) Scientific Opinion, 2011. Guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain. EFSA J. 9 (5), 2140.
Egerton, R.F., 2011. Electron Energy-Loss Spectroscopy in the Electron Microscope, third ed. Springer, New York.
Gilliland, D., Gibson, P., Hempelmann, U., Vicent Pena, J.B., Kund, K., Wohlleben, W., Koch, T., Burke, A., Mcnulty, G., Hartl-Gunselmann, A., Knobl, S., Reisinger, M., Sokull-Klüttgen, B., Stamm, H., Liewald, H., 2014. Basic Comparison of Particle Size Distribution Measurements of Pigments and Fillers Using Commonly Available Industrial Methods, EUR 26916. doi: 10.2788/21024.
Glatter, O., Kratky, O., 1982. Small-Angle X-Ray Scattering. Academic Press, London.
Graewert, M.A., Svergun, D.I., 2013. Impact and progress in small and wide angle X-ray scattering (SAXS and WAXS). Curr. Opin. Struct. Biol. 23 (5), 748–754.
Grant, T., Briggs, D., 2003. Surface Analysis by Auger and X-Ray Photoelectron Spectroscopy. IM Publications, Chichester.
Grobelny, J., Del Rio, F.W., Pradeep, N., Kim, D.I., Hackley, V.A., Cook, R.F., 2011. Size measurement of nanoparticles using atomic force microscopy. Methods Mol. Biol. 697, 71–82.
Hartmann, N.B., Jensen, K.A., Baun, A., Rasmussen, K., Rauscher, H., Tantra, R., Cupi, D., Gilliland, D., Pianella, F., Riego Sintes, J.M., 2015. Techniques and protocols for dispersing nanoparticle powders in aqueous media—is there a rationale for harmonization? J. Toxicol. Environ. Health B Crit. Rev. 18 (6), 299–326.
Health Canada, 2011. Policy Statement on Health Canada's Working Definition for Nanomaterial. Available from: http://www.hc-sc.gc.ca/sr-sr/pubs/nano/pol-eng.php
Hota, G., Idage, S.B., Khilar, K.C., 2007. Characterization of nano-sized CdS–Ag2S core-shell nanoparticles using XPS technique. Coll. Surf. A 293, 5–12.
ISO, 2006. Pore size distribution and porosity of solid materials by mercury porosimetry and gas adsorption—Part 2: Analysis of mesopores and macropores by gas adsorption. International Organization for Standardization, ISO 15901-2.
ISO, 2008a. Nanotechnologies—terminology and definitions for nano-objects—nanoparticle, nanofibre and nanoplate. International Organization for Standardization, ISO/TS 27687.

ISO, 2008b. Terms and definitions used in connection with reference materials. International Organization for Standardization, ISO Guide 30 1992/Amd.1.
ISO, 2008c. Representation of results of particle size analysis—Part 6: descriptive and quantitative representation of particle shape and morphology. International Organization for Standardization, ISO 9276-6.
ISO, 2010. Determination of the specific surface area of solids by gas adsorption-BET method. International Organization for Standardization, ISO 9277.
ISO, 2012a. Nanotechnologies—guidance on physico-chemical characterization of engineered nanoscale materials for toxicologic assessment. International Organization for Standardization, ISO TR ISO/TR 13014.
ISO, 2012b. Nanomaterials—preparation of material safety data sheet (MSDS). International Organization for Standardization, ISO/TR 13329.
ISO, 2013. Particle characterization of particulate systems—vocabulary. International Organization for Standardization, ISO 26824.
ISO, 2015. Surface chemical analysis—use of total reflection X-ray fluorescence spectroscopy in biological and environmental analysis. International Organization for Standardization, ISO/TS 18507.
Jacobsen, N.R., Pojano, G., Wallin, H., Jensen, K.A., 2010. Nanomaterial dispersion protocol for toxicological studies in ENPRA. Internal ENPRA Report. National Research Centre for the Working Environment, 8 pp.
Jarvis, P., Jefferson, B., Gregory, J., Parsons, S.A., 2005. A review of floc strength and breakage. Water Res. 39 (14), 3121–3137.
Johnston, H., Brown, D., Kermanizadeh, A., Gubbins, E., Stone, V., 2012. Investigating the relationship between nanomaterial hazard and physicochemical properties: Informing the exploitation of nanomaterials within therapeutic and diagnostic applications. J. Control. Release 164, 307–313.
Juillerat-Jeanneret, L., 2008. The targeted delivery of cancer drugs across the blood–brain barrier: chemical modifications of drugs or drug-nanoparticles? Drug Discov. Today 13 (23/24), 1099–1106.
Klimisch, H.-J., Andreae, M., Tillmann, U., 1997. A systematic approach for evaluating the quality of experimental toxicological and ecotoxicological data. Regul. Toxicol. Pharmacol. 25, 1–5.
Klobes, P., Meyer, K., Munro, R.G., 2006. Porosity and Specific Surface Area Measurements for Solid Materials. NIST Recommended Practice Guide, Special Publication 960-17. U.S. Dept. of Commerce, Technology Administration, National Institute of Standards and Technology, Gaithersburg, MD.
Laborda, F., Bolea, E., Jiménez-Lamana, J., 2014. Single particle inductively coupled plasma mass spectrometry: a powerful tool for nanoanalysis. Anal. Chem. 86, 2270–2278.
Laborda, F., Jiménez-Lamana, J., Bolea, E., Castillo, J.R., 2011. Selective identification, characterization and determination of dissolved silver(I) and silver nanoparticles based on single particle detection by inductively coupled plasma mass spectrometry. J. Anal. Atom. Spectrom. 26, 1362–1371.
Lee, J.Y., Kim, J.S., An, K.H., 2005. Electrophoretic and dynamic light scattering in evaluating dispersion and size distribution of single walled carbon nanotubes. J. Nanosci. Nanotechnol. 5, 1045–1049.
Linsinger, T.P.J., Peters, R., Weigel, S., 2014. International interlaboratory study for sizing and quantification of Ag nanoparticles in food simulants by single-particle ICPMS. Anal. Bioanal. Chem. 406 (16), 3835.
Linsinger, T.P.J., Roebben, G., Gilliland, D., Calzolai, L., Rossi, F., Gibson, N., Klein, C., 2012. Requirements on Measurements for the Implementation of the European Commission Definition of the Term 'Nanomaterial'. European Union, Luxembourg, 52 p.
Linsinger, T.P.J., Roebben, G., Solans, C., Ramsch, R., 2011. Reference materials for measuring the size of nanoparticles. Trends Analyt. Chem. 30, 1827.
Lövestam, G., Rauscher, H., Roebben, G., Sokull-Klüttgen, B., Gibson, N., Putaud, J.-P., Stamm, H., 2010. Considerations on a Definition of Nanomaterial for Regulatory Purposes. European Commission Joint Research Centre. Publications Office of the European Union, Luxembourg.
Lubinski, L., et al., 2013. Evaluation criteria for the quality of published experimental data on nanomaterials and their usefulness for QSAR modelling. SAR QSAR Environ. Res. 24, 995–1008.
Marchese Robinson, R.L., et al., 2016. How should the completeness and quality of curated nanomaterial data be evaluated? Nanoscale 8 (19), 9919–9943.
Marchini, S., Gunther, S., Wintterlin, J., 2007. Scanning tunneling microscopy of graphene on Ru(0001). Phys. Rev. B 76, 075429.
Marina, webpage. Homepage for the project "Managing risk of nanomaterials." Available from: http://www.marina-fp7.eu/publications/

Naha, P.C., Casey, A., Tenuta, T., Lynch, I., Dawson, K.A., Byrne, H.J., et al., 2009. Preparation, characterization of NIPAM and NIPAM/BAM copolymer nanoparticles and their acute toxicity testing using an aquatic test battery. Aquat. Toxicol. 92, 146–154.

NanoDefine, 2016. Technical Report D3.1:Techniques evaluation report for selection of characterization methods. Available from: http://www.nanodefine.eu/publications/reports/NanoDefine_TechnicalReport_D3.1.pdf

Nanogenotox, 2011. Nanogenotox dispersion protocol. Available from: http://www.nanogenotox.eu/files/PDF/Deliverables/nanogenotox%20deliverable%203_wp4_%20dispersion%20protocol.pdf

Nanogenotox, 2012. NanoGenoTox project, Deliverable 4.6: Dustiness of NANOGENOTOX nanomaterials using the NRCWE small rotating drum and the INRS Vortex shaker. 2012. Available from: http://www.nanogenotox.eu/files/PDF/Deliverables/d4.6_dustiness.pdf

National Science and Technology Council, 2014. Committee on Technology. Subcommittee on Nanoscale Science, Engineering, and Technology National Nanotechnology Initiative. Strategic Plan, USA. Available from: http://www.nano.gov/sites/default/files/pub_resource/2014_nni_strategic_plan.pdf

Oberdörster, G., Maynard, A., Donaldson, K., Castranova, V., Fitzpatrick, J., Ausman, K., et al., 2005. Principles for characterizing the potential human health effects from exposure to nanomaterials: Elements of a screening strategy. Part. Fibre Toxicol. 2, 8, Acc. Chem. Res. 46(3), 607–621.

OECD, webpage. Safety of manufactured nanomaterials. Available from: http://www.oecd.org/science/nanosafety/. Harmonised templates for reporting chemical test summaries: OHT 101-113: Additional physicochemical properties of nanomaterials. Organisation for Economic Co-operation and Development. Available from: http://www.oecd.org/ehs/templates/templates.htm. OECD Good Laboratory Practice. Available from: http://www.oecd.org/chemicalsafety/testing/goodlaboratorypracticeglp.htm

OECD, 2009. Preliminary review of OECD test guidelines for their applicability to manufactured nanomaterials. ENV/JM/MONO(2009)21. Organisation for Economic Co-operation and Development. Available from: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?doclanguage=en&cote=env/jm/mono

OECD, 2010. Guidance Manual for the Testing of Manufactured Nanomaterials: OECD's Sponsorship Programme; first revision. ENV/JM/MONO(2009)20/REV. Organisation for Economic Co-operation and Development. Available from: http://www.oecd.org/officialdocuments/displaydocumentpdf/?cote=env/jm/mono(2009)20/rev&doclanguage=en

OECD, 2012a. Manual for the Assessment of Chemicals. Data Gathering. Organisation for Economic Co-operation and Development (Chapter 2). Available from: http://www.oecd.org/chemicalsafety/testing/49944183.pdf

OECD, 2012b. Guidance on Sample Preparation and Dosimetry for the Safety testing of Manufactured Nanomaterials. ENV/JM/MONO(2012)40. Organisation for Economic Co-operation and Development.

OECD, 2013. Recommendation of the Council on the Safety Testing and Assessment of Manufactured Nanomaterials. OECD. C(2013)107. Organisation for Economic Co-operation and Development.

OECD, 2014. Report of the OECD Expert Meeting on the Physical Chemical Properties of Manufactured Nanomaterials and Test Guidelines. ENV/JM/MONO(2014)15. Organisation for Economic Co-operation and Development. Available from: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2014)15&docLanguage=En

OECD, 2016a. Physical-Chemical Properties of Nanomaterials: Evaluation of Methods Applied in the OECD-WPMN Testing Programme. ENV/JM/MONO(2016)7. Organisation for Economic Co-operation and Development.

OECD, 2016b. Physical-Chemical Parameters: Measurements and Methods Relevant for the Regulation of Nanomaterials. OECD Workshop Report. ENV/JM/MONO(2016)2. Organisation for Economic Co-operation and Development.

OECD, 2016c. Publication of Dossiers From the WPMN Testing Programme. Organisation for Economic Co-operation and Development. Available from: http://www.oecd.org/chemicalsafety/nanosafety/testing-programme-manufactured-nanomaterials.htm

Ohdaira, T., Suzuki, R., Kobayashi, Y., Akahane, T., Dai, L., 2002. Surface analysis of a well-aligned carbon nanotube film by positron-annihilation induced Auger-electron spectroscopy. Appl. Surf. Sci. 194, 291–295.

Okitsu, K., 2013. UV-VIS and photoluminescence spectroscopy for nanomaterials characterization. In: Challa, S.S., Kumar, R. (Eds.), UV-VIS and Photoluminescence Spectroscopy for Nanomaterials. Springer, pp. 151–177.

Oomen, A.G., Bennink, M., van Engelen, J.G.M., 2011. Nanomaterial in consumer products—detection, characterization and interpretation. Report 320029001/2011. Sips RIVM Report 320029001/2011.

Pace, H.E., Rogers, N.J., Jarolimek, C., Coleman, V.A., Gray, E.P., Higgins, C.P., Ranville, J.F., 2012. Single particle inductively coupled plasma-mass spectrometry: a performance evaluation and method comparison in the determination of nanoparticle size. Environ. Sci. Technol. 46, 12272–12280.

Philippe, A., Gangloff, M., Rakcheev, D., Schaumann, G.E., 2014. Evaluation of hydrodynamic chromatography coupled with inductively coupled plasma mass spectrometry detector for analysis of colloids in environmental media-effects of colloid composition, coating and shape. Anal. Methods 6, 8722–8728.

Powers, K.W., Brown, S.C., Krishna, V.B., Wasdo, S.C., Moudgil, B.M., Roberts, S.M., 2006. Research strategies for safety evaluation of nanomaterials. Part VI. Characterization of nanoscale particles for toxicological evaluation. Toxicol. Sci. 90 (2), 296–303.

PROSPEcT, 2010. Protocol for Nanoparticle Dispersion. Available from: http://www.nanotechia.org/sites/default/files/files/PROSPECT_Dispersion_Protocol.pdf

Rahman, M., et al., 2013. Protein-nanoparticle interactions. Nanoparticle and Protein Corona. Springer Series in Biophysics, 15, Springer-Verlag, Berlin, Heidelberg, (Chapter 2).

Ramallo-Lopez, J.M., Giovanetti, L., Craievich, A.F., 2007. XAFS, SAXS and HREM characterization of PD nanoparticle capped with n-alkyl thiol molecule. Phys. B Condens. Mater. 389, 150–154.

Rasmussen, K., et al. 2013. Synthetic amorphous silicon dioxide (NM-200, NM-201, NM-202, NM-203, NM-204): characterization and physico-chemical properties. JRC Repository: NM-series of Representative Manufactured Nanomaterials. EUR Report 26046.

Rasmussen, K., Gonzalez, M., Kearns, P., Riego Sintes, J., Rossi, F., Sayre, P., 2016. Review of achievements of the OECD Working Party on Manufactured Nanomaterials' Testing and Assessment Programme. From exploratory testing to test guidelines. Regul. Toxicol. Pharmacol. 74, 147–160.

Rauscher, H., et al., 2014. Towards a review of the EC Recommendation for a definition of the term "nanomaterial." Part 1 Compilation of information concerning the experience with the definition. EUR Report 26567 EN.

Ray, S., Shard, A.G., 2011. Quantitative analysis of adsorbed proteins by X-ray photoelectron spectroscopy. Anal. Chem. 83 (22), 8659–8666.

Roco, M.C., 2001. International strategy for nanotechnology research and development. J. Nanopart. Res. 3, 353–360.

Roebben, G., Emons, H., Reiners, G., 2011. Nanoscale reference materials. In: Murashov, V., Howard, J. (Eds.), Nanostructure Science and Technology. Springer, New York, pp. 53–75.

Roebben, G., Rasmussen, K., Kestens, V., Linsinger, T.P.J., Rauscher, H., Emons, H., Stamm, H., 2013. Reference materials and representative test materials: the nanotechnology case. J. Nanopart. Res. 15, 1455.

Roming, M., Feldmann, C., Avadhut, Y.S., Schmedt auf der Günne, J., 2008. Characterization of noncrystalline nanomaterials: NMR of zinc phosphate as a case study. Chem. Mater. 20 (18), 5787–5795.

Rong, M.Z., Zhang, M.Q., Liang, H.C., Zeng, H.M., 2004. Surface derivatization of nano-CdS clusters and its effect the performance of CdS quantum dots in solvents and polymeric matrices. Appl. Surf. Sci. 228, 176–190.

Royal Society and Royal Academy of Engineering, 2004. Nanoscience and Nanotechnologies: Opportunities and Uncertainties. RS Policy Document 19/04. Royal Society, London. Available from: http://www.nanotec.org.uk/finalReport.htm

Sales, D.L., Beltran, A.M., Lozano, J.G., Manuel, J.M., Guerrero-Lebrero, M.P., Ben, T., Herrera, M., Morales, F.M., Pizarro, J., Sanchez, A.M., Galindo, P.L., Gonzalez, D., Garcia, R.S., Molina, S.I., 2012. In: Patane, A., Baltan, N. (Eds.), High-Resolution Electron Microscopy of Semiconductor Heterostructures and Nanostructures, vol. 150. pp. 23–62 (Chapter 2). ISBN978-3-642-23351-7.

Saw, C.K., 2005. X-ray scattering techniques for characterization of nanosystems in lifescience in nanotechnologies for life sciences. In: Challa, S.S., Kumar, R. (Eds.), Nanosystem. Characterization Tools in the Life Sciences, vol. 3. UCRL-JRNL-211387.

Samori, P. (Ed.), 2006. Scanning Probe Microscopies Beyond Imaging: Manipulation of Molecules and Nanostructures. Wiley-VCH, ISBN: 9783527608515.

SCENIHR, 2007. Opinion on the Appropriateness of the Risk Assessment methodology in accordance with the technical guidance documents for new and existing substances for assessing the risks of nanomaterials. Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR). Available from: http://ec.europa.eu/health/ph_risk/committees/04_scenihr/docs/scenihr_o_010.pdf

SCENIHR, 2009. Risk Assessment of Products of Nanotechnologies. Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR). Available from: http://ec.europa.eu/health/archive/ph_risk/committees/04_scenihr/docs/scenihr_o_023.pdf

Smith, D.J., 2015. Characterization of nanomaterials using transmission electron microscopy. Nanocharacterization (2), pp. 1–29, ISBN:978-1-78262-186-7 (Chapter 1).

Stefaniak, A.B., Hackley, V.A., Roebben, G., Ehara, K., Hankin, S., Postek, M.T., Lynch, I., Fu, W.-E., Linsinger, T.P.J., Thünemann, A.F., 2013. Nanoscale reference materials for environmental, health and safety measurements: needs, gaps and opportunities. Nanotoxicology 7 (8), 1325–1337.

Stintz, M., Babick, F., Roebben, G., 2010. Metrology for Nanoparticle Characterization: Instruments, Standard Methods and Reference Materials (Workshop Report, Nuremberg, 28./29.04.2010). Co-nanomet Consortium, c/o euspen, Cranfield.

Tantra, R., 2016. Nanomaterial Characterization: An Introduction. Wiley.

Tantra, R., Sikora, A., Hartmann, N., Riego Sintes, J., 2014. Comparison of the effects of different protocols on the particle size distribution of TiO_2 dispersions. Particuology 19, 35–44.

Terrones, M., Jorio, A., Endo, M., 2004. New direction in nanotube science. Mater. Today 7, 30–45.

Thomas, et al., 2013. ISA-TAB-Nano: a specification for sharing nanomaterial research data in spreadsheet-based format. BMC Biotechnol. 13, 2.

Tiede, K., Boxall, A.B.A., Tear, S.P., Lewis, J., David, H., Hassellöv, M., 2008. Detection and characterization of engineered nanoparticles in food and the environment. Food Addit. Contam. 25 (7), 795–821.

Torres, T., Bottari, G., 2013. Organic Nanomaterials: Synthesis, Characterization, and Device Applications. Wiley, ISBN: 978-1-118-01601-5, 622 p.

TSI, 2004. TSI incorporated 3800 ATOFMS. Available from: http://www.tsi.com/uploadedFiles/Product_Information/Literature/Spec_Sheets/3800SeriesPN1933798RevD.pdf

van Doren, E.F., de Temmerman, P.J., Francisco, M.A., Mast, J., 2011. Determination of the volume-specific surface area by using transmission electron tomography for characterization and definition of nanomaterials. J. Nanobiotechnol. 9, 17.

Walczyk, D., Bombelli, F.B., Monopoli, M.P., Lynch, I., Dawson, K.A., 2010. What the cell "sees" in bionanoscience. J. Am. Chem. Soc. 132, 5761–5768.

Wiedensohler, A., et al., 2012. Mobility particle size spectrometers: harmonization of technical standards and data structure to facilitate high quality long-term observations of atmospheric particle number size distributions. Atmos. Meas. Tech. 5, 657–685.

Williams, S.K.R., Runyon, J.R., Ashames, A.A., 2011. Field-flow fractionation: addressing the nano challenge. Anal. Chem. 83, 634–642.

Wilson, S.C., Baryza, J.L., Reynolds, A.J., Bowman, K., Keegan, M.E., Standley, S.M., Gardner, N.P., Parmar, P., Agir, V.O., Yadav, S., Zunic, A., 2015. Real time measurement of peg shedding from lipid nanoparticles in serum via NMR spectroscopy. Mol. Pharm. 12 (2), 386–392.

Wohlfart, S., Gelperina, S., Kreuter, J., 2012. Transport of drugs across the blood–brain barrier by nanoparticles. J. Control. Release 161, 264–327.

Xu, R., 2000. Particle Characterization: Light Scattering Methods. Kluwer Academic Publishers, Dordrecht.

Zensi, A., Begley, D., Pontikis, C., Legros, C., Mihoreanu, L., Wagner, S., Büchel, C., von Briesen, H., Kreuter, J., 2009. Albumin nanoparticles targeted with Apo E enter the CNS by transcytosis and are delivered to neurons. J. Control. Release 137, 78–86.

CHAPTER

3

Toxicity Tests: In Vitro and In Vivo

Maria Dusinska, Elise Rundén-Pran*, Jürgen Schnekenburger**, Jun Kanno†*

**NILU-Norwegian Institute for Air Research, Kjeller, Norway; **Biomedical Technology Center, Westfälische Wilhelms-Universität Muenster, Muenster, Germany; †Japan Bioassay Research Center, Japan Organization of Occupational Health and Safety, Hirasawa, Hadano, Kanagawa, Japan

OUTLINE

Introduction 51

Methods for Visualizing Cellular Uptake and Biodistribution 52

Evaluation of Transport and Uptake of ENM In Vitro and In Vivo 58

In Vitro Toxicity Tests 60
- *Cytotoxicity Tests* 62
- *Limitations of In Vitro Tests* 62
- *New In Vitro Test Principles* 63
- *Validated Cytotoxicity Tests* 64
- *Genotoxicity Tests* 65
- *Ex Vivo Models* 68

In Vivo Toxicity Testing 68
- *Toxicokinetics* 69
- *Immunological Responses* 72
- *Chronic Toxicity and Carcinogenicity* 72

Considerations for Selection of Toxicity Tests: In Vitro/In Vivo Comparison 73

Conclusions 76

Take-Home Messages 76

Acknowledgments 76

References 76

INTRODUCTION

Nanotoxicology is an emerging field, dealing with human and environmental hazard and risk assessment caused by structures smaller than 100 nm (Haynes, 2010; Maynard et al., 2011). Progress within nanotechnology is important, but the benefits of nanotechnology must be

Adverse Effects of Engineered Nanomaterials. http://dx.doi.org/10.1016/B978-0-12-809199-9.00003-3
Copyright © 2017 Elsevier Inc. All rights reserved.

weighed against its risks. People have been exposed to ambient nanoscale particles, such as volcano ashes or diesel exhaust for a long time. Although this field provides some knowledge, engineered nanomaterials (ENMs) are designed with structural benefits and specific modifications that introduce totally different features, and thus should be handled accordingly (Seaton et al., 2010). Data obtained for the bulk compound cannot be directly applied to the scaled-down nanosize material (Feliu and Fadeel, 2010). Toxicity testing of ENM is not straightforward and needs special attention and consideration (Dusinska et al., 2015). Generally, chemicals are regulated under REACH (registration, evaluation, authorization, and restriction of chemicals) and nanopharmaceuticals and nanomedical products and devices under regulatory directives of EMEA in Europe, or FDA in the United States. However, ENMs may require adjustments to the legislation (Dusinska et al., 2009, 2015; Seaton et al., 2010) (and see Chapter 7 for a detailed discussion). There is also a demand for rapid and reliable ways of testing ENMs preferably using in vitro and in silico approaches. However, enormous sets of data are needed for developing and verifying intelligent testing strategies for risk assessment of ENMs, based on grouping and read-across approaches (Oomen et al., 2015). Robust and reliable data can be provided by applying high throughput methods. The adoption of high-throughput screening and high content analysis for ENM toxicity testing allows the testing of numerous ENMs at different concentrations, types of cells, and exposure conditions, reduces the effect of interexperimental variation, and makes substantial savings in time and cost (Collins et al., 2016).

In vitro and in vivo tests used to assess toxicity of chemicals and underlying mechanisms (e.g., oxidative stress, immunotoxicity, genotoxicity) can also be applied to ENMs (Dusinska et al., 2009, 2015). Some of these methods are already developed into OECD guidelines and can be used for regulatory purposes (Tables 3.1 and 3.2). However, there are methodological concerns about ENMs regarding dosimetry, dispersion, lack of washout, uptake, and interaction of ENMs with cells and tissues. It is becoming clear that for development of standardized and validated tests for ENMs, OECD-recommended or scientifically validated tests need to be adapted to fit the unique features of nanosize material, and thus implement characterization of ENM, uptake by cells and detection in biological systems. Some of these issues will be addressed and discussed later.

METHODS FOR VISUALIZING CELLULAR UPTAKE AND BIODISTRIBUTION

Since uptake through barriers (e.g., skin, blood–brain barrier (BBB), pulmonary mucosa, placenta) and toxicokinetics for a compound may change considerably with a decrease in size, toxicological data must be obtained specifically for nanosized particles (Lehr et al., 2011; Schleh et al., 2012; Simko and Mattsson, 2010). As a particulate matter, NPs can be carried by foreign body scavenging systems of the body and redistributed in the body. Macrophages, and other cell types that show phagocytic activities against NPs, are the important player as carriers as they may recognize NPs as a general foreign body.

Reactivity increases concomitantly with a decrease in size and corresponding increased surface area. Surface area and composition strongly determine reactivity, dispersion, interaction with biological environments including cellular macromolecules and thus toxicity of

TABLE 3.1 OECD Guidelines for In Vitro Tests for Chemical Compounds

Type of toxicity	In vitro test	Test guideline
Cytotoxicity (starting doses for acute oral systemic toxicity tests)	Neutral red uptake (NRU) test with human cells [normal human epidermal keratinocytes (NHK)] (NHK NRU assay) and mouse BHK 3T3 fibroblasts 3T3 NRU tests	OECD Draft Guidance 129 (2009)
Cytotoxicity	Colony forming efficiency assay (CFE) http://publications.jrc.ec.europa.eu/repository/handle/JRC92910	Validated by JRC
Phototoxicity	3T3 Neutral Red Uptake for identifying substances with acute oral LD50 > 2000 mg/kg b.w.	ECVAM validated—(2009)
	3T3 NRU Phototoxicity Test (photo-irritation); 3T3 NRU Phototoxicity Test: Application to UV Filter Chemicals	OECD TG 432 (2004) EU Test Method B.41
	Tiered testing strategy to predict phototoxicity (3T3 NRU PT and reconstructed human epidermis models)	ECVAM feasibility study completed
	Produced reactive oxygen species (ROS) and photostability study	JaCVAM
	Test method battery to predict phototoxicity (yeast growth inhibition; phototoxicity assay and red blood cell photohemolysis assay)	JaCVAM
Eye irritation	Short time exposure in vitro test method for identifying (1) chemicals inducing serious eye damage and (2) chemicals not requiring classification for eye irritation or serious eye damage	OECD 491
	Reconstructed human cornea-like epithelium (RhCE) test method for identifying chemicals not requiring classification and labeling for eye irritation or serious eye damage	OECD 492
	Performance-Based Test Guideline for Human Recombinant Estrogen Receptor (hrER) In Vitro Assays to Detect Chemicals with ER Binding Affinity	OECD 493
	Bovine corneal opacity and permeability test method for identifying (1) chemicals inducing serious eye damage and (2) chemicals not requiring classification for eye irritation or serious eye damage	OECD 437
	Isolated chicken eye test method for identifying (1) chemicals inducing serious eye damage and (2) chemicals not requiring classification for eye irritation or serious eye damage	OECD 438
	Fluorescein leakage test method for identifying ocular corrosives and severe irritants	OECD 460
Skin absorption	Skin absorption: in vitro method	OECD 428
Skin corrosion	In vitro skin corrosion: transcutaneous electrical resistance test method (TER)	OECD 430
	Test No. 431: in vitro skin corrosion: reconstructed human epidermis (RHE) test method	OECD 431
	In vitro membrane barrier test method for skin corrosion	OECD 435

(*Continued*)

TABLE 3.1 OECD Guidelines for In Vitro Tests for Chemical Compounds (*cont.*)

Type of toxicity	In vitro test	Test guideline
	In vitro skin irritation: reconstructed human epidermis test method	OECD 439
Skin sensitisation	In chemico skin sensitisation	OECD 442C
	In vitro skin sensitisation. *ARE-Nrf2 Luciferase test method*	OECD 442D
Endocrine disruption	Performance-based test guideline for stably transfected transactivation in vitro assays to detect estrogen receptor agonists and antagonists	OECD 455
	BG1Luc estrogen receptor transactivation test method for identifying estrogen receptor agonists and antagonists	OECD 457
	H295R steroidogenesis assay	OECD 456
Genotoxicity	Bacterial reverse mutation test	OECD 471
	In vitro mammalian chromosome aberration test	OECD 473
	In vitro mammalian cell gene mutation test	OECD 476
	Genetic toxicology: in vitro sister chromatid exchange assay in mammalian cells	OECD 479
	Genetic toxicology: saccharomyces cerevisiae, gene mutation assay	OECD 480
	Genetic toxicology: sacharomyces cerevisiae, mitotic recombination assay	OECD 481
	Genetic toxicology: DNA damage and repair, unscheduled DNA synthesis in mammalian cells in vitro	OECD 482
	In vitro mammalian cell MN test	OECD 487
	In vitro comet assay [single-cell gel electrophoresis (SCGE)]	JaCVAM ECVAM/ICVAM
	In vitro thymidine kinase mutation test	OECD 492
Carcinogenicity in vitro: cell transformation assay (Alternative to 451) http://www.oecd.org/dataoecd/59/29/36069919.pdf	Syrian hamster embryo (SHE) assay	OECD draft review paper (No 31); EU B.21; OECD Guidance document; Series on Testing & Assessement No. 214, May 2015
	Balb/c assay (anchorage independent grow)	OECD draft review paper (No 31)
	C3H/10T1/2 assay—Bhas42 cell system based on morphologically transformed foci	OECD Guidance document; Series on Testing & Assessment No. 231, January 8, 2016

TABLE 3.2 In Vivo OECD Guideline Tests for Chemical Compounds

Type of toxicity	In vivo test	OECD test guideline no
Toxicokinetics	ADME	417
	Skin absorption	427
Acute toxicity	Inhalation	403, 436
	Oral	401, 420, 423, 425
	Dermal	402
Sub-acute toxicity	Oral, 28 days	407
	Inhalation, 28 days	412
	Dermal, 21/28 days	410
Sub-chronic toxicity	Oral, 90-days rodents	408
	Inhalation, 90-days rodents	413
	Oral 90-days nonrodents	409
	Combined repeated dose toxicity (54 days) with reproduction/developmental toxicity screening	422
	Dermal 90 days	411
Reproduction and teratogenicity	Reproduction and developmental toxicity	414, 415, 416
	1-Generation reproduction	415
	2-Generation reproduction	416
	Prenatal	414
	Combined repeated dose toxicity with reproduction/developmental toxicity screening	422
	Reproduction/developmental toxicity screening test	421
	Extended 1-generation reproductive toxicity study	443
Neurotoxicity	Developmental neurotoxicity	426
	Rodents	424
Chronic	Carcinogenicity, 24 months	451
	Chronic toxicity, 12 months	452
	Chronic toxicity and carcinogenicity 12/24 months	453
Genotoxicity	In vivo MN	474
	In vivo mammalian alkaline comet assay	489
	Transgenic rodent somatic and germ cell gene mutation assay	488
	Mammalian erythrocyte MN test	474
	Mammalian bone marrow chromosome aberration test	475
	Mammalial spermatogonial chromosome aberration test	483
	Unscheduled DNA synthesis test with mammalian liver cells in vivo	486

(*Continued*)

TABLE 3.2 In Vivo OECD Guideline Tests for Chemical Compounds (*cont.*)

Type of toxicity	In vivo test	OECD test guideline no
	Mouse spot test	484
	Mouse heritable translocation assay	485
	Rodent dominant lethal test	478
Skin sensitization	Dermal	406, 429, 442A, 442B
Irritation/corrosion	Eye	405
	Dermal	404
Endocrine effects	Hershberger bioassay: short-term screening test for androgenic properties in rat	441
	Uterothropic bioassay: short-term screening test for estrogenic properties in rats	440

ENMs (Huk et al., 2015; Kunzmann et al., 2011; Warheit et al., 2008). Other factors including zeta-potential of the surface may also affect the reactivity (Huk et al., 2015). ENMs may be designed to deliberately overcome biological barriers (e.g., for drug delivery) or they may cross them unintentionally as a result of environmental exposure. Once ENMs have been taken up, they can potentially be deposited in any region of the body (Borm and Muller-Schulte, 2006; Oberdorster et al., 2005). Due to their small size or specific functionalization, ENMs may cross-important biological barriers like the placenta or the BBB and cause toxicity to very sensitive systems, such as the developing fetus or the brain, respectively. Knowledge from thorotrast, a contrast enhancer used during World War I, indicates that insoluble NPs of a size 3–10 nm in diameter are taken up by the reticuloendothelial system, that is, liver, spleen, and lymphnodes, and stay there almost for life.

Methods of NP detection need to be established in order to study both targeted delivery and distribution of ENM in exposed cells and tissues, as well as in the body. Detection and accurate determination of NP concentrations are particularly important for in vivo toxicity studies, as the concentration of systemically applied NPs will vary between tissues. A summary of the most commonly used NP detection methods is presented in Table 3.3 and discussed later (for a more detailed discussion on nanomaterial characterization, refer to Chapter 2).

Cellular uptake of ENMs is commonly studied using microscopy. Some relatively large particles, such as multiwalled carbon nanotubes and fullerene aggregates, made of regular repeated or crystalline-like structures may show birefringence as indicated by a pair of polarizing filters on a regular light microscope. A dark field system, which claims to achieve resolution beyond the diffraction limit, that is, less than the wave-length of the illumination light, is available.

Confocal and electron microscopy (EM) deliver very high resolution images, making them suitable tools for visualizing nanosized particles in cells or tissues. For confocal imaging, ENMs are often labeled with a fluorescent tag which allows real time tracking of particles (Seo et al., 2011) or determining their localization in tissue samples (Kuo and Chen, 2010; Thurn et al., 2011). However, bleaching or loss of fluorescence is a challenge for long-term experiments. In addition, the tag can possibly alter the behavior and the toxic potential of

TABLE 3.3 Selection of Most Commonly Used Techniques for Nanoparticle Detection In Vitro and Ex Vivo

Method	Principle	Sample	Benefits	Limitations	Example
Electron microscopy (EM)	Electron beam illuminates sample to produce a magnified, high resolution image	Sample needs to be dried and placed on support grid; if NP exposed cells/tissues are imaged specific fixing/staining is required	Very high resolution; accurate determination of nanoparticle shape and intracellular localization possible	Drying of sample may alter behavior of the particle; quantification of NP difficult	Localization of silver NP in histological samples (Garza-Ocanas et al., 2010); intracellular localization of carbon nanotubes (CNT) (Monteiro-Riviere et al., 2005), TiO_2 (Wang et al., 2010a) and CeO NP (Raemy et al., 2011)
Confocal microscopy	Point illumination and a spatial pinhole used to eliminate out-of-focus light in specimen, increasing resolution of the image	Sample in suspension; if NP exposed cells/tissues are imaged specific fixing/staining is required	Very high resolution; 3D reconstruction of specimen possible	Particles may need labeling with fluorophore; quantification of NP difficult	Intracellular localisation of fluorescently labelled TiO_2 (Thurn et al., 2011) and cationic solid lipid NP (Kuo and Chen, 2010)
Raman spectroscopy	Identification of molecular vibrations characteristic of distinct functional groups that comprise the molecules	Sample in suspension or associated with cells/tissues	Label-free NP tracking; minimal sample preparation; high sensitivity,spatial resolution	Poor penetration into body, therefore limited to ex vivo or in vivo surface analyses	Cellular uptake of gold (Shah et al., 2011) and PLGA NP (Xu et al., 2009), CNT distribution in tissues (Al Faraj et al., 2011)
Spectrophotometry	Particle concentration estimated as a measure of amount of light transmission, reflection or fluorescence intensity of a sample	Sample in suspension; standard curve to be included for concentration determination	Simple, noninvasive technique; further analysis and quantification of sample possible	Relatively low sensitivity; not for qualitative use	Accumulation of silver NP in cells (Trickler et al., 2010); transport of fluorescently labelled silica NP across BBB (Ragnaill et al., 2011)
Mass spectrometry (MS)	Particles are ionized, ions separated on basis of their mass-to-charge ratio; signal proportional to concentration	Sample in suspension	High sensitivity; qualitative and quantitative use	Most suitable for metal based NP; protocols may need adaption for nanosize material	Silver (Garza-Ocanas et al., 2010) and TiO_2 (Chen et al., 2011)NP in tissues; intracellular concentration of CeO NP (Raemy et al., 2011)

the particle itself. Electron-dense ENMs can be visualized label-free using EM techniques (Garza-Ocanas et al., 2010). EM is useful to visualize the location of particles but also to characterize their shape in tissue samples (Monteiro-Riviere et al., 2005; Raemy et al., 2011; Wang et al., 2010a). Both these microscopic methods require ex situ sample preparation and are therefore mainly of use for in vitro and ex vivo imaging. Artifacts, such as particle agglomerates can be induced through transition of particles from dispersed to dried state in sample preparation for EM. Moreover, visualization of cellular particle uptake using confocal microscopy struggles to distinguish between internalized particles and those merely adhering to the cell surface. A promising tool to overcome these issues is Raman spectroscopy, which is more often associated with determination of molecular structure and qualitative chemical analysis. When combined with optical microscopy (often referred to as Raman microscopy) this is also a very powerful tool for fast, noninvasive, and label-free study of cellular binding and uptake of NPs (Collins et al., 2016). The technique is based on the identification of molecular vibrations that are characteristic of distinct functional groups. Therefore, ENM can be detected on the basis on their intrinsic physical properties rather than relying on extrinsic labels, such as fluorescent tags (Shah et al., 2011). This direct detection method may lead to a more accurate assessment of NP behavior over both the short and long term. Xu et al. (2009) compared cellular uptake of fluorescently labeled and unlabelled poly(lactic-*co*-glycolic acid) (PLGA) NP by confocal and Raman microscopy. Analysis with confocal microscopy revealed intracellular fluorescence, which indicates particle uptake. However, coherent anti-Stokes Raman scattering (CARS) microscopy revealed that unlabeled PLGA NPs were not taken up by cells. The authors concluded that the observed intracellular fluorescence was a result of dye transfer rather than NP uptake. Similar to EM and confocal microscopy, Raman microscopy is more adapted to ex vivo investigations. Confocal and Raman microscopy can also be applied in vivo for surface investigations, but have poor tissue penetration (Collins et al., 2016). New techniques for tissue clearing may allow for a more refined observation of particle distribution in the near future (Susaki and Ueda, 2016).

For in vivo imaging of particle distribution, medical imaging techniques, such as magnetic resonance imaging (MRI) may be used (Al Faraj et al., 2011).

EVALUATION OF TRANSPORT AND UPTAKE OF ENM IN VITRO AND IN VIVO

It is important for both risk assessment and therapeutic advances to determine the mechanisms by which ENMs may cross biological barriers, and reach areas, such as the brain and fetus. Preliminary investigation of transport and uptake of ENMs can be undertaken in vitro. Cells can be grown to confluence on semipermeable membranes in specialized inserts, for example, Transwells, placed in tissue culture wells to provide access to both apical and basal compartments. Samples can be collected at time-intervals and ENM concentration determined using techniques as mentioned later. Integrity of the cell barrier can be determined by measuring marker transfer, for example, Lucifer Yellow dye or radioactive mannitol, combined with measurement of *trans*-epithelial/endothelial electrical resistance (TEER) (Saunders, 2009). Careful preparation of the ENMs to avoid agglomeration is important as this will impact on uptake and transport. In principle, any adherent cell line can be used

as a barrier model, but selection requires careful thought. Some cell lines do not form an intact monolayer (Saunders, 2009) and others have been shown not to be representative of the organ of origin (Brown et al., 2000). Elucidation of the transport mechanisms and cellular uptake can be determined by energy inhibition studies under varying metabolic conditions and use of appropriate pathway inhibitors, for example, sucrose or chlorpromazine (clathrin-dependent), filipin or nystatin (caveolin-dependent), 5-ethylisopropyl amiloride (EIPA), or cytochalasin D (macropinocytosis) in combination with imaging by confocal or EM as appropriate (Saovapakhiran et al., 2009). However, these approaches can only provide an insight into the potential mechanisms of biological interaction with ENM and should be considered as a screening approach prior to further investigation in vivo.

In vivo studies of NP biodistribution add further complexity to NP detection due to biological interactions, for example, opsonization. Sampling from the sites of interest will provide data on uptake and transport, but the time-points selected need to be appropriate to the material and organism under study. The pharmacokinetics and biodistribution of NPs within tissues will be dependent upon the NP characteristics, such as size, surface charge and physical chemistry, and the tissue concentration should be monitored for long enough to obtain the full profile (usually 3*x* half-life) (Li and Huang, 2008). Longer retention times may be necessary, and should be considered in experimental design.

Inductively coupled plasma mass spectrometry (ICP-MS) can be used to determine tissue levels of a range of metal (and some nonmetal) NPs down to nanogram per gram (Chen et al., 2011; Garza-Ocanas et al., 2010). The use of radiolabeled NPs also provide a quantitative approach to in vivo biodistribution studies. It is possible to determine the NP uptake not only in the key organs but also in the remainder of the tissue, thus enabling total mass balance calculations to be done. Dependent upon the isotope selected, further tissue processing may be required after sample removal, for example, tissue solubilization or ashing. It is also possible to undertake real-time imaging and follow the biodistribution in vivo using techniques, such as positron-emission tomography (PET), computed tomography (CT), and single photon emission computed tomography (SPECT) with appropriate labeling of NPs (Zheng et al., 2009). This is an area of great clinical interest for delivery of imaging and therapeutic agents (Pillai, 2014).

Other imaging approaches can be used for biodistribution studies (Skotland et al., 2010) but tend to be less sensitive compared with radiolabeling due to less specific target tissue uptake. Contrast agents, such as iron oxide or gadolinium NPs, and gold NPs can be imaged using MRI or X-ray, respectively and are of particular interest for tumor detection and therapy. Fluorescent contrast agents, such as quantum dots (QD) can be utilized for optical imaging with ex vivo pharmacokinetic data obtained through cryosectioning of tissues followed by fluorescence microscopy (Robe et al., 2008); however, the risk of systemic toxicity from QD administration is a concern for longer-term studies. Development of longer wavelength new generation fluorescent probes enables whole body imaging, for example, near infrared imaging (Burns et al., 2009; Tasciotti et al., 2011). Advances in ENM chemistry mean that combined labeling techniques can be used with the same material allowing increased sensitivity of detection and reduction in toxicity. Examples, include neutron activation combined with EM, fluorescence, and MRI (Vaccaro et al., 2008); MRI, PET, and fluorescence imaging (Stelter et al., 2010); multiplex Raman imaging (Wang et al., 2010b). Such advances will play a vital role in determining the precise uptake, transport, and biodistribution of NPs in vivo.

Concentrations of detected ENMs can be determined in tissue samples, cells or cell culture supernatants. ENMs are then often labeled with a fluorescent or radioactive tag, as fluorescence intensity and radioactivity are proportional to particle concentration. Fluorescence intensity of labeled particles (Ragnaill et al., 2011) or light absorption through a nonfluorescent NP suspension can be detected by spectrophotometric methods (Trickler et al., 2010). Spectrophotometry is a simple and noninvasive method to determine particle concentration, but limited in terms of sensitivity. A more sensitive method is the use of mass spectrometry (MS). With MS, particles are ionized and the ions are separated on the basis of their mass-to-charge ratio. The produced ions generate a signal, which is proportional to the concentration of the particles. MS may also be used to identify the chemical composition of an unknown sample. Although this is a widely used analytical technique, it should be borne in mind that existing protocols for MS analysis of bulk materials may need to be validated according to the specific features for NPs. Federici et al. (2007) performed calibrations with TiO_2 NP and Ti metal standards for ICP-MS. They found that TiO_2 NP solutions had a shallower slope than calibrations performed with Ti metal standards, indicating that ICP-MS responds differently to NPs compared with pure metal solutions.

Ion beam microscopy (IBM) techniques, such as microproton-induced X-ray emission (μPIXE) and micro-Rutherford backscattering (μRBS), are powerful tools for spatially resolved elemental imaging and quantitative analysis at the single-cell level. A proton beam is used for scanning the sample in the *xy*-plane at a resolution of about 1 μm. With the combination of PIXE and RBS it becomes possible to determine the concentration of cellular elements (e.g., P, S, Ca, K, Zn, and Fe), with a sensitivity in the parts per million range (Collins et al., 2016).

The techniques discussed previously and summarized in Table 3.3 are not an exhaustive list, but represent the most commonly used ENM detection methods in in vitro and in vivo nanotoxicology (and refer to Chapter 2). Choosing the most appropriate detection method depends greatly upon what question is addressed. For short-term tracking of NPs in vitro it may be most suitable to attach a fluorescent label and visualize particles with confocal microscopy or quantify samples spectrophotometrically. For long-term follow-up, however, the use of Raman spectroscopy may be more appropriate. If an unknown sample is to be analyzed, for example, for human exposure assessments, a method with both qualitative and quantitative use, like MS, would be the most suitable. Needless to say, the durability of the label in the organism should be checked for long-term studies. For particles for which it is important to monitor size and shape distribution, unfortunately, the most reliable measurement could be the most labor-intensive EM morphology based approach. Image analysis programs may facilitate the measurement.

IN VITRO TOXICITY TESTS

In vitro testing is a crucial part of an innovative testing strategy, using tiered approaches to identify ENM, which should undergo all testing steps including animal tests. An approved testing scheme would allow new regulations and test guidelines to reduce testing efforts. The existing in vitro toxicity tests have still present major obstacles toward integration, into a tiered approach. First, many studies were done with immortalized cell lines, many of them

derived from tumors. These cell lines have undergone dedifferentiation and many of them are insensitive to drugs but also to other toxic agents, such as ENM. Studies using these cell lines often require higher doses and do not reflect the biological potential of ENM properly (Kroll et al., 2011). The widespread use of cell lines for toxicity testing reflects the fact that they provide a more standardized system compared to primary cell cultures, as well as higher throughput. Primary cultures often diverge between different laboratories, and are much more susceptible to environmental changes. However, primary cultures have a normal karyotype and offer the advantage of being closer to the in vivo situation. Novel three dimensional (3D) cell culture systems and ex vivo organotypical slice cultures are very interesting in regard to toxicity testing in vitro, as cells are in an environment where they are better able to mimic their in vivo counterpart (Breslin and O'Dridcoll, 2013; Runden et al., 1998; Roy et al., 2016; Susewind et al., 2016; Walczak et al., 2015).

In vivo, cells are organized three dimensionally and very often, the function of individual cells depends on interactions of proteins and neighboring cells within this 3D organization (Susewind et al., 2016). In common cell cultures used for toxicity testing, this important level of complexity is reduced to a two dimensional (2D) culture on a flat substrate that does not exactly recapitulate the structure, function, or physiology of living tissues. Therefore, toxicity tests carried out in 2D culture may not accurately reflect in vivo cellular response. There is evidence that toxic effects of gold and cadmium telluride (CdTe) NP are reduced in 3D spheroid cultures of liver cells compared with conventional 2D liver cell culture (Lee et al., 2009). However, for existing 3D systems especially appropriate complex scaffolds for 3D culture need to be further developed to overcome certain limitations with the spatial distributions of oxygen, metabolites, and signaling molecules found in tissues (Derda et al., 2009) and for broader use in in vitro toxicity testing. So-called organ-on-a-chip microdevices represent even more sophisticated in vitro models, implementing not only structural but also mechanical features of the whole living organ. Huh et al. (2010) developed a biomimetic microsystem that represents key structural, functional, and mechanical properties of the alveolar-capillary interface of the human lung. Within this system, the authors were able to mimic the stretching of the alveolar epithelium during breathing, which proved to be a crucial factor influencing the toxic and inflammatory response of the lung to silica NPs.

The extent to which NPs are dispersed will influence the outcome of cytotoxicity testing of ENMs, and is affected by physicochemical properties of NPs, dispersion medium, temperature, ultrasound energy used for dispersion, dispersing agents, and sequence of dispersion preparation steps (Byrne et al., 2010). NP dispersion/agglomeration is a concentration dependent phenomenon (Dusinska et al., 2015), and hence agglomeration state, uptake mechanisms and toxic effects may vary nonlinearly with dose. A currently unanswered question is whether tests should be performed on dispersed ENM or under the most natural conditions with minimal processing, allowing natural formation of aggregates and agglomerates. Additionally, ENMs often interact with the cell growth medium, eliciting in itself a toxic response that may have little or no physiological relevance in vivo. Many assays need to be adapted for testing of ENMs (Dusinska et al., 2015; Guadagnini et al., 2015) and there is also a need to develop NPs that can serve as standards and as positive and negative controls for a range of relevant biological end-points (Dusinska et al., 2015). Here, we briefly discuss cytotoxicity and genotoxicity tests.

Cytotoxicity Tests

Cytotoxicity or cell viability assessment is a cornerstone in in vitro toxicity testing. Cell lines of different origins, such as intestine or lung cells can be used to predict acute toxicity by estimation of LC_{50} values (lethal concentration for 50% of the cells) and usually both 2D and 3D systems can be used to address specific endpoints. Most cytotoxicity assays are based on colorimetric assays involving optical activity of organic dyes (Guadagnini et al., 2015). In Trypan blue exclusion assay, only damaged cells take up the dye and the proportion of damaged cells can be counted. However, it should be noted that the assay detects cell membrane damage and not necessarily cell death. The lactate dehydrogenase (LDH) assay measures cellular enzyme activity as damaged cells release LDH into the supernatant. This assay also measures cell membrane damage and not necessarily total cell death. The result will be affected by cell number, and so if there is any inhibition of proliferation during exposure, the decrease in cell concentration must be taken into account to avoid a false estimate of cytotoxicity. Tetrazolium assays, such as MTT, XTT, and WST are based on enzymatic formation of formazan salts. Cellular dehydrogenases catalyze conversion of a tetrazolium salt into formazan with a resultant color change, which can be measured by spectrophotometry. As these assays occur inside cells, they are a measure of metabolic activity related to cell viability. In the Alamar Blue assay a resazurin dye (dark blue, nonfluorescent) is reduced to resorufin (pink and highly fluorescent) through a redox reaction. Fluorescence intensity corresponds with the cellular metabolic activity and is proportional to the number of living cells. Damaged and dead cells have lower innate metabolic activity and thus generate a proportionally lower signal than viable cells.

Limitations of In Vitro Tests

Recent studies suggest that standard cell-based assays designed for testing chemicals may not provide reliable data since ENMs can interfere with assay components and optical readout systems (Guadagnini et al., 2015; Kroll et al., 2009; Monteiro-Riviere et al., 2009). Due to their large surface area and high surface energy, NPs can adsorb assay reagents or reporter dyes resulting in assay outcome changes. For carbon ENMs, such as single-walled carbon nanotubes (SWCNTs) or carbon black (CB), adsorption of MTT-formazan, a reporter dye that indicates metabolic activity in MTT assays, has been described by different groups of investigators (Casey et al., 2007; Guadagnini et al., 2015; Kroll et al., 2009; Monteiro-Riviere et al., 2009; Worle-Knirsch et al., 2006). Additionally, SWCNTs seem to adsorb essential nutrients from cell culture media resulting in indirect cytotoxic effects (Guo et al., 2008). In both cases, the high adsorption capacity of carbon ENMs generates false positive data resulting in an overestimation of their toxicity. Notably, CB also seems to adsorb proinflammatory cytokines leading to false negative results in some immunoassays and, consequently, to an underestimation of its potential toxicity (Brown et al., 2010). This demonstrates ENM interference with assay systems as a cause of contradictory testing results. Verifying cytotoxicity data with at least two or more independent test systems as has been suggested earlier (Monteiro-Riviere et al., 2009) may thus not be sufficient to eliminate misleading results and incorrect interpretations.

Recent studies indicate that also metal oxide ENMs interact with assay components or reporter dyes of in vitro test systems. Ultrafine superparamagnetic iron oxide ENMs seem to

interfere with MTS-based cell viability assays (Doak et al., 2009; Guadagnini et al., 2015), a confounding factor that results in an underestimation of their potential toxicity. Moreover, superparamagnetic iron oxide ENMs can cause significant changes in the cell culture medium, such as depletion of Cl^- ions or denatureation of proteins that, in turn, can result in toxic effects on cells (Mahmoudi et al., 2010). TiO_2 and SiO_2 can cause artifacts in immunoassays by adsorbing the proinflammatory cytokine IL-6 (Guadagnini et al., 2015; Veranth et al., 2007). Additional challenges for ENM cytotoxicity screening arise from the intrinsic optical absorbance of ENM dispersions. As most of the classic in vitro toxicity assays are based on optical read-outs, the high absorption or scattering of nanoparticles may interfere with the detection method. Carbon ENMs are known to absorb light of the visible spectrum (Wells and Smith, 1941) and may therefore generate an absorbance at the same wavelength as that used to detect the colored assay product. Metal oxide ENMs frequently found in sunscreens, such as TiO_2 and ZnO absorb and scatter UV radiation, as well as visible light and may possibly influence absorbance read-outs of colorimetric assays. In fact, TiO_2 ENMs have been recently shown to change the color and increase the opalescence of experimental resin matrices (Yu et al., 2009). However, the influence of ENMs on optical detection is difficult to estimate in advance, as optical activity depends not only on the chemical composition but also on other properties, such as particle size, shape and crystallinity, as well as on the dispersion agent. A number of ENMs also seem to interact directly with fluorescent dyes thereby altering their emission intensity. Silver ENMs, for instance, have been shown to quench fluorescent dyes presumably by binding them onto their surface (Sabatini et al., 2007). Gold NPs have been found to quench DCF fluorescence used to determine ENM-induced oxidative stress (Pfaller et al., 2010). Dextran-coated iron oxide ENMs displayed interference with the fluorescence emission of DCFH. The interference was more pronounced with Fe_3O_4 than with Fe_2O_3 ENMs demonstrating that even differences in the oxidation state can impact the test results (Doak et al., 2009). Unfortunately, all colorimetric assays are prone to interference due to interaction between the dye and NPs, and washout can be difficult due to interactions (Dusinska et al., 2015; Monteiro-Riviere et al., 2009). Guadagnini et al. (2015) showed that many NP characteristics (composition, size, coatings, and agglomeration) interfere with a range of in vitro cytotoxicity assays (WST-1, MTT, LDH, neutral red, propidium iodide, 3H-thymidine incorporation, and cell counting), proinflammatory response evaluation (ELISA for GM-CSF, IL-6, and IL-8), and oxidative stress detection (monoBromoBimane, dichlorofluorescein, and NO assays). Interferences were both assay, as well as NP specific. The authors proposed ways to avoid the interference with testing systems and suggested integrating testing for interference into a screening strategy for testing NPs toxicity.

Thus, many standard cytotoxicity assays have to be further developed, validated for each NP tested and restricted to ENM concentrations below interfering levels. Assay protocols have to be validated by a series of control experiments for each single NP variant to obtain reliable NP toxicity data (Kroll et al., 2012).

New In Vitro Test Principles

Since ENM interference with in vitro test systems potentially creates read-out artefacts independent of the particle hazard potential, there is a crucial need for new in vitro assays using test principles, which reduce ENM interference. Especially ENMs with no or low toxicity potential

can often not be tested up to the regulatory relevant LOEL concentration levels due to test interference at higher concentrations. Many in vitro assays involve the detection of light, which is disturbed by many types of NPs. ENM toxicity testing should therefore ideally be performed using test systems that cannot be influenced by nanospecific properties. The EU-funded project FP7-MARINA has used digital holographic microscopy (DHM) as a nonintensity-based and label-free tool to analyze parameters, such as refractive index, which can be correlated to the protein content, cell thickness and dry mass. Furthermore, this technique can monitor the development of cellular dry mass over time (www.marina-fp7.eu). In DHM, the sample is illuminated by a light wave with a certain phase. By passing through the cell, this phase changes due to the density and therefore the refractive index. In detail, the light passes dense parts of the cell more slowly than less dense ones. The resulting phase shift $\Delta\phi$ is detected by interfering this object wave with a reference wave that did not pass the sample. Fourier transformation calculates the quantitative phase image and the optical path length changes are determined for further calculations of cell specific parameters. A second indicator for cytotoxicity is cell viability. Therefore, DHM was used to determine the cell area as a measure of total cell count and cell proliferation. RAW 264.7 macrophages were exposed to different concentrations of silver nanoparticles (NM 300) and cell area development was monitored for at least 24 h, by taking a hologram every 3 min. The effect on NM 300 leads to a decreased occupied surface area and dry mass development over time. These data show the same tendency as those determined by a WST-8 assay, but give a more detailed insight on the development over time. ENMs do not interfere with the method and higher concentrations of ENMs might be applied.

DHM and other new technologies have the potential to be integrated in high-throughput platforms and to play a crucial role in the application of in vitro testing systems in a tiered toxicity testing approach (Mues et al., 2016; Rommel et al., 2014).

Validated Cytotoxicity Tests

Existing OECD guidelines for in vitro tests (Table 3.1) have limitations with respect to assessment of biological hazards of ENMs and do not include characterization. Several OECD guidelines have been developed for the Neutral Red assay based on neutral red uptake. Neutral red dye is weakly cationic and can diffuse across the membrane and accumulate in cellular lysosomes. Increased cell permeability and lysosome fragility are associated with the latter stages of cell death; thus, the level of cellular dye is indicative of the level of viability. This assay is more sensitive than other cytotoxicity tests and dye uptake is readily measured using a plate reader. However, as internalized NPs can be engulfed in lysosomes, increased lysosomal activity does not always reflect decreased cell viability. It is important to note that different endpoints represent distinct metabolic (dys)functions. Thus, assays can differ significantly in terms of their sensitivity.

One of the most reliable assays for cytotoxicity testing of ENMs to be used in both 2D and 3D systems appear to be the proliferation assay and clonogenic or colony forming efficiency assay (CFE) (adapted from OECD test guideline 476) that has been recently validated for ENMs (Ponti et al., 2014). In the CFE assay, single cells are seeded at low density, and colonies (with minimum 50 cells) are counted at fixed time points after exposure. Thus, both viability (colony number) and proliferative capacity (colony size) are measured in this assay (Ponti et al., 2014) and the assay is used to determine LC_{50}.

Genotoxicity Tests

Genotoxicity is a key parameter in toxicity testing of ENMs. The assays for genotoxicity testing have different endpoints, such as single- and double-strand breaks, point mutations, deletions, chromosomal aberrations, micronuclei formation, DNA repair, and cell-cycle interactions (Magdolenova et al., 2014). These endpoints are related to genotoxicity in vitro (human and mammalian cells), as well as in vivo (population biomonitoring and animal studies). Use of the same endpoints in various biological systems in different species enables comparison of interspecies effects. In vitro genotoxicity assays represent simple, robust and time- and cost-effective testing of targeted toxicity and underlying mechanisms. The in vitro approach is appropriate for testing primary genotoxicity, while in vivo models can also give information on secondary genotoxicity, for example, via inflammation. Better selection of appropriate cells can be based upon identified target organs in in vivo studies or by using novel in vitro techniques (such as 3D coculture of different cell types), simulating in vivo conditions.

The most commonly applied methods for detecting genotoxicity of bulk chemicals include the bacterial Ames test, DNA strand break measurements in cells (e.g., comet assay, alkaline unwinding and hydroxyapatite chromatography, alkaline elution), and cytogenetic assays [micronucleus (MN) and chromosomal aberration assays, including the use of fluorescence in situ hybridization and chromosome painting]. Most of them can be applied also for testing ENMs or can be adopted but some of them, such as the Ames test are not recommended, as discussed further.

The Ames test (bacterial reverse mutation assay) (Mortelmans and Zeiger, 2000) is the most commonly used genotoxicity assay in regulatory toxicology. It is based on induction of reverse mutation in the histidine gene, which enables the bacteria to synthesize histidine and form visible colonies in minimal histidine medium. There are a number of concerns regarding the suitability of the Ames test for NP testing due to the bacterial cell wall composition and the fact that larger NPs cannot cross the cell membrane. It is also unknown how ENMs could possibly interfere with histidine synthesis and induce false negative (down regulation) or positive (up regulation) results. In spite of serious limitations, the Ames test has been used to assess genotoxicity of NPs (Magdolenova et al., 2014), such as C60 NPs (Shinohara et al., 2009); MWCNT (Di Sotto et al., 2009), aluminum oxide nanomaterials (Balasubramanyam et al., 2010), iron–platinum FePt NPs (Maenosono et al., 2009), and ZnO NPs (Yoshida et al., 2009) and has so far given largely negative results for ENMs (Magdolenova et al., 2014). The regulatory committees thus, do not accept genotoxicity results obtained with the Ames test.

The comet assay or single-cell gel electrophoresis is one of the most common tests for exploring the genotoxic potential of NPs. It detects DNA damage at the level of a single cell. DNA breaks cause relaxation of supercoiled DNA, which then extends to form comet-like tails under electrophoresis. The relative intensity of DNA in the tail can be quantified and indicates the DNA break frequency. This method represents a rapid, sensitive, reliable, robust, and relatively inexpensive way to study DNA damage (including DNA oxidation), and repair in different cell types both in vitro, as well as in vivo. DNA damage should be assessed at concentrations that are not cytotoxic; a threshold of 80% viability is recommended (Cowie et al., 2015; Huk et al., 2014, 2015). The assay has been validated by ECVAM/JaCVAM, and a guideline protocol for regulatory purposes was approved for in vivo testing (OECD, 2014). A high-throughput version was suggested for ENM testing (Collins et al., 2016). Specific DNA lesions can be analyzed by incubation of agarose-embedded nuclei with lesion-specific DNA

repair enzymes, such as bacterial endonuclease III, (Endo III), or formamidopyrimidine DNA glycosylase (Fpg). Fpg recognizes oxidized purine bases, and thus detects oxidative damage to DNA (Collins et al., 1996; OECD, 2014). The comet assay modified with Fpg or Endo III is useful to detect induction of oxidative damage by ENMs, which would otherwise be missed when only the standard alkaline version is used. Additionally, photogenotoxic effects of NPs can be measured in combination with ultraviolet radiation (Dusinska and Collins, 1996). Any cell type with a nucleus can be used, and thus the comet assay can assess both cell- and tissue-specific DNA damage induced by NPs. Responses to ENMs can vary depending on the cell type selected for testing (Cowie et al., 2015). Both short- and long-term exposure should always be performed as different mechanisms can be involved in DNA damage induction (Cowie et al., 2015; Huk et al., 2014, 2015; Magdolenova et al., 2015). The use of a high-throughput version of the comet assay to measure DNA damage increases robustness and reduces experimental variation (Collins et al., 2016; Harris et al., 2015). The interference reported by some authors seems not to be significant especially when appropriate controls are included (Karlsson et al., 2015; Magdolenova et al., 2012).

The H2AX assay is an assay for double strand breaks based on the fact that a cellular response to these breaks is the phosphorylation of one of the core nucleosomal histones, H2AX, has been also used for testing genotoxicity of ENMs. The phosphorylated form is known as γ-H2AX, and the concentration in the proximity of a double strand break is sufficient to form a focus, visible by immunohistochemistry using a fluorescence-tagged antibody to the phosphorylated form. Alternatively, γ-H2AX can be measured by flow cytometry. The γ-H2AX assay is also suitable for automated procedures (Collins et al., 2016).

Mammalian gene mutation assays are suitable for testing ENMs. They detect a range of mutations from point gene mutations to small and large deletions. The most commonly tests are tests using the *HPRT* or *TK*+/− locus, such as the mouse lymphoma assay which detects deletions with higher efficiency compared with the *HPRT* gene mutation test. HPRT is a purine salvage enzyme, which phosphorylates "waste" purines and adds them to the cellular DNA precursor nucleotide pool. The *HPRT* gene is X-linked, with only one active copy per cell, so that a mutation in only one allele is needed for phenotypic expression (Magdolenova et al., 2014). Recently, the in vitro Pig-a (phosphatidylinositol glycan anchor biosynthesis, class A) gene mutation assay was suggested as an additional in vitro mammalian mutagenicity test (Krüger et al., 2015).

The MN assay can be applied both in vitro and in vivo and has been recommended for assessing mutagenic and clastogenic effects of pollutants (OECD, 1997). The MN assay can easily be applied in different target cells and tissues (Laingam et al., 2008; OECD, 1997) and has been used also in nanotoxicology (Fenech et al., 2011; Magdolenova et al., 2014). Micronuclei are formed during anaphase from chromosomal fragments or whole chromosomes that are left behind when the nucleus divides. After telophase these fragments may not be included in the nuclei of daughter cells but instead form single or multiple micronuclei in the cytoplasm (OECD, 1997). The MN test detects both clastogenic and aneugenic effects (OECD, 1997) and is suitable for detection of genotoxicity of a wide range of compounds, including NPs. Formation of nucleoplasmic bridges and binucleated cells provides a complementary detection of chromosome rearrangement. Increased assay sensitivity can be obtained with cytochalasin-B, a cytokinesis-blocking agent that inhibits cell division, and differential staining methods (e.g., use of fluorescent acridine orange). This method is now routinely used for measuring chromosome breakage, impaired DNA repair, chromosome loss, nondisjunction, necrosis,

apoptosis, and cytostasis (Fenech et al., 2011). The MN assay is easy to score and has advantages over other cytogenetic methods, such as sister chromatid exchanges, or chromosome aberrations, being less time consuming. However, the protocol for the MN assay needed modification. As cytochalasin B inhibits endocytosis and may thus prevent uptake of NPs, the cells need to be exposed to ENMs several hours before adding cytochalasin B (Dusinska et al., 2015; Gonzalez et al., 2011; Magdolenova et al., 2012). Additionally, 24 h treatment is necessary to cover at least 1–1.5 cell cycles, as some compounds including NPs might be active only at a specific cell cycle stage and also access to nuclear DNA will be facilitated by the absence of the nuclear membrane during mitosis (Dusinska et al., 2015). In order to reduce the risk of falsely identifying ENM aggregates as MN fragments, histological staining of positively identified nucleic acids can be performed.

The chromosomal aberration test is often applied in mammalian systems, both in vitro and in vivo, and has been used also for testing of genotoxicity of ENMs (Galloway et al., 1987). The in vitro chromosomal aberration test identifies agents that cause structural chromosomal or chromatid breaks in cultured mammalian cell lines or primary cultures (Galloway et al., 1987). Chromosomal aberrations are the cause of many human genetic diseases, and there is substantial evidence that chromosomal damage and related events causing alterations in oncogenes and tumor suppressor genes of somatic cells are involved in cancer induction in humans and experimental animals. Cell cultures are exposed to the test substance both with and without metabolic activation and are treated with a metaphase-arresting substance (e.g., colcemid or colchicine) before harvest. Metaphase cells are analyzed microscopically for presence of chromosomal aberrations.

Cell transformation assays (CTA). The most frequently used endpoint for cell transformation is morphological transformation of mammalian cells in culture. Morphologically transformed cells are characterized by the loss of density-dependent regulation of growth and the formation of colonies with criss-crossed cells or foci of piled-up cells that are not observed in untreated controls (Sasaki et al., 2014). Recently, CTAs have been used to test NMs (and larger particles and fibers) (Demir et al., 2015; Ponti et al., 2009; Sasaki et al., 2014; Stoccoro et al., 2016; Uboldi et al., 2016). CTAs are in vitro tests measuring the conversion from normal to transformed phenotype of mammalian cells (primary Syrian hamster embryo (SHE) cells, or stable cell lines, such as mouse BALB/c3T3 or C3H/10T1/2) exposed to test chemicals. CTAs based on SHE and BALB/c3T3 cells have the potential to detect nongenotoxic, as well as genotoxic carcinogens. The SHE cells are normal diploid, metabolically and p53-competent primary cells, which retain the ability to biotransform a wide range of xenobiotics. Bhas42, derived from BALB/c3T3 clone transfected with v-Ha-ras are suitable for high throughput modification. OECD Guidance documents on both the in vitro SHE, as well as Bhas42 cell transformation assay have been approved (OECD, 2015, 2016).

Recent reviews on nanogenotoxicity (Magdolenova et al., 2014; Singh et al., 2009) show that the most frequently used genotoxicity test is the comet assay, followed by the MN test, chromosome aberrations and the Ames test. Some of these assays have been applied in both 2D and 3D culture systems. However, the results from the Ames test were almost always negative, indicating that the method may not be suitable for ENM testing (Magdolenova et al., 2014). Overall, the most promising assays for nanogenotoxicology testing in vitro seem to be the MN, comet and mammalian gene mutation assays, covering all genotoxicity endpoints. The CTAs are promising to be included into a battery of in vitro tests as they can assess several steps of carcinogenesis, such as initiation and promotion (Sasaki et al., 2014).

For hazard assessment of nanogenotoxicity in human/mammalian systems, a range of cell types from different organs is recommended unless target organs for the specific ENM have been identified. In vitro assays should incorporate appropriate standards and controls (dispersion controls, positive ENM relevant controls, nearest equivalent bulk chemical for bench marking). Current experiments have often used high microgram or milligram concentrations of ENMs and acute exposure instead of long term or chronic, perhaps of little relevance in risk assessment given the lower microgram or nanogram circulating levels of ENMs more likely to occur in vivo.

For regulatory genotoxicity/mutagenicity testing of chemicals, all relevant genotoxicity endpoints should be included covering mutagenicity at the gene level, structural chromosomal breakage (clastogenicity) and numerical chromosomal aberrations (aneuploidy). In this case, that one of the tests is positive, the compound is potentially genotoxic, and is also likely to be carcinogenic. Genotoxicity in vitro should be confirmed in vivo (often applying the MN assay and DNA repair assays to liver samples). However, in vivo testing is not always possible for ethical or economical reasons or time constraints. Also several regulatory committees, such as the scientific committee for consumer safety (SCCS) no longer accept in vivo studies for the risk of ENMs in cosmetic products and so alternative (in vitro and in some cases in silico) approaches must be used.

The tiered approach and the associated technical difficulties with ENMs have been recently reviewed (Dusinska et al., 2015; Karlsson et al., 2015). It is becoming clear that testing of ENMs will have to be revised and an additional tier will be needed to incorporate information on particle characterization, dispersion and uptake, and to determine whether or not the nano form can be demonstrated to have a different hazard from the equivalent bulk chemical. The use of dispersion controls and an appropriate bulk powder or mineral salt control should also be incorporated in the test methods.

Ex Vivo Models

Ex vivo, tissue cultures represent a model between in vitro and in vivo (Clift et al., 2011), where whole tissue slices are cultured, such as organotypical slice cultures (Runden et al., 1998). In these ex vivo models based on tissue slices, the cytoarchitecture is retained, as well as many of the intercellular connections and interplays. Thus, metabolic processes more closely represent the in vivo situation. Studies can also be performed ex vivo with blood samples and cultivation of lymphocytes. Ex vivo tests can be applied, for instance, for testing of ocular corrosives on bovine corneas from slaughtered animals (OECD test guideline 437). Use of coculture systems enduring different cell types is also a possibility for enhanced resemblance of the in vivo approach. These culture systems can be constructed to resemble barriers and used to investigate mechanisms of NP uptake (see above).

IN VIVO TOXICITY TESTING

Interactions of ENMs with macromolecules and other biological components can only be explored properly in in vivo models, making in vivo testing a prerequisite for toxicological risk assessment for ENMs (Clift et al., 2011; EFSA Scientific committee, 2011).

Toxicokinetics

Toxicokinetic studies are important not least in regard to the differences in toxicokinetics between ENMs and the bulk compound. Pharmacokinetic studies exploring absorption, distribution, metabolism, and excretion (ADME) (OECD guideline 471) of NPs are needed as a basis to determine toxicity and perform risk assessments, as well as for designing ENMs for diagnostics and therapy. Only kinetics studies in vivo can provide data about concentrations in target organs and cells, uptake into blood and distribution in the body. The concentration at the target cells is dependent on metabolism and elimination. ENMs that are not easily excreted will accumulate in the body, and this needs to be tested in vivo with repeated administration of ENM, for example, in subchronic 90 day study in rats (Table 3.2). Toxicokinetic studies should preferably be carried out in the same species as used for other toxicological experiments with the ENM, most commonly rat.

In reality, most ENMs are still very difficult to identify and measure in organs and tissues. Before determining the toxicokinetics and ADME of the applied ENMs, there is very limited, or almost no information on what kind of toxicity would be induced at what target organ/tissue by the ENMs. As mentioned previously, the only exception is MWCNT, tested for its mesotheliomagenic potency due to its similarity to asbestos in characteristic shape and size distribution.

In order to identify the toxicity of ENMs for which no biological information exists, classical toxicity testing using a proper route of exposure is the default approach. Oral, dermal, and inhalation are the routes of exposure to be considered for ENMs to enter the human body. The duration of the toxicity testing is another important factor for the identification of its toxicity. Most ENMs are chemically and physiologically stable, and therefore mostly biopersistent, and virtually insoluble in biological hydrophilic cytosolic and extracellular fluids, and also insoluble in hydrophobic components of the cells, such as fatty acids, cell membrane lipids, and organelle membrane lipids. Insoluble particles tend to show minimal acute toxicity. The major problem, which is historically proven for some industrial materials in the past that ended up being banned, is that some of those materials show significant chronic toxicity. MWCNT has been shown to induce mesotheliomas and lung adenocarcinoma in rats and mice in 2-years or 1.5-years carcinogenesis studies. This result was predicted by the fact that intraperitoneal injection models, used in the past for asbestos-alternative man-made fibers has identified similar properties in MWCNTs sharing the same size and shape distribution. In general, aspects of chronic toxicity including carcinogenic endpoints are detected by long-term bioassay, up to 2 years in rats and at least 1.5 years in mice. Noncarcinogenic toxicity may also become overt in long-term studies up to 2 years.

Oral route toxicity can be tested by gavage or dietary administration. The absorption of ENMs is expected to be, in general, low. ENMs with a size small enough to pass through gap junctions or tight junctions of the epithelial lining of the gastrointestinal tract mucosa may be absorbed. For ENMs subject to pinocytosis this may also be the case. Another postulated mechanism is via dendritic cells in Peyer's patches. Dendritic cells are known to show phagocytic activity and to carry the particles out to the local lymphatic system and eventually the systemic circulation.

The dermal route of entry is either direct penetration of epidermal layer/epidermal basement membrane or phagocytosis by dermal Langherhans cells to local lymph nodes. Abrasion

of the skin is known to have no effect on penetration of titanium dioxide in rat (Sagawa et al., 2012).

Inhalation is the major and most important route of exposure when testing ENMs for human safety. Whole body inhalation, nose-only inhalation and intratracheal instillation methods as surrogates for inhalation are performed on rats and mice.

Whole body inhalation is considered as the gold standard for ENMs. However, facilities are limited, and the amount of test sample needed for a long-term study can be very large, only feasible for those already in mass production. Even for shorter studies, the amount can be too high in the case of newly produced ENMs. Nose-only inhalation apparatus could be used with lesser amounts of ENMs. Recently reported small-scale whole-body inhalation systems (Taquann system, etc.) may facilitate faster whole body inhalation studies (Taquahashi et al., 2013). Standard methods for generation of aerosols are proposed and in use (ISO). It is experienced that the large aggregates/agglomerates are trapped by the upper respiratory tract and relatively small particles in the aerosol reach the peripheral region of the lower respiratory system. Even so, aerosols with larger aggregates/agglomerates tend to induce bronchiolar lesions.

Intratracheal instillation methods are reportedly easier and less expensive methods that supplement inhalation methods. This method differs from inhalation in two ways. First, the sample bypasses the size-dependent filtering function of the upper respiratory system, that is, nasal cavity, trachea, and bronchi. Therefore, larger components in the sample, which would have been filtered out by the upper respiratory tract reach the peripheral part of the lung, such as bronchiole and alveoli, inducing lesions that cannot be seen in the inhalation studies. Second, the medium of the suspension reaches the periphery of the lung and induces specific responses, at least in the acute phase before it is totally absorbed by the lung tissue. In reality, monitored by bronchoalveolar lavage (BAL), intratracheal instillation induces an acute response that is much stronger than that induced by inhalation and such reactions not seen by the inhalation assay can last for months (Morimoto et al., 2016). Therefore, the acute phase pathology is, in general, very different in intratracheal instillation studies compared with inhalation studies (Morimoto et al., 2016). An important factor in reporting intratracheal instillation studies is the status of aggregation/agglomeration of the suspension, as shown by light microscopy immediately preceding instillation into the trachea, and immediately after the treatment of the last animal (Morimoto et al., 2016).

Toxicity is generally dose-dependent, although some substances induce nonthreshold low dose effects (e.g., genotoxic/carcinogenic compounds including asbestos-like particles). Ideally, at least the highest dose group should be high enough for detection of ENMs in blood, plasma, or excreta, and it is important to investigate retention in gut or lung epithelium in addition to distribution to secondary target organs or tissues. However, as the methods for measurement in tissues and organs are not always available, it is advisable to avoid unrealistically high doses, that is, overload doses in inhalation and dose levels that clog the bronchioles in IT studies.

A minimum of two doses with four animals per group should be tested in an ADME study. Administration route and doses should be selected based upon relevant human exposure to the EMNs. For investigations of accumulation potential in the body, both in vivo repeated dose, 28 or 90 day toxicity studies in rodents could be performed (Table 3.2). As a minimum, the percentage of applied dose in tissues (lung burden) should be measured at the end of

the study. Rate and extent of excretion, and thus potential for accumulation, should be determined by measuring the percentage of the administered dose recovered in urine, feces and expired air (OECD guideline 417). Demonstrated toxicity in vitro is only relevant if it is verified that the cells represent a target organ for the NP, and at realistic concentrations (OECD, 1997). Toxicokinetics can also to some extent be modeled and provide a basis for extrapolation across species, exposure routes, dosimetry, and human risk assessment (OECD guideline 417).

Metabolism is not fully accounted for in cell lines or cell cultures, and information should be attained from toxicokinetic studies in vivo by use of radiolabeled test substance for mass balance and metabolite identification (OECD guideline 417). Some information can be obtained from in vitro studies in, for example, microsomal fractions of the target tissue or S9 mitochondrial fraction. ENMs can be subjected to metabolic changes and thus encounter new features and mechanisms of actions, as well as changed distribution pattern. Though some metabolism will also be present in in vitro systems, it can be fully explored only in animal models. However, animals differ in metabolism from humans and thus extrapolation from animal models to humans could also be questioned.

In inhalation studies with particulate matter, determining dose is not a simple matter. In the case of inhalation studies with gaseous substances, it is assumed that the concentration of the gas in the air is the "dose" to which the animals in the inhalation chamber are exposed. The concentration of the gas in the body reaches an equilibrium state within a relatively short time compared to the duration of inhalation, normally 6 h a day. Therefore, the "area under the curve" (AUC) of the concentration of gaseous test chemical during exposure is close to rectangular in shape when drawn in a "dose *x* time" graph. This AUC is, in general, considered to be proportional to the toxicity in the lung and other target organs. On the other hand, in the case of particulate matter including insoluble/biopersistent ENMs, the effect of the test substances (TS) is considered to be in proportion to the amount of TS present in the lung, or the lung burden of the TS. Even if the concentration of the TS in the air is constant, the lung burden increases in proportion to not only the concentration however, the duration of exposure. The AUC (as a "dose *x* time" graph) in this case is triangular in shape. The steepness of the slope of the AUC graph is considered to be a complex, or polynomial function, made of at least two factors, such as deposition rate and clearance rate. Both rates are relative to the anatomy of the lung and the size of the particle and its agglomerate/aggregate. It is known that particles floating and flowing in the air stream can pass through a certain size of hole. The size is not the same as the diameter of the particles but follows aerodynamic principles, named "aerodynamic diameter (AD)." Depending on the AD, the place where TS are trapped in the respiratory tract (which becomes narrower toward the distal part) can be determined. Each place in the respiratory system has a different clearance rate. The TS with large AD will be trapped in the nasal cavity and go down the GI tract instead of the respiratory tract. Those with smaller AD will be trapped by the trachea, bronchi, or bronchiole, where ciliate epithelial cells are waiting to carry the trapped particles toward the larynx and eventually to the GI tract through the esophagus. Particles with AD around 1 μm reach the alveolar region of the lung, where the clearance mechanisms are maintained by alveolar macrophages and other less known systems, such as lymphatic drainage and penetration to the pleural cavity. At each level, the high rate of deposition can overload the clearance mechanisms. In the acute phase, the airway can be clogged by the deposition. When local tissue reactions are induced by

the deposition, the pathology gets complicated and progressive. Inflammation in bronchiolar area may induce obstructive pneumonia. Early on, a foreign body reaction may be initiated, giving rise to the formation of foreign body granulomas with Langerhans giant cells. In the alveolar area, various responses can be seen according to the dose and the type of TS; they include pulmonary edema, fibrotic thickening of the alveolar septum, and type II cell hyperplasia. The kinetics of the TS exposure is therefore also a function of the histological reaction, which can further slow down the clearance by trapping the TS inside the fibrotic or granulomatous lesion. The BAL cytology can reflect the pathology, taking place inside the lung with high sensitivity. It is most valid at an early stage where all the recruited inflammatory cells are freely floating. Once the parenchymal reaction takes place, the BAL will no longer reflect the picture of lung lesion. It has been argued whether histology can always replace BAL cytology. Measurement of proteins, cytokines, and other soluble factors in the BAL cannot be replaced. This argument plays a role in setting up the feasibility study for OECD inhalation protocol(s). The shape of the AUC graph aforementioned can help in devising the dosing regimen of an ENM inhalation study. Daily exposure to a constant concentration of ENMs results in a triangular AUC graph. In order to match with the rectangular AUC of gaseous inhalation studies, a relatively high concentration can be given daily at the beginning to increase the lung burden to a planned level and lower concentrations given thereafter at longer intervals simply to maintain the lung burden by compensating for the loss by the clearance mechanisms.

Immunological Responses

Another aspect that should be addressed in vivo is immunological responses. Many of the serum proteins that interact with ENMs, such as complement and immunoglobulins, are immunoactive (Kunzmann et al., 2011). In addition, many target organs, as spleen, lymph node, and bone marrow, contain large amounts of macrophages, which are part of the mononuclear phagocyte system (or reticuloendothelial system) together with monocytes. Many ENMs are shown to activate this system, and it is important to investigate a possible activation of the immune system in its entirety in in vivo studies (Dusinska et al., 2015). However, some aspects of immunotoxicity can also be addressed in in vitro models.

In terms of particulate matter toxicity, the immunological reaction of ENMs, particularly in the chronic phase, can be considered as a blend of innate immune and acquired immune (including self-tolerance) responses influenced by or influencing the apoptotic signaling of alveolar epithelial cells. In Chapters 9 and 10, animal studies focusing on immunological parameters and inflammation following pulmonary exposure to ENMs are discussed.

Chronic Toxicity and Carcinogenicity

The need to perform in vivo chronic toxicity and carcinogenicity studies should be considered on the basis of results from in vivo ADME and in vitro genotoxicity studies (see aforementioned). However, as aforementioned, in practice, ADME data are often unavailable at the time when the toxicity results are needed. Short-term toxicity studies are less effective to predict the chronic toxicity of ENMs, as most are insoluble and biopersistent in nature. The dilemma cannot easily be addressed by the current series of in vitro studies and short-term in vivo studies. The definition of genotoxicity for particulate matter is another issue to

be carefully handled in the future. Compared with soluble chemicals, in vitro genotoxicity studies of ENMs are sensitive to the mode of interaction of the cells with the particles, that is, touching the surface membrane, penetrating the cell cytoplasm, being phagocytized, internalized by pinocytosis, and so on. The endpoint of asbestos-like needle shaped particles is often clastogenesis, considered to be a result of interference with mitotic processes. The biocorona effect (see Chapter 1) is another issue to consider for such tests, as a part of the general problem with any in vitro studies.

If there is evidence of toxic effects and/or accumulation in organs or tissues, chronic toxicity testing may be appropriate in order to reveal progressive or delayed toxicity with repeated exposure. Data from these types of study are commonly used for determination of no observable adverse effect level (NOAEL) or no observable effect level (NOEL) and lowest observable adverse effect level/lowest observable effect level (LOAEL/LOEL) used in risk assessment (EFSA Scientific committee, 2011). If the EMNs are shown to cross the placenta in ADME studies, testing for reproductive and developmental toxicity should be considered in the light of existing toxicity data (Ema et al., 2010). Positive results from in vitro genotoxicity tests normally require further testing in vivo, unless it can be demonstrated that the positive in vitro findings are not relevant for the in vivo situation. However, the results should be assessed and evaluated together with other relevant information on the substance, such as chemical reactivity, bioavailability, metabolism, toxicokinetics, and target organ specificity. If in vivo genotoxicity and carcinogenicity testing is considered necessary, the tests should be designed according to appropriate target organs or tissues, as well as in vitro genotoxicity endpoints. There are several possible in vivo tests for genotoxicity (see Table 3.2 for OECD guidelines), and the type of test should be carefully selected (EFSA Scientific committee, 2011).

CONSIDERATIONS FOR SELECTION OF TOXICITY TESTS: IN VITRO/IN VIVO COMPARISON

There are advantages and disadvantages of both in vitro and in vivo studies. Mechanistically investigations can better be explored in cell culture systems, which can be more easily controlled and yield more reproducible factors than in vivo systems. Although high-throughput analysis for nanotoxicology can best be performed in monolayer or suspension cultures, information obtained on interactions between NPs and tissues and organs are limited. Such high-throughput toxicity screening models for ENMs could be useful for selection of ENMs for further testing, including animal testing. OECD guidelines for testing of chemicals are generally used in regulatory safety testing and chemical registration. When applying established test methods for ENMs one should keep in mind that these methods may need methodological modifications to be applied in a reliable manner to testing of ENMs (Dusinska et al., 2015; Guadagnini et al., 2015; Magdolenova et al., 2014; Monteiro-Riviere et al., 2009; Oomen et al., 2015).

One of the most important modifications is a consequence of the need to define the lung burden of the tested animals at the end of exposure, and preferably with additional data on clearance rates. Recent OECD activity includes planning of a feasibility test protocol for the short-term toxicity inhalation assay. As the inhalation chambers available worldwide are

designed for gaseous chemicals, there is a strict limitation in numbers of animals per chamber that can be exposed to a certain concentration at the same time. Compared with the gaseous chemical studies, as mentioned earlier, the inhalation of PM including ENMs has to monitor the lung burden in addition to the concentration of the aerosol in the chamber. This means that lung tissue or lung lobe or lung as a whole is used for such measurement and the same sample cannot be used for other investigation, such as histology slide preparation. Currently, there are multiple proposals as to how to use animals; how many animals per group, for what measurement, which side of the lung, or which lobe of a lung is to be used for lung burden, histology, or BAL. The idea of using the lung from one animal for more than one measurement raises the question of a decrease in precision of measurement and diagnosis by the smaller amount of lung tissue available for monitoring. As mentioned earlier, a trade-off between histology and BAL analysis is suggested. In the chronic phase, BAL is typically considered to have less value over a histopathological diagnosis. The OECD effort on this matter is in progress (https://www.oecd.org/env/ehs/testing/TG%20413%20Revised%2016-OCT-2015.pdf).

ENMs could induce toxic effects, which are not caught by the endpoints under the current protocols (Guadagnini et al., 2015). There might also be a need for additional endpoints, as cardiovascular, immunological, and neurological (Walczak et al., 2015). The fact that NPs can interact with the endpoints of test assays, especially those based upon detection of light, addresses the need of careful analysis of these interactions, as well as the benefit of applying different test systems to validate the findings (Dusinska et al., 2015; Guadagnini et al., 2015). NPs could also interact with components in the test system, such as absorption to the wall of cell culture flasks or drinking vessels in oral in vivo studies, and consequently the dose will be considerably lower than expected. Thus, measurements of actual concentration of NPs in media, as well as inside the cells should be performed. Similarly, in in vivo experiments it is important to measure the amount taken up by the gastrointestinal tract, as well as blood concentration and concentration in target organs. Attention should be given toward comprehensive characterization, both for in vivo experiments and in vitro studies (Dusinska et al., 2015; Guadagnini et al., 2015). Stability and physicochemical characteristics of the ENM should be determined after synthesis but also in the applied vehicle (Dusinska et al., 2009, 2015; Guadagnini et al., 2015; Sayes and Warheit, 2009). This is a prerequisite for appropriate testing in vivo, as well as in vitro (Sayes and Warheit, 2009). Currently, there are no fully validated methods for testing of ENMs, and it can be difficult to draw conclusions across different in vitro and in vivo studies due to discrepancies in experimental conditions and lack of test standardization (Dusinska et al., 2015; Khlebtsov and Dykman, 2011; Sayes et al., 2007). An example of a change in physicochemical features when shrinking a compound to nanosize, is given by TiO_2, which appears in sunscreen formulations for UV protection. Nanosized TiO_2 on the other hand, makes the formulation transparent and less thick, and thus more convenient for the consumer (Warheit et al., 2008).

Data from in vivo studies with bulk compound should always be taken into consideration in view of the need for long-term in vivo studies. The scientific testing strategy should always be in accordance with the 3 R's—refine, replace, and reduce—for animal experiments. One should always use as few animals as possible, and refine the methods to minimize the suffering of the animals involved in the study. The replacement of in vivo studies with in vitro

studies in cell cultures should always be considered. Use of human blood is also an alternative. An intermediate strategy would be to apply coculture systems, 3D cell cultures systems or ex vivo tissue slice cultures (Clift et al., 2011). Nonetheless, in vivo studies have to be performed at least for ADME, interactions with cells, tissues, and proteins, hormonal effects and long-term chronic effects.

The size-specific properties of ENMs introduce a need for validated screening tests for toxicity, as interference with endpoints in general cytotoxicity tests must be precluded. Use of new endpoints with marker-free detection (such as the CFE assay) is a possibility. Another challenge is dosimetry, as this is not yet standardized when it comes to mass, number, surface area, or other metrics, and this makes comparisons and objective analysis difficult (Seaton et al., 2010). Reference NPs and standardized test protocols are highly desirable. Dosimetry is still a challenge for in vivo studies, as the dose should reflect the actual dose to which humans are exposed. It is technically very difficult to determine the concentration or amount of NPs in air, water, soil, or any consumer products due to their small size and quantity. When no information is available, a worst-case scenario should be applied, assuming presence, absorption, and systemic distribution of ENMs (EFSA Scientific committee, 2011). However, if the dose is too high, agglomeration will occur, and this can introduce a different toxicological profile (Tang et al., 2010). It is also important to ensure that the vehicle is suitable for good dispersion of the NPs. NPs are prone to agglomerate at physiological conditions due to their large surface area. Selection of an appropriate vehicle will also depend upon the route of exposure and source of NPs and standardization of tests should be performed.

For proper hazard and risk assessment, all available toxicity data from in vitro and in vivo experiments should be evaluated together with exposure data and other relevant information from read-across of other ENMs or from bulk chemicals. The output should be the overall assessment of safety of ENMs in its intended use. The materials should be thoroughly characterized to ensure that the test results reflect intrinsic toxicity of the ENMs and do not interact with the selected test assay. When it comes to exposure, the whole life cycle of the ENMs should be taken into consideration (Feliu and Fadeel, 2010). There will always be uncertainties when extrapolating from animal models to humans, as well as from laboratory experiments to complex systems (Canady, 2010). The risk of ENMs will be determined by interaction with cells and tissues, ADME and potential exposure levels, and all these factors are likely influenced by physicochemical properties (such as shape, reactivity, size, surface coating, surface charge) of the ENM (EFSA Scientific committee, 2011; Huk et al., 2014, 2015; Magdolenova et al., 2015). For design of both in vivo and in vitro experiments, exposure assessment is of uttermost importance. Relevant exposure routes should always be taken into consideration for selection of administration route in studies and choice of cell lines or cultures for in vitro testing.

Finally, one should also investigate whether in silico modeling and QSAR can be applied successfully to ENMs, and thus determine which features influence toxicity the most (see Chapter 4). This approach could be used as a basis for grouping of NPs (Oomen et al., 2015). Gene expression profiles (Williams and Halappanavar, 2015) as well epigenetic markers (Smolkova et al., 2015) could also aid in hazard assessment of ENMs. Until this can be done, ENMs should be treated case-by-case when it comes to toxicity testing and risk assessment.

CONCLUSIONS

The rapid development of nanotechnology outstrips our current understanding of the potential risks to human and environmental health posed by nanoscale materials. Altered behavior at the nanoscale compared with bulk material requires the development of improved tiered testing strategies to determine the impact of biological interaction with nanomaterials both in vitro and in vivo. The target toxicity of ENMs is not fully understood. For fiber ENMs, lessons from asbestos and its substitutes had facilitated the risk assessment in a small area of toxicity, that is the indcution of mesothelioma and lung adenocarcinoma. Little is yet known about the systemic chronic toxicity of other fiber ENMs (nor it is fully understood for asbestos). The fate of biopersistent NPs, in general, is predicted from the knowledge of a historical compound known as trotrast; all was deposited in the reticuloendotherial system and was retained for life. Examples of diseases linked to nanotoxicity can be counted on the fingers of one hand: cardiovascular toxicity of metal fumes, TiO_2 rat lung adenocarcinoma, crystalline silica for lung damage, MWCNT mesothlioma/lung adenocarcinoma. Nothing else is known yet of chronic targets of ENMs. Before dosimetry becomes possible, for the newest ENMs, hazard identification should be performed to prevent human casualties in workers and in consumers, and to prevent polluting the environment. We must learn from the history of products that were ultimately banned.

TAKE-HOME MESSAGES

- Challenges arise from the fact that unique features of ENMs may be coupled to toxicity.
- Existing validated and standardized tests for toxicity testing of NPs both in vitro and in vivo need to be adopted for ENMs testing while new methods also need to be developed.
- Physicochemical characterization of the test substance is an important step, as well as an agreement on validated reference materials and appropriate controls for tests.
- Detection techniques for studying cellular uptake and biodistribution of NPs need to be an integral part of the testing strategy and have to be improved and validated.
- Toxicity testing of NPs should be in accordance with the 3R principles (reduce, refine, and replace).

Acknowledgments

Supported by European Commission—FP7 (www.marina-fp7.eu; www.nanotest-fp7.eu).

References

Al Faraj, A., Fauvelle, F., Luciani, N., Lacroix, G., Levy, M., Cremillieux, Y., Canet-Soulas, E., 2011. In vivo biodistribution and biological impact of injected carbon nanotubes using magnetic resonance techniques. Int. J. Nanomed. 6, 351–361.

Balasubramanyam, A., Sailaja, N., Mahboob, M., Rahman, M.F., Hussain, S.M., Grover, P., 2010. In vitro mutagenicity assessment of aluminium oxide nanomaterials using the *Salmonella*/microsome assay. Toxicol. In Vitro 24, 1871–1876.

Borm, P.J., Muller-Schulte, D., 2006. Nanoparticles in drug delivery and environmental exposure: same size, same risks? Nanomedicine 1, 235–249.

Breslin, S., O'Dridcoll, L., 2013. Three-dimensional cell culture: the missing link in drug discovery. Drug Discov. Today 18, 240–249.

Brown, J., Reading, S.J., Jones, S., Fitchett, C.J., Howl, J., Martin, A., Longland, C.L., Michelangeli, F., Dubrova, Y.E., Brown, C.A., 2000. Critical evaluation of ECV304 as a human endothelial cell model defined by genetic analysis and functional responses: a comparison with the human bladder cancer derived epithelial cell line T24/83. Lab. Invest. 80, 37–45.

Brown, D.M., Dickson, C., Duncan, P., Al-Attili, F., Stone, V., 2010. Interaction between nanoparticles and cytokine proteins: impact on protein and particle functionality. Nanotechnology 21, 215104.

Burns, A.A., Vider, J., Ow, H., Herz, E., Penate-Medina, O., Baumgart, M., Larson, S.M., Wiesner, U., Bradbury, M., 2009. Fluorescent silica nanoparticles with efficient urinary excretion for nanomedicine. Nano Lett. 9, 442–448.

Byrne, H.J., Lynch, I., de Jong, W.H., Kreyling, W., Loft, S., Park, M.V.D.Z., Riediker, M., Warheit, D.B., 2010. Protocols for assessment of biological hazards of engineered nanoparticles. European Network on the Health and Environmental Impact of Nanomaterials.

Canady, R.A., 2010. The uncertainty of nanotoxicology: report of a Society for Risk Analysis Workshop. Risk Anal. 30, 1663–1670.

Casey, A., Herzog, E., Davoren, M., Lyng, F.M., Byrne, H.J., Chambers, G., 2007. Spectroscopic analysis confirms the interactions between single walled carbon nanotubes and various dyes commonly used to assess cytotoxicity. Carbon 45, 1425–1432.

Chen, J., Dong, X., Xin, Y., Zhao, M., 2011. Effects of titanium dioxide nano-particles on growth and some histological parameters of zebrafish (*Danio rerio*) after a long-term exposure. Aquat. Toxicol. 101, 493–499.

Clift, M.J., Gehr, P., Rothen-Rutishauser, B., 2011. Nanotoxicology: a perspective and discussion of whether or not in vitro testing is a valid alternative. Arch. Toxicol. 85, 723–731.

Collins, A.R., Dusinska, M., Gedik, C.M., Stetina, R., 1996. Oxidative damage to DNA: do we have a reliable biomarker? Environ. Health Perspect. 104 (Suppl. 3), 465–469.

Collins, A.R., Annangi, B., Rubio, L., Marcos, R., Dorn, M., Merker, C., Estrela-Lopis, I., Cimpan, M.R., Ibrahim, M., Cimpan, E., Ostermann, M., Sauter, A., El Yamani, N., Shaposhnikov, S., Chevillard, S., Paget, V., Grall, R., Delic, J., Goñi- de-Cerio, F., Suarez-Merino, B., Fessard, V., Hogeveen, K.N., Fjellsbø, L.M., Runden-Pran, E., Brzicova, T., Topinka, J., Silva, M.J., Leite, P.E., Ribeiro, A.R., Granjeiro, J.M., Grafström, R., Prina-Mello, A., Dusinska, M., 2016. High throughput toxicity screening and intracellular detection of nanomaterials. WIREs Nanomed. Nanobiotechnol.doi: 10.1002/wnan.1413.

Cowie, H., Magdolenova, Z., Saunders, M., Drlickova, M., Correia Carreira, S., Halamoda Kenzaoiu, B., Gombau, L., Guadagnini, R., Lorenzo, Y., Walker, L., Fjellsbo, L.M., Huk, A., Rinna, A., Tran, L., Volkovova, K., Boland, S., Juillerat-Jeanneret, L., Marano, F., Collins, A.R., Dusinska, M., 2015. Suitability of human and mammalian cells of different origin for the assessment of genotoxicity of metal and polymeric engineered nanoparticles. Nanotoxicology 9 (S1), 57–65.

Demir, E., Akça, H., Turna, F., Aksakal, S., Burgucu, D., Kaya, B., Tokgün, O., Vales, G., Creus, A., Marcos, R., 2015. Genotoxic and cell-transforming effects of titanium dioxide nanoparticles. Environ. Res. 136, 300–308.

Derda, R., Laromaine, A., Mammoto, A., Tang, S.K., Mammoto, T., Ingber, D.E., Whitesides, G.M., 2009. Paper-supported 3D cell culture for tissue-based bioassays. Proc. Natl. Acad. Sci. USA 106, 18457–18462.

Di Sotto, A., Chiaretti, M., Carru, G.A., Bellucci, S., Mazzanti, G., 2009. Multi-walled carbon nanotubes: lack of mutagenic activity in the bacterial reverse mutation assay. Toxicol. Lett. 184, 192–197.

Doak, S.H., Griffiths, S.M., Manshian, B., et al., 2009. Confounding experimental considerations in nanogenotoxicology. Mutagenesis 24, 285–293.

Dusinska, M., Collins, A.R., 1996. Detection of oxidised purines and UV-induced photoproducts in DNA, by inclusion of lesion-specific enzymes in the comet assay (single cell gel electrophoresis). ATLA 24, 405–411.

Dusinska, M., Fjellsbo, L., Magdolenova, Z., Rinna, A., Runden Pran, E., Bartonova, A., Heimstad, E., Harju, M., Tran, L., Ross, B., Juillerat, L., Halamoda Kenzaui, B., Marano, F., Boland, S., Guadaginini, R., Saunders, M., Cartwright, L., Carreira, S., Whelan, M., Kelin, C., Worth, A., Palosaari, T., Burello, E., Housiadas, C., Pilou, M., Volkovova, K., Tulinska, J., Kazimirova, A., Barancokova, M., Sebekova, K., Hurbankova, M., Kovacikova, Z., Knudsen, L., Poulsen, M., Mose, T., Vila, M., Gombau, L., Fernandez, B., Castell, J., Marcomini, A., Pojana, G., Bilanicova, D., Vallotto, D., 2009. Testing strategies for the safety of nanoparticles used in medical applications. Nanomedicine 4, 605–607.

Dusinska, M., Boland, S., Saunders, M., Juillerat-Jeanneret, L., Tran, L., Pojana, G., Marcomini, A., Volkovova, K., Tulinska, J., Knudsen, L.E., Gombau, L., Whelan, M., Collins, A.R., Marano, F., Housiadas, C., Bilanicova, D., Halamoda Kenzaoui, B., Correia Carreira, S., Magdolenova, Z., Fjellsbø, L., Huk, A., Handy, R., Walker, L., Barancokova, M., Bartonova, A., Burello, E., Castell, J., Cowie, H., Drlickova, M., Guadagnini, R., Harris, H., Harju, M., Heimstad, E.S., Hurbankova, M., Kazimirova, A., Kovacikova, Z., Kuricova, M., Liskova, A., Milcamps, A., Neubauerova, E., Palosaari, T., Papazafiri, P., Pilou, M., Poulsen, M.S., Ross, B., Runden-Pran, E., Sebekova, K., Staruchova, M., Vallotto, D., Worth, A., 2015. Towards an alternative testing strategy for nanomaterials used in nanomedicine: lessons from NanoTEST. Nanotoxicology 9 (S1), 118–132.

EFSA Scientific committee, 2011. Guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain. EFSA J. 9, 2140–2176.

Ema, M., Kobayashi, N., Naya, M., Hanai, S., Nakanishi, J., 2010. Reproductive and developmental toxicity studies of manufactured nanomaterials. Reprod. Toxicol. 30, 343–352.

Federici, G., Shaw, B.J., Handy, R.D., 2007. Toxicity of titanium dioxide nanoparticles to rainbow trout (*Oncorhynchus mykiss*): gill injury, oxidative stress, and other physiological effects. Aquat. Toxicol. 84, 415–430.

Feliu, N., Fadeel, B., 2010. Nanotoxicology: no small matter. Nanoscale 2, 2514–2520.

Fenech, M., Kirsch-Volders, M., Natarajan, A.T., Surralles, J., Crott, J.W., Parry, J., Norppa, H., Eastmond, D.A., Tucker, J.D., Thomas, P., 2011. Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. Mutagenesis 26, 125–132.

Galloway, S.M., Armstrong, M.J., Reuben, C., Colman, S., Brown, B., Cannon, C., Bloom, A.D., Nakamura, F., Ahmed, M., Duk, S., et al., 1987. Chromosome aberrations and sister chromatid exchanges in Chinese hamster ovary cells: evaluations of 108 chemicals. Environ. Mol. Mutagen. 10 (Suppl. 10), 1–175.

Garza-Ocanas, L., Ferrer, D.A., Burt, J., Diaz-Torres, L.A., Ramirez Cabrera, M., Rodriguez, V.T., Lujan Rangel, R., Romanovicz, D., Jose-Yacaman, M., 2010. Biodistribution and long-term fate of silver nanoparticles functionalized with bovine serum albumin in rats. Metallomics 2, 204–210.

Gonzalez, L., Sanderson, B.J., Kirsch-Volders, M., 2011. Adaptations of the in vitro MN assay for the genotoxicity assessment of nanomaterials. Mutagenesis 26, 185–191.

Guadagnini, R., Halamoda Kenzaoui, B., Cartwright, L., Pojana, G., Magdolenova, Z., Bilanicova, D., Saunders, M., Juillerat, L., Marcomini, A., Huk, A., Dusinska, M., Fjellsbø, L.M., Marano, F., Boland, S., 2015. Toxicity screenings of nanomaterials: challenges due to interference with assay processes and components of classic in vitro tests. Nanotoxicology 9 (S1), 13–24.

Guo, L., Von Dem Bussche, A., Buechner, M., Yan, A., Kane, A.B., Hurt, R.H., 2008. Adsorption of essential micronutrients by carbon nanotubes and the implications for nanotoxicity testing. Small 4, 721–727.

Harris, G., Palosaari, T., Magdolenova, Z., Mennecozzi, M., Gineste, J.M., Saavedra, L., Milcamps, A., Huk, A., Collins, A.R., Dusinska, M., Whelan, M., 2015. Iron oxide nanoparticle toxicity testing using high throughput analysis and high content imaging. Nanotoxicology 9 (S1), 87–94.

Haynes, C.L., 2010. The emerging field of nanotoxicology. Anal. Bioanal. Chem. 398, 587–588.

Huh, D., Matthews, B.D., Mammoto, A., Montoya-Zavala, M., Hsin, H.Y., Ingber, D.E., 2010. Reconstituting organ-level lung functions on a chip. Science 328, 1662–1668.

Huk, A., Izak-Nau, E., Reidy, B., Boyles, M., Duschl, A., Lynch, I., Dusinska, M., 2014. Is the toxic potential of nanosilver dependent on its size? Part. Fibre Toxicol. 11, 65.

Huk, A., Izak-Nau, E., el Yamani, N., Uggerud, H., Vadset, M., Zasonska, B., Duschl, A., Dusinska, M., 2015. Impact of nanosilver on various DNA lesions and HPRT gene mutations. Part. Fibre Toxicol. 12 (1), 25.

Karlsson, H.L., Di Bucchianico, S., Collins, A.R., Dusinska, M., 2015. Can the comet assay be used reliably to detect nanoparticle-induced genotoxicity? Environ. Mol. Mutagen. 56, 82–96.

Khlebtsov, N., Dykman, L., 2011. Biodistribution and toxicity of engineered gold nanoparticles: a review of in vitro and in vivo studies. Chem. Soc. Rev. 40, 1647–1671.

Kroll, A., Pillukat, M.H., Hahn, D., Schnekenburger, J., 2009. Current in vitro methods in nanoparticle risk assessment: limitations and challenges. Eur. J. Pharm. Biopharm. 72, 370–377.

Kroll, A., Dierker, C., Rommel, C., Hahn, D., Wohlleben, W., Schulze-Isfort, C., Göbbert, C., Voetz, M., Hardinghaus, F., Schnekenburger, J., 2011. Cytotoxicity screening of 23 engineered nanomaterials using a test matrix of ten cell lines and three different assays. Part. Fibre Toxicol. 8, 9.

Kroll, A., Pillukat, M.H., Hahn, D., Schnekenburger, J., 2012. Interference of engineered nanoparticles with in vitro toxicity assays. Arch. Toxicol. 86, 1123–1136.

Krüger, C.T., Hofmann, M., Hartwig, A., 2015. The *in vitro* PIG-A gene mutation assay: mutagenicity testing via flow cytometry based on the glycosylphosphatidylinositol (GPI) status of TK6 cells. Arch. Toxicol. 89, 2429–2443.
Kunzmann, A., Andersson, B., Thurnherr, T., Krug, H., Scheynius, A., Fadeel, B., 2011. Toxicology of engineered nanomaterials: focus on biocompatibility, biodistribution and biodegradation. Biochim. Biophys. Acta 1810, 361–373.
Kuo, Y.C., Chen, H.H., 2010. Effect of electromagnetic field on endocytosis of cationic solid lipid nanoparticles by human brain-microvascular endothelial cells. J. Drug Target. 18, 447–456.
Laingam, S., Froscio, S.M., Humpage, A.R., 2008. Flow-cytometric analysis of in vitro micronucleus formation: comparative studies with WIL2-NS human lymphoblastoid and L5178Y mouse lymphoma cell lines. Mutat. Res. 656, 19–26.
Lee, J., Lilly, G.D., Doty, R.C., Podsiadlo, P., Kotov, N.A., 2009. In vitro toxicity testing of nanoparticles in 3D cell culture. Small 5, 1213–1221.
Lehr, C.M., Daum, N., Schneider, M., Schafer, U.F., 2011. Biological barriers—a need for novel tools in nanotoxicology and nanomedicine. Preface. Eur. J. Pharm. Biopharm. 77, 337.
Li, S.D., Huang, L., 2008. Pharmacokinetics and biodistribution of nanoparticles. Mol. Pharm. 5, 496–504.
Maenosono, S., Yoshida, R., Saita, S., 2009. Evaluation of genotoxicity of amine-terminated water-dispersible FePt nanoparticles in the Ames test and in vitro chromosomal aberration test. J. Toxicol. Sci. 34, 349–354.
Magdolenova, Z., Lorenzo, Y., Collins, A., Dusinska, M., 2012. Can standard genotoxicity tests be applied to nanoparticles? J. Toxicol. Environ. Health A 75, 800–806.
Magdolenova, Z., Collins, A.R., Kumar, A., Dhawan, A., Stone, V., Dusinska, M., 2014. Mechanisms of genotoxicity. Review of recent in vitro and in vivo studies with engineered nanoparticles. Nanotoxicology 8, 233–278.
Magdolenova, Z., Drlickova, M., Henjum, K., Rundén-Pran, E., Tulinska, J., Bilanicova, D., Pojana, G., Kazimirova, A., Barancokova, M., Kuricova, M., Liskova, A., Staruchova, M., Ciampor, F., Vavra, I., Lorenzo, Y., Collins, A., Rinna, A., Fjellsbø, L., Volkovova, K., Marcomini, A., Amiry-Moghaddam, M., Dusinska, M., 2015. Coating-dependent induction of cytotoxicity and genotoxicity of iron oxide nanoparticles. Nanotoxicology 9 (S1), 44–56.
Mahmoudi, M., Simchi, A., Imani, M., et al., 2010. A new approach for the in vitro identification of the cytotoxicity of superparamagnetic iron oxide nanoparticles. Colloids Surf. 75, 300–309.
Maynard, A.D., Warheit, D.B., Philbert, M.A., 2011. The new toxicology of sophisticated materials: nanotoxicology and beyond. Toxicol. Sci. 120 (Suppl. 1), S109–S129.
Monteiro-Riviere, N.A., Nemanich, R.J., Inman, A.O., Wang, Y.Y., Riviere, J.E., 2005. Multi-walled carbon nanotube interactions with human epidermal keratinocytes. Toxicol. Lett. 155, 377–384.
Monteiro-Riviere, N.A., Inman, A.O., Zhang, L.W., 2009. Limitations and relative utility of screening assays to assess engineered nanoparticle toxicity in a human cell line. Toxicol. Appl. Pharmacol. 234, 222–235.
Morimoto, Y., Izumi, H., Yoshiura, Y., Fujishima, K., Yatera, K., Yamamoto, K., 2016. Usefulness of intratracheal instillation studies for estimating nanoparticle-induced pulmonary toxicity. Int. J. Mol. Sci. 17, 165.
Mortelmans, K., Zeiger, E., 2000. The Ames *Salmonella*/microsome mutagenicity assay. Mutat. Res. 455, 29–60.
Mues, S., Antunovic, J., Ketelhut, S., Kemper, B., Schnekenburger, J., 2016. Novel optical approaches for label-free quantification of nano-cytotoxic effects. Proceedings of SPIE 9719-19.
Oberdorster, G., Oberdorster, E., Oberdorster, J., 2005. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environ. Health Perspect. 113, 823–839.
OECD, 1997. Guidelines for testing chemicals. Mammalian erythrocyte micronucleus test, Vol. Guideline 474.
OECD, 2014. Guidelines for testing chemicals. *In vivo* mammalian alkaline comet assay Guideline TG 489. Available from: http://www.oecd-ilibrary.org/docserver/download/9714511e.pdf?expires=1470669663&id=id&accname=guest&checksum=2DC70EECFA92367CE1B756C201964E10
OECD, 2015. Guidance document on the in vitro Syrian hamster embryo (SHE) cell transformation assay Series on Testing & Assessement No. 214.
OECD, 2016. Guidance document on the in vitro Bhas 42 cell transformation assay Series on Testing & Assessment No. 231.
Oomen, A.G., Bleeker, E.A., Bos, P.M., van Broekhuizen, F., Gottardo, S., Groenewold, M., Hristozov, D., Hund-Rinke, K., Irfan, M.A., Marcomini, A., Peijnenburg, W.J., Rasmussen, K., Jiménez, A.S., Scott-Fordsmand, J.J., van Tongeren, M., Wiench, K., Wohlleben, W., Landsiedel, R., 2015. Grouping and read-across approaches for risk assessment of nanomaterials. Int. J. Environ. Res. Public Health 12, 13415–13434.
Pfaller, T., Colognato, R., Nelissen, I., et al., 2010. The suitability of different cellular in vitro immunotoxicity and genotoxicity methods for the analysis of nanoparticle-induced events. Nanotoxicology 4, 52–72.

Pillai, G, 2014. Nanomedicines for cancer therapy: an update of FDA approved and those under various stages of development. SOJ Pharm. Pharm. Sci. 1 (2), 13.

Ponti, J., Sabbioni, E., Munaro, B., et al., 2009. Genotoxicity and morphological transformation induced by cobalt nanoparticles and cobalt chloride: an in vitro study in Balb/3T3 mouse fibroblasts. Mutagenesis 24 (5), 439–445.

Ponti, J., Kinsner-Ovaskainen, A., Norlén, H., Altmeyer, S., Andreoli, C., Bogni, A., Chevillard, S., De Angelis, I., Chung, S.-T., Eom, I., Fujita, K., Gilliland, D., Grollino, M.G., Gulumian, M., Hirsch, C., Ichiraku, K., Igarashi, T., Jeong, J., Jo, E., Kim, D.-Y., Kaiser, J.-P., Lagache, D., La Spina, R., Kwon Lee, J., Lee, J., Lovera, A., Mäder-Althaus, X., Nesslany, F., Ojea Jimenez, I., Pacchierotti, F., Pianella, F., Paget, V., Rim Kim, T., Roszak, J., Rosenkranz, P., Simar, S., Stępnik, M., Vetten, M., Woong Song, N., Yang, J.-Y., Rossi, F., 2014. Interlaboratory Comparison Study of the Colony Forming Efficiency Assay for Assessing Cytotoxicity of Nanomaterials. In: Kinsner-Ovaskainen, A., Ponti, J. (Eds.), Report EUR 27009 EN.

Raemy, D.O., Limbach, L.K., Rothen-Rutishauser, B., Grass, R.N., Gehr, P., Birbaum, K., Brandenberger, C., Gunther, D., Stark, W.J., 2011. Cerium oxide nanoparticle uptake kinetics from the gas-phase into lung cells in vitro is transport limited. Eur. J. Pharm. Biopharm. 77, 368–375.

Ragnaill, M.N., Brown, M., Ye, D., Bramini, M., Callanan, S., Lynch, I., Dawson, K.A., 2011. Internal benchmarking of a human blood–brain barrier cell model for screening of nanoparticle uptake and transcytosis. Eur. J. Pharm. Biopharm. 77, 360–367.

Robe, A., Pic, E., Lassalle, H.P., Bezdetnaya, L., Guillemin, F., Marchal, F., 2008. Quantum dots in axillary lymph node mapping: biodistribution study in healthy mice. BMC Cancer 8, 111.

Rommel, C.E., Dierker, C., Vollmer, A., Ketelhut, S., Kemper, B., Schnekenburger, J., 2014. Multimodal label-free in vitro toxicity testing with digital holographic microscopy. Proceedings of SPIE—The International Society for Optical Engineering, 9129, pp. 912–940.

Roy, S., Kulkarni, R., Hewitt, N.J., Aardema, M.J., 2016. The EpiDerm™ 3D human reconstructed skin micronucleus (RSMN) assay: historical control data and proof of principle studies for mechanistic assay adaptations. Mutat. Res. Genet. Toxicol. Environ. Mutagen. 805, 25–37.

Runden, E., Seglen, P.O., Haug, F.M., Ottersen, O.P., Wieloch, T., Shamloo, M., Laake, J.H., 1998. Regional selective neuronal degeneration after protein phosphatase inhibition in hippocampal slice cultures: evidence for a MAP kinase-dependent mechanism. J. Neurosci. 18, 7296–7305.

Sabatini, C.A., Pereira, R.V., Gehlen, M.H., 2007. Fluorescence modulation of acridine and coumarin dyes by silver nanoparticles. J. Fluoresc. 17, 377–382.

Sagawa, Y., Futakuchi, M., Xu, J., Fukamachi, K., Sakai, Y., Ikarashi, Y., Nishimura, T., Suzui, M., Tsuda, H., Morita, A., 2012. Lack of promoting effect of titanium dioxide particles on chemically-induced skin carcinogenesis in rats and mice. J. Toxicol. Sci. 37 (2), 317–327.

Saovapakhiran, A., D'Emanuele, A., Attwood, D., Penny, J., 2009. Surface modification of PAMAM dendrimers modulates the mechanism of cellular internalization. Bioconjug. Chem. 20, 693–701.

Sasaki, K., Huk, A., El Yamani, N., Tanaka, N., Dusinska, M., 2014. Bhas 42cell transformation assay for genotoxic and non-genotoxic carcinogens. In: Sierra, L.M., Gavaivago, I. (Eds.), Genotoxicity and Repair. A Practical Approach. Series: Methods in Pharmacology and Toxicology. Springer Protocols, Humana Press, Springer New York, Heidelberg Dordrecht London, pp. 343–362.

Saunders, M., 2009. Transplacental transport of nanomaterials. Wiley Interdiscip Rev Nanomed Nanobiotechnol 1, 671–684.

Sayes, C.M., Warheit, D.B., 2009. Characterization of nanomaterials for toxicity assessment. Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol. 1, 660–670.

Sayes, C.M., Reed, K.L., Warheit, D.B., 2007. Assessing toxicity of fine and nanoparticles: comparing in vitro measurements to in vivo pulmonary toxicity profiles. Toxicol. Sci. 97, 163–180.

Schleh, C., Semmler-Behnke, M., Lipka, J., Wenk, A., Hirn, S., Sch Ffler, M., Schmid, G.N., Simon, U., Kreyling, W.G., 2012. Size and surface charge of gold nanoparticles determine absorption across intestinal barriers and accumulation in secondary target organs after oral administration. Nanotoxicology 6 (1), 36–46.

Seaton, A., Tran, L., Aitken, R., Donaldson, K., 2010. Nanoparticles, human health hazard and regulation. J. R. Soc. Interface 7 (Suppl. 1), S119–S129.

Seo, J.H., Cho, K., Lee, S.Y., Joo, S.W., 2011. Concentration-dependent fluorescence live-cell imaging and tracking of intracellular nanoparticles. Nanotechnology 22, 235101.

Shah, N.B., Dong, J., Bischof, J.C., 2011. Cellular uptake and nanoscale localization of gold nanoparticles in cancer using label-free confocal Raman microscopy. Mol. Pharm. 8, 176–184.

Shinohara, N., Matsumoto, K., Endoh, S., Maru, J., Nakanishi, J., 2009. In vitro and in vivo genotoxicity tests on fullerene C60 nanoparticles. Toxicol. Lett. 191, 289–296.
Simko, M., Mattsson, M.O., 2010. Risks from accidental exposures to engineered nanoparticles and neurological health effects: a critical review. Part Fibre Toxicol. 7, 42.
Singh, N., Manshian, B., Jenkins, G.J., Griffiths, S.M., Williams, P.M., Maffeis, T.G., Wright, C.J., Doak, S.H., 2009. NanoGenotoxicology: the DNA damaging potential of engineered nanomaterials. Biomaterials 30, 3891–3914.
Skotland, T., Iversen, T.G., Sandvig, K., 2010. New metal-based nanoparticles for intravenous use: requirements for clinical success with focus on medical imaging. Nanomedicine 6, 730–737.
Smolkova, B., El Yamani, N., Collins, A.R., Gutleb, A.C., Dusinska, M., 2015. Nanoparticles in food. epigenetic changes induced by nanomaterials and possible impact on health. Food Chem. Toxicol. 77, 64–73.
Stelter, L., Pinkernelle, J.G., Michel, R., Schwartlander, R., Raschzok, N., Morgul, M.H., Koch, M., Denecke, T., Ruf, J., Baumler, H., Jordan, A., Hamm, B., Sauer, I.M., Teichgraber, U., 2010. Modification of aminosilanized superparamagnetic nanoparticles: feasibility of multimodal detection using 3T MRI, small animal PET, and fluorescence imaging. Mol. Imaging Biol. 12, 25–34.
Stoccoro, A., Di Bucchianico, S., Uboldi, C., Coppedè, F., Ponti, J., Placidi, C., Blosi, M., Ortelli, S., Costa, A.L., Migliore, L., 2016. A panel of in vitro tests to evaluate genotoxic and morphological neoplastic transformation potential on Balb/3T3 cells by pristine and remediated titania and zirconia nanoparticles. Mutagenesis 31 (5), 511–529.
Susaki, E.A., Ueda, H.R., 2016. Whole-body and whole-organ clearing and imaging techniques with single-cell resolution: toward organism-level systems biology in mammals. Cell Chem. Biol. a23, 137–157.
Susewind, J., De Souza Carvalho-Wodarz, C., Repnik, U., Collnot, E.-M., Schneider-Daum, N., Griffiths, G.W., Lehr, C.-M., 2016. A 3D co-culture of three human cell lines to model the inflamed intestinal mucosa for safety testing of nanomaterials. Nanotoxicology 10, 53–62.
Tang, M., Zhang, T., Xue, Y., Wang, S., Huang, M., Yang, Y., Lu, M., Lei, H., Kong, L., Yuepu, P., 2010. Dose dependent in vivo metabolic characteristics of titanium dioxide nanoparticles. J. Nanosci. Nanotechnol. 10, 8575–8583.
Taquahashi, Y., Ogawa, Y., Takagi, A., Tsuji, M., Morita, K., Kanno, J., 2013. Improved dispersion method of multiwall carbon nanotube for inhalation toxicity studies of experimental animals. J. Toxicol. Sci. 38 (4), 619–628.
Tasciotti, E., Godin, B., Martinez, J.O., Chiappini, C., Bhavane, R., Liu, X., Ferrari, M., 2011. Near-infrared imaging method for the in vivo assessment of the biodistribution of nanoporous silicon particles. Mol. Imaging 10, 56–68.
Thurn, K.T., Arora, H., Paunesku, T., Wu, A., Brown, E.M., Doty, C., Kremer, J., Woloschak, G., 2011. Endocytosis of titanium dioxide nanoparticles in prostate cancer PC-3M cells. Nanomedicine 7, 123–130.
Trickler, W.J., Lantz, S.M., Murdock, R.C., Schrand, A.M., Robinson, B.L., Newport, G.D., Schlager, J.J., Oldenburg, S.J., Paule, M.G., Slikker, Jr., W., Hussain, S.M., Ali, S.F., 2010. Silver nanoparticle induced blood–brain barrier inflammation and increased permeability in primary rat brain microvessel endothelial cells. Toxicol. Sci. 118, 160–170.
Uboldi, C., Urbán, P., Gilliland, D., Bajak, E., Valsami-Jones, E., Ponti, J., Rossi, F., 2016. Role of the crystalline form of titanium dioxide nanoparticles: rutile, and not anatase, induces toxic effects in Balb/3T3 mouse fibroblasts. Toxicol. In Vitro 31, 137–145.
Vaccaro, D.E., Yang, M., Weinberg, J.S., Reinhardt, C.P., Groman, E.V., 2008. Cell tracking using nanoparticles. J. Cardiovasc. Transl. Res. 1, 217–220.
Veranth, J.M., Kaser, E.G., Veranth, M.M., Koch, M., Yost, G.S., 2007. Cytokine responses of human lung cells (beas-2b) treated with micron-sized and nanoparticles of metal oxides compared to soil dusts. Part. Fibre Toxicol. 4, 2.
Walczak, A.P., Kramer, E., Hendriksen, P.J.M., Helsdingen, R., van der Zande, M., Rietjens, I.M.C.M., Bouwmeester, H., 2015. *In vitro* gastrointestinal digestion increases the translocation of polystyrene nanoparticles in an in vitro intestinal coculture model. Nanotoxicology 9, 886–894.
Wang, Y., Wang, J., Wu, M., Deng, X., Wen, T., Chen, C., Zhang, H., Liu, Y., Jiao, Z., 2010a. Internalization, translocation and biotransformation of silica-coated titanium dioxide nanoparticles in neural stem cells. J. Nanosci. Nanotechnol. 10, 7121–7125.
Wang, Y., Seebald, J.L., Szeto, D.P., Irudayaraj, J., 2010b. Biocompatibility and biodistribution of surface-enhanced Raman scattering nanoprobes in zebrafish embryos: in vivo and multiplex imaging. ACS Nano 4, 4039–4053.
Warheit, D.B., Sayes, C.M., Reed, K.L., Swain, K.A., 2008. Health effects related to nanoparticle exposures: environmental, health and safety considerations for assessing hazards and risks. Pharmacol. Ther. 120, 35–42.
Wells, A.J., Smith, W.R., 1941. The absorption spectrum of suspensions of carbon black. J. Phys. Chem. 45, 1055–1060.
Williams, A., Halappanavar, S., 2015. Application of biclustering of gene expression data and gene set enrichment analysis methods to identify potentially disease causing nanomaterials. Beilstein. J. Nanotechnol. 6, 2438–2448.

Worle-Knirsch, J.M., Pulskamp, K., Krug, H.F., 2006. Oops they did it again! Carbon nanotubes hoax scientists in viability assays. Nano Lett. 6, 1261–1268.

Xu, P., Gullotti, E., Tong, L., Highley, C.B., Errabelli, D.R., Hasan, T., Cheng, J.X., Kohane, D.S., Yeo, Y., 2009. Intracellular drug delivery by poly(lactic-*co*-glycolic acid) nanoparticles, revisited. Mol. Pharm. 6, 190–201.

Yoshida, R., Kitamura, D., Maenosono, S., 2009. Mutagenicity of water-soluble ZnO nanoparticles in Ames test. J. Toxicol. Sci. 34, 119–122.

Yu, B., Ahn, J.S., Lim, J.I., Lee, Y.K., 2009. Influence of TiO_2 nanoparticles on the optical properties of resin composites. Dent. Mater. 25, 1142–1147.

Zheng, J., Jaffray, D., Allen, C., 2009. Quantitative CT imaging of the spatial and temporal distribution of liposomes in a rabbit tumor model. Mol. Pharm. 6, 571–580.

CHAPTER

4

Computational Approaches

*Tu C. Le**, Vidana Epa***, Lang Tran*[†], Dave Winkler***,[‡],[§],[¶]

***CSIRO Manufacturing, Clayton, VIC, Australia; **CSIRO Manufacturing, Parkville, VIC, Australia; [†]Institute of Occupational Medicine, Edinburgh, United Kingdom; [‡]Monash Institute of Pharmaceutical Science, Parkville, VIC, Australia; [§]La Trobe Institute for Molecular Science, La Trobe University, Bundoora, VIC, Australia; [¶]Flinders University, Adelaide, SA, Australia**

OUTLINE

Introduction 83

Challenges in Modeling Nanoparticle Biological Effects 85

Examples of Nanotoxicology Modeling Approaches 85

Quantum Chemical Studies of Toxicity of Nanoparticles 86

Statistical Modeling and Machine Learning Methods 88

Nanotoxicological Fingerprints to Classify Nanomaterials 91

Molecular Modeling and Molecular Dynamics Methods 92

Elucidating Interactions Between Nanomaterials and Proteins 94

Other Modeling Approaches for Nanotoxicology 97

Conclusions 97

Take-Home Messages 98

References 98

INTRODUCTION

Our understanding of the novel properties of materials and particles with dimensions in the nanometer range is growing rapidly, and commercial nanomaterials are reaching the marketplace at an increasing rate [approximately 50,000 products available now contain nanomaterials, and the Nanotechnology Consumer Products Inventory currently lists 1,814 consumer products (Vance et al., 2015)]. However, we still understand very little about how nanomaterials interact with mammalian systems or the environment. The precautionary

Adverse Effects of Engineered Nanomaterials. http://dx.doi.org/10.1016/B978-0-12-809199-9.00004-5
Copyright © 2017 Elsevier Inc. All rights reserved.

principle demands care in handling and distributing nanomaterials in the workplace, marketplace, or environment until we better understand their interactions with biological systems (Hansen et al., 2008; Maynard and Rejeski, 2009; Morris et al., 2011). For example, titanium dioxide nanoparticles generate free radicals in light, a property exploited for water photolysis reactions and for developing self-cleaning surfaces. However, titanium dioxide and zinc oxide nanoparticles are also used in sunscreens as they absorb UV radiation and effectively prevent sunburn (Barnard, 2010a; Gulson et al., 2008). The potential for generating free radicals that would adversely affect skin cells is a cause for concern, particularly as sunscreen is mandated for childcare centers in some countries, such as Australia. A further example is high-aspect ratio carbon nanotubes that have been shown to have detrimental health effects similar to asbestos (Sakamoto et al., 2009; Takagi et al., 2008). There is also increasing general concern about the occupational health and safety, and public health impacts of nanoparticles. Their size allows them to be inhaled, penetrate the skin, mucosa, or lung tissue, be transported around the body, and enter cells where they could interact with biomolecules and interfere with cellular biochemistry. We have grossly inadequate knowledge of how cells, organs, and tissues interact with nanoparticles, particularly for chronic exposure. There is, therefore, an urgent need to determine whether nanoparticles impact negatively on human and environmental health, and to develop robust and reliable methods for predicting nanotoxicity proactively, rather than reactively, once a serious public health or environmental problem appears.

As experiments on biological effects of nanomaterials are absolutely necessary, but time-consuming and expensive, there is a strong need to develop cheaper, predictive methods for rapidly assessing potential toxicities of new and modified nanomaterials. Computational modeling techniques have a relatively long history of performance, and have obvious synergy with experimental toxicology, in assessing the risk of industrial chemicals, agrochemical, and drugs. Chemical regulatory agencies around the world are increasingly using computational tools, particularly statistical modeling and machine learning methods embodied in quantitative structure–activity relationships (QSAR) modeling (Ahlers et al., 2008; Jaworska et al., 2010; Nabholz, 1991; Zeeman et al., 1995). Recent reviews and commentaries in high-impact journals (Hansen et al., 2008; Maynard and Rejeski, 2009; Morris et al., 2011) have called for computational methods to be applied to understanding nanotoxicology, and regulators are calling for these tools to be developed as a matter of urgency. As with industrial chemicals, high throughput methods can provide large volumes of data on the biological effects of nanomaterials, although experimental programs are just starting to embrace these technologies. Increased used of gene arrays and related technologies to more rapidly assess the impact of chemical and nanomaterials on biological systems should also be of major significance. Computational tools and the necessary informatics and ontological tools that must accompany them, will be necessary to manage nanotoxicological data in the future. Hopefully large-scale scientific consortia will be formed [similar to ToxCAST (Dix et al., 2007) and the HPV challenge (Nicholson et al., 2004) with industrial chemicals] that will provide data for risk assessment and modeling. Other initiatives for data consolidation similar to ChEMBL, ACToR (Judson et al., 2008), data fusion, MIAME (Brazma, 2009; Brazma et al., 2001) for small molecules and gene arrays, and embracing new information technologies, such as FaceBook, RSS feeds will also be important for efficient data sharing between research groups and regulatory agencies. In this chapter, we explain why in silico methods are an essential addition to

the nanomaterials research toolkit, why nanomaterials may be more difficult to model than single molecules, and give examples of recent successful models of the biological effects of nanoparticles.

CHALLENGES IN MODELING NANOPARTICLE BIOLOGICAL EFFECTS

Unlike small organic molecules, which have been studied computationally for a long period, a number of important issues arise when attempting to model the biological effects of complex materials like nanoparticles. Nanoparticles are complex materials with diverse and important surface features that play a dominant role in their biological properties. It is now known that the particle surface area is an important descriptor because this is how particles come into contact with cells. Thus, other microscopic surface-related descriptors, such as surface charge densities (and related zeta potentials), surface coatings and modifications, crystal faces and edges, electrochemical properties, etc., are all likely to affect not only how a nanoparticle interacts with cells or the environment, but also on its photochemical properties, aggregation potential, and type of protein corona formed on the particle in various biological fluids. In addition, the possibility of soluble metal-ion leaching, and the difficulties in accurately characterizing manufactured nanoparticle sizes, shapes, morphologies, crystal phases, etc. adds an extra dimension of complexity in understanding the biological effects and identifying what is in fact the biologically relevant entity. Finally, lack of knowledge about how nanoparticles are taken up, distributed, and transformed in the body, how they enter cells and affect cellular processes, and how they are eliminated, make traditional methods of pharmacokinetic and pharmacodynamic models, that work well for small discrete molecules, very problematic. Clearly, in vitro assays also need to be chosen that generate results that are indicative of in vivo effects of nanomaterials.

Mechanism-based toxicity models are the ideal way of understanding and predicting likely biological effects. However, given the significant increase in complexity of nanoparticles and their interaction with their environment and the paucity of knowledge described earlier, it is clear that very robust modeling tools are required. The modeling methods summarized in the following sections fall into two main types. First are those that provide precise information on likely modes of toxicity (quantum chemical and molecular dynamics), but which cannot deal with large data sets or make predictions about most in vitro or in vivo effects. The second are the pattern recognition or machine learning methods (quantitative structure–activity relationships, or QSARs, classifiers, and fingerprints) that provide limited mechanistic information but can extract useful information from large data sets and make predictions of properties of new nanomaterials not yet assessed experimentally.

EXAMPLES OF NANOTOXICOLOGY MODELING APPROACHES

There have been a very small number of publications reporting models for the adverse biological effects of nanoparticles, in spite of the importance of such models to regulators. Clearly the issues discussed in the previous section, coupled with the relatively small number

of expert molecular-level modeling groups in the materials area, are the main reasons for this dearth of published work. Four main approaches have been taken in the literature:

- The use of quantum chemical methods to understand the fundamental principles of nanoparticle structure, surface, properties, photocatalytic effects, and likely toxicity.
- Statistical modeling or machine learning (QSAR) methods applied to existing nanotoxicological data.
- Development of nanotoxicological fingerprints or indices to classify groups of nanomaterials with similar biological properties.
- The application of molecular modeling and molecular dynamics methods to understand the kinetics and mechanisms of the interaction of nanomaterials with biological systems at the molecular level.

Recent reviews have captured some of this work, but the field is moving ahead rapidly so a summary of recent work in these four areas is provided here.

Quantum Chemical Studies of Toxicity of Nanoparticles

Barnard's group has been very active in the application of quantum chemical methods to understanding and predicting the adverse effects of nanomaterials. Recent reviews and papers by the group provide accessible summaries of the methods and recent results (Barnard, 2009a,b, 2010b, 2014a,b; Barnard and Snook, 2011a,b). We provide an example of a recent study of the biological effects of nanoparticles used in sunscreens. ZnO and TiO_2 nanoparticles in sunscreens are successful examples of engineered nanoparticles in increasing use in everyday life. Here, the nanometer-sized particles contribute to the high sun-protection factors and transparency afforded by these sunscreens when applied on the skin. This makes them much more popular with consumers compared to more conventional sunscreens which appear as "unsightly" opaque white creams when applied to the skin and explains their commercial success. However, the high surface-to-volume ratios of these nanoparticles also mean that large numbers of potentially reactive moieties on the particles may be exposed. As Nel et al. (2006) point out, the decreased size of the nanoparticles could expose reactive functional groups by increasing surface structural defects and altering the electronic configuration through the creation of discontinuous crystal planes. The TiO_2 nanoparticles in the sunscreen can, on exposure to UV light, produce electrons and positive "holes" by excitation of valence band electrons. These electrons and holes are capable of reacting with adsorbed oxygen and water on the particle surface to produce hydroxyl, $OH\bullet$ and superoxide, $HO_2\bullet$ free radicals (Brayner, 2008). These radicals can then go on to produce more ROS in a cascade of Fenton chemistry-like reactions.

The seminal paper in using computational simulations to estimate the toxicity due to the production of ROS from titanium dioxide (TiO_2) nanoparticles was published by Barnard (2010a). In this paper, Barnard calculated the quantity of ROS production by these nanoparticles as a function of the particle diameter, and obtained a very striking "saw-toothed" graph with a maximum in the ROS production at 33 nm (Fig. 4.1). Barnard estimates the amount of ROS produced by these nanoparticles by simply counting the number of reactive sites on the most reactive surface planes of the two TiO_2 polymorphs, anastase [001], and rutile [110]. The fractional surface areas of these polymorphs present on a nanoparticle at a given size,

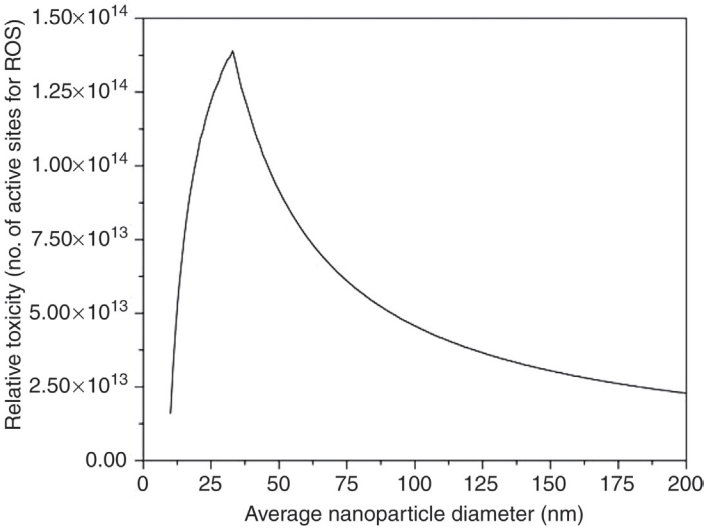


FIGURE 4.1 Relative phototoxicity of TiO_2 nanoparticles as a function of particle size. *Source: Reprinted from Barnard, A.S., 2010. One-to-one comparison of sunscreen efficacy, aesthetics and potential nanotoxicity. Nat. Nanotechnol. 5, 271–274, with permission from Nature Publishing Group.*

temperature, and surface adsorbate is computed using a thermodynamic nanomorphology model (Barnard, 2006; Barnard and Xu, 2008):

$$G_x(T) = \Delta G_f^0(T) + \frac{M}{\rho_x}\left\langle 1 - \frac{2\sum_i f_i\,\sigma_{xi}(T,\mu_y)}{B_0 < R >} + \frac{P_{ex}}{B_0}\right\rangle\left[q\sum_i f_i \gamma_{xi}(T,\mu_y)\right]$$

where the free energy of TiO_2 in phase x at temperature T is dependent on isotropic surface stresses σ_{xi} (T, μ_y), external pressure P_{ex}, bulk modulus B_0, average particle radius $<R>$, and total surface-to-volume ratio q. This equation contains the information necessary for what is in effect an "environmentally sensitive" phase map of TiO_2 (Barnard and Xu, 2008). Density functional theory is used to calculate the energies from this equation for all the relevant facets of anatase and rutile in the presence of the adsorbates OH and H_2O.

Acknowledging the fact that the reason TiO_2 nanoparticles are used in sunscreens that are commercially popular is their transparency, as well as the sun-protection factor, Barnard also computes the latter two quantities as a function of particle size. Combining these two quantities with the toxicity (assumed proportional to ROS levels), Barnard concludes that the TiO_2 in the sunscreen will need to be of high concentration of nanoparticles smaller than 13 nm in diameter for maximal efficacy and transparency and minimal toxicity. However, the body has significant capacity to tolerate ROS and it is only when this is overwhelmed that toxicity is observed.

The major caveat of this work is, of course, the assumption that the only source of toxicity from these nanoparticles comes from their photo-induced production of ROS; other mechanisms of toxicity or adverse effects may become operational particularly at these very small

sizes. We note that the majority of published literature indicates that nanoparticles do not penetrate normal healthy skin (Brayner, 2008; Osmond and McCall, 2010), although other reports from human trials show that zinc from ZnO particles in sunscreens does enter the body but could not determine whether it was still in a nanoparticulate form (Gulson et al., 2008). However damaged skin, hair follicles, and even flexing of normal skin are potential routes for such penetration (Barnard, 2010a; Brayner, 2008; Nel et al., 2006). We also note in passing that nanoparticles have been shown to penetrate the blood–brain barrier and affect the aggregation of proteins, as well as potentially causing ROS-associated damage (Oberdorster et al., 2009). Both of these mechanisms have been implicated in the neurotoxicity occurring in Alzheimer's disease (Cappai and Barnham, 2008); however, this is normally caused by Cu^{2+} bound to amyloid-beta peptide reacting with endogenous reducing agents (Chapter 12).

Statistical Modeling and Machine Learning Methods

The use of QSAR approaches for nanomaterials toxicity prediction has been increasing rapidly over the last 5 years. These cost and time effective techniques can be applied to relate different types of toxic effects or biological activities of the materials to measurable or calculable descriptors, such as particle size, shape, surface area, or zeta potential. A schematic representation of the QSAR workflow is given in Fig. 4.2.

Fourches et al. (2010) reported one of the first investigations of the potential of cheminformatics methods to establish statistically significant relationships between the biological profiles of nanoparticles and their physical, chemical, and geometric properties. They studied

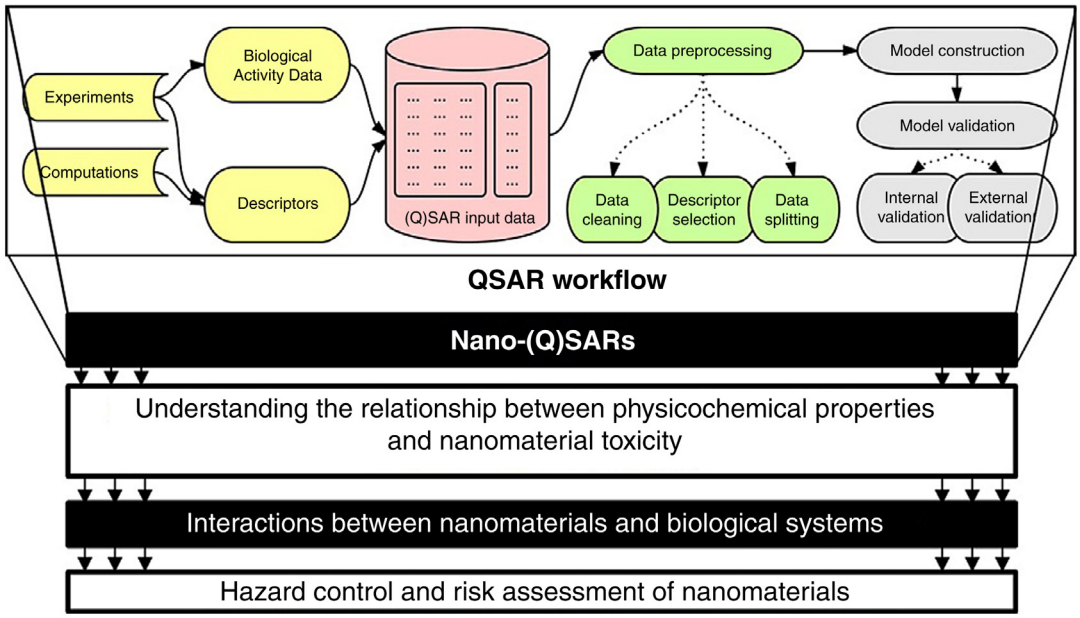


FIGURE 4.2 QSAR modeling of nanomaterial toxicity. *Source: Oksel, C., Ma, C.Y., Liu, J.J., Wilkins, T., Wang, X.Z., 2015. (Q)SAR modelling of nanomaterial toxicity: a critical review. Particuology 21, 1–19, with permission from Elsevier.*

two data sets reported by the Harvard groups (Weissleder et al., 2005; Shaw et al., 2008). Weissleder et al. (2005) used fluorescent magnetic nanoparticles to screen a library of nanoparticles decorated with various small molecules against a number of different cell lines, that is, human umbilical vein endothelial cells (HUVEC), human pancreatic adenocarcinoma cells (PaCa2), a human macrophage-like cell line (U937), primary resting human macrophages (RestMph), and human macrophages activated by granulocyte macrophage colony stimulating factor (GMCSF_Mph). Shaw et al. (2008) published a proof-of-concept study of the effects of 50 different nanomaterials at 4 different doses in 4 cell types, using 4 physiologic assays, a total of 3200 data points. The 50 nanoparticles possessed varying core compositions, coatings, and surface attachments. For the first data set consisting of biochemical assay data for 50 nanoparticles, they conducted a binary classification study by transforming the 64 assay results for each nanoparticle into a single value by calculating their arithmetic mean. They defined two binary classes using an arbitrary threshold to split the set into two groups each containing the same number of nanoparticles. Support vector machines were used to develop a classification model that had an external prediction accuracy of 56%–88%. For the second set consisting of cellular uptake data for 109 nanoparticles with different small molecules covalently attached to their surfaces, they modeled uptake of the particles using molecular descriptors and a *k*-nearest neighbors modeling approach. These models had prediction accuracies expressed as the r^2 value of 0.72 on average. This seminal paper showed that nanoparticle properties could be modeled successfully using classification and quantitative methods.

In a similar vein, Puzyn et al. (2011) reported a very simple nano-QSAR model relating the toxicity of 17 types of metal oxide nanoparticles toward *Escherichia coli*. They found an excellent relationship between a single nanoparticle property, $\Delta H_{\mathrm{Me+}}$, and the concentration of nanoparticles causing 50% loss in viability of the bacteria, EC_{50}.

$$\log \mathrm{EC}_{50} = 2.59 - 0.50\,\Delta H_{\mathrm{Me+}}$$

$\Delta H_{\mathrm{Me+}}$ is the enthalpy of formation of a gaseous cation having the same oxidation state as the metal ion structure. This descriptor combines information about two processes involved in toxicity, dissolution, and the sum of the ionization potential of the metals. The model performed very well on the training data (predicting 85% of the variance in the data), and internal and external validation sets (77 and 83% of the variance in the data).

Two of us (Epa and Winkler) have employed sparse feature selection methods, and parsimonious nonlinear methods for quantitative modeling of structure–property relationships (Burden and Winkler, 1999, 2009a,b). We conducted a more intensive study of the nanoparticle data sets from the Harvard groups (Weissleder et al., 2005; Shaw et al., 2008) using sparse nonlinear methods employing Bayesian neural networks and sparse Bayesian feature selection methods reported recently in the literature (Figueiredo, 2003; Mackay, 1992). We studied all of the data quantitatively, rather than using a single measure of biological activity (for the biochemical assay data) or a linear cluster analysis method for uptake in a single cell type as the previous modeling study did (Fourches et al., 2010). These techniques generated high quality, nonlinear, predictive quantitative structure–activity models for both data sets. Apart from improving model predictivities, we also elucidated the mechanisms by which nanoparticles are taken up by cells, or influence cellular physiology (Epa et al., 2011). The models based on data from Shaw et al. (2008) describing the effect of 50 diverse hard nanoparticles on apoptosis of 4 cells types

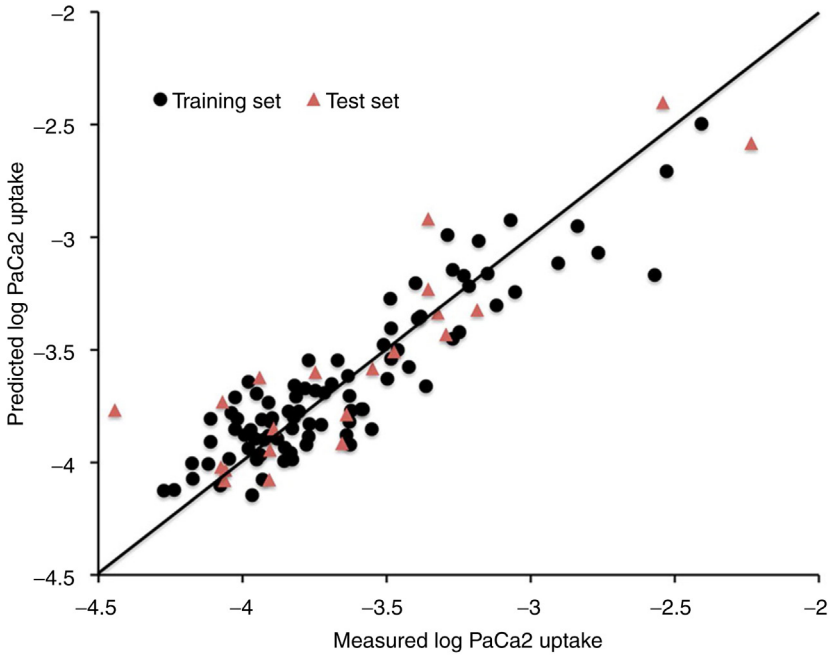


FIGURE 4.3 **Performance of PaCa2 nanoparticle uptake model for the training set *(dots)* and test set *(triangles)***
Source: Reprinted from Epa, V.C., Burden, F.R., Shaw, S., Tassa, C., Weissleder, R., Winkler, D.A., 2011. Modelling cellular uptake and apoptosis by nanoparticles. J. Am. Chem. Soc., with permission from ACS.

yielded a simple QSAR model. This explained smooth muscle apoptosis quantitatively in terms of the relaxivities and zeta potentials of the 50 nanoparticles. The models of the second nanoparticle uptake data set (Weissleder et al., 2005) yielded excellent quantitative nano-QSAR models for uptake in pancreatic cancer and human umbilical endothelial cell (HUVEC) lines. Uptake in macrophages and macrophage-like cells was smaller than in the cancer and HUVEC lines, and showed little variation with the identity of the small molecules functionalizing the nanoparticle surfaces. The models were able to successfully predict the cellular uptake of nanoparticles in an independent test set of nanoparticles. Fig. 4.3 shows the performance of the pancreatic cancer nanoparticle uptake model for 108 nanoparticles.

These three early studies have shown that statistical modeling and machine learning methods embodied in QSAR methods are capable of modeling data on the biological effects of nanoparticles, sometimes with relative simplicity. One reason for this may be that methods, such as neural networks are capable of modeling very complex biological phenomena using pattern recognition, albeit with loss of mechanistic clarity. An increasing number of studies on QSAR modeling of nanomaterial toxicity and biological properties have been reported over the last 5 years. A recent comprehensive review of the state of the art in QSAR modeling of nanoparticle biological properties was published by Oksel et al. (2015). Another review which only focused on carbon-based nanomaterials was written by Salahinejad (2015). The development of descriptors for nanomaterials has been covered in a number of papers by Toporov and Toporova (Toropov and Toropova, 2014, 2015a,b;

Toropova and Toropov, 2015; Toropov et al., 2015a,b; Toropova et al., 2014, 2015a,b; Veselinovic et al., 2015). Winkler (2015) also recently published a paper on advances and unresolved issues in the application of computational modeling to the prediction of the biological effects of nanomaterials.

So far, a relatively large number of successful QSAR models have been reported for the biological effects of nanoparticles with metal oxide cores. Electronic properties, such as conduction band and frontier orbital energies, and hydration energies have been used as descriptors in most of these studies as they provided a mechanistic link to reactive oxygen species causing cell toxicity (Kar et al., 2014a,b; Kaweeteerawat et al., 2015; Puzyn et al., 2011). For example, Le et al. (2015a) reported a large experimental and modeling study of the toxicity of ZnO nanoparticles that had been modified in various ways to ameliorate any potentially toxic effects. Cell damage and stress was evaluated using three biological assays The models showed how the type and concentration of metal oxide doping in the ZnO nanoparticle, the particle shape and size, and the types of surface coating influence the toxic effects on HUVEC and HepG2 cells.

In another recent study, the interactions of surface functionalized gold nanoparticles with specific, common human enzymes, and different types of cancer cells were modeled using QSAR methods. The aim of these studies was to understand and control how surface chemistry modulates the specific and nonspecific interactions of nanoparticles with enzymes, and the uptake in cells (Le et al., 2015b; Liu et al., 2015b). They showed how the nanoparticle surface functionality identified as having a major influence on the inhibition of acetylcholinesterase by the QSAR models could interact with the active site of the enzyme. They also described how decorating the surface of gold nanoparticles with two families of chemical substituents could selectively target cancer cells, opening the door to better selective toxicity toward these cells.

The prediction of nanoparticle transport has been successfully performed using random forest classification and regression methods (Goldberg et al., 2015). The interaction of pristine and surface-modified nanoparticles with proteins and enzymes has also been modeled using machine learning or other statistical approaches (Chen et al., 2014; Kamath et al., 2015; Liu et al., 2015a,c; Rihn and Joubere, 2015; Walkey et al., 2014; Wang et al., 2015; Winkler et al., 2014). A brief review of the use of molecular dynamics to model nanoparticle—protein interactions is also provided in a later section in the chapter.

Nanotoxicological Fingerprints to Classify Nanomaterials

Regulatory agencies assessing the risk of chemicals in the workplace, marketplace, or environment use classification schemes to group chemicals with similar biological profiles. Two groups have reported studies aimed at generating similar classification thresholds or indices for nanomaterials. If suitable indices could be found, the hazard (intrinsic toxicity) and risk (intrinsic toxicity and likely exposure) assessment of nanomaterials for regulatory purposes could be simplified. While the two recent reports represent the first attempts to achieve these aims, and they relate to relatively narrow biological effects, they show that such a scheme may be achievable.

Xia et al. (2010) described an index for characterizing adsorption of small molecules onto the surfaces of nanoparticles. They defined five indices or descriptors that described the

adsorption properties of 28 small organic molecules onto multiwalled carbon nanotubes (MWCNT). The indices described a wide range of physical interactions between the molecules and the nanotube surface: molar refractivity R, the lipophilicity π, effective hydrogen bond acidity α, effective hydrogen bond basicity β, and the molecular volume V. The aim of the study was to define a set of "probe" molecules whose interactions with nanoparticles could be used as indices or classification descriptors for predicting various specific biological effects of nanoparticles. The study effectively reported a quantitative structure–property relationship that could predict 93% of the variance in the data used to build the model, and 88% of the variance in an independent test set of molecules not used in training. The adsorption of the small molecules onto the MWCNTs correlated strongly with the lipophilicity (logP octanol/water partition coefficient).

More recently, Liu et al. described classification nanoSAR models for the effects of nine metal oxide nanoparticles against bronchial epithelial cells. They used a high throughput screening approach to generate the data at nanoparticle concentrations between 0.375 and 200 mg/L, and a set of 14 intuitive physicochemical descriptors to model the toxicity (Liu et al., 2011). Logistic regression was used to determine the probability that a given nanoparticle at a specified concentration will be toxic or nontoxic. The best performing model, containing only three descriptors, had 100% classification accuracy for both internal and external validation data sets. These key descriptors were the nanoparticle size, and the nanoparticle metal period and oxide atomization energy. The authors propose that, although this simple model performed well, a much larger data set would be required to increase the domain of applicability (range of nanomaterials types) and confidence of data-driven nanoSAR models.

Molecular Modeling and Molecular Dynamics Methods

Molecular dynamics is a physics-based modeling method that provides detailed information on the fluctuations and conformational changes of atoms and molecules in materials. Liu and Hopfinger (2008) were the first to report the use of molecular dynamics simulations to explore four possible sources of cellular toxicity due to the insertion of a carbon nanotube into a dimyristoylphosphatidylcholine (DMPC) membrane bilayer. Comparisons of (1) the structural organization of the membrane bilayer, (2) dynamical features of the membrane bilayer, and (3) transport of small polar molecules across the membrane bilayer were carried out with, and without, a carbon nanotube inserted into the bilayer (Fig. 4.4). A fourth study was performed to determine how the transport of solvated ions through the inserted nanotube might alter the structure of the membrane bilayer.

Comprehensive reviews on the use of molecular dynamics, as well as Monte Carlo and dissipative particle dynamics simulations to model the uptake of pristine nanoparticles by biomembranes have been published recently by Ding and Ma (2015) and Qu et al. (2013). Most of the research reported so far has focused on the effect of nanoparticle properties in cellular uptake. The size of nanoparticles has been found to greatly influence the cellular uptake pathways. Smaller nanoparticles near atomic dimension can enter cells through direct transmembrane diffusion or via protein channels whereas larger nanoparticles enter cells via endocytic pathways (Ding and Ma, 2015). Nanoparticle shape was proved to be another important factor in cellular uptake. Yang and Ma (2010) used a coarse-grained method to

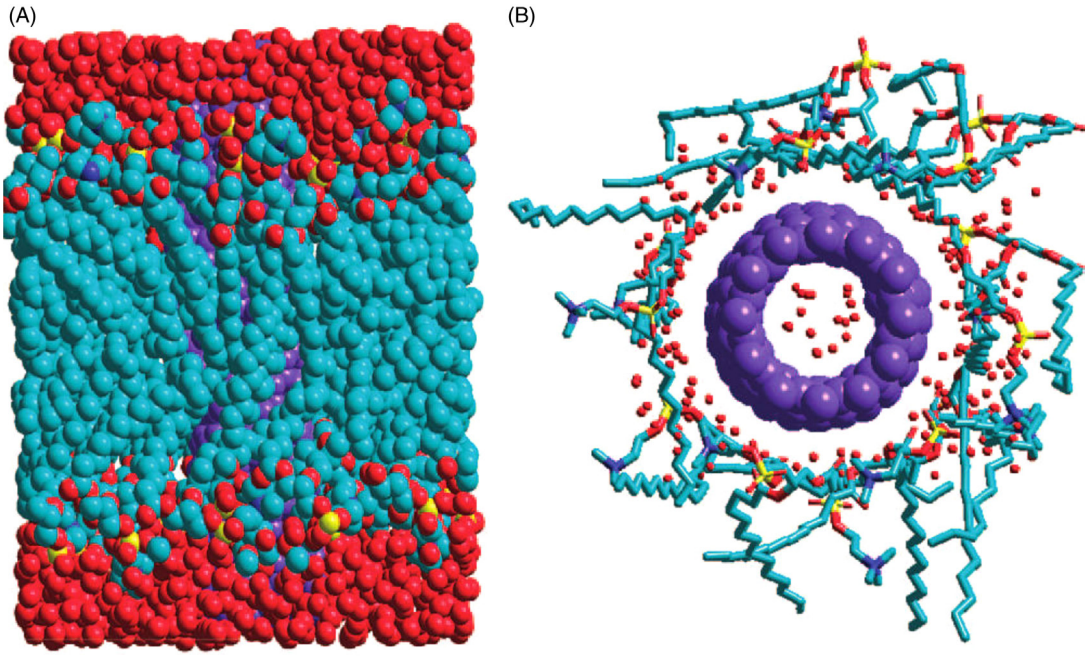


FIGURE 4.4 Effect of a carbon nanotube inserted into a phospholipid bilayer (A), showing exclusion of lipids from the zone around the nanotube (B). *Source: Reprinted from Liu, J.Z., Hopfinger, A.J., 2008. Identification of possible sources of nanotoxicity from carbon nanotubes inserted into membrane bilayers using membrane interaction quantitative structure-activity relationship analysis. Chem. Res. Toxicol. 21, 459–466, with permission from ACS.*

simulate the direct penetration of nanoparticles of different shapes across a lipid bilayer. They found that the shape anisotropy and initial orientation of the nanoparticle were crucial to the nature of the interaction between the nanoparticle and lipid bilayer. The nanoparticle can rotate to present a sharp edge when penetrating the membrane. For the endocytosis, the effect of shape is more complicated. Vacha et al. (2011) showed that spherocylinders can lead to a more efficient delivery than spheres of the same diameter because of the smaller mean curvature. Some other works showed that it was more difficult for ellipsoidal particles to be internalized than spherical ones due to higher local curvature (Bahrami, 2013; Dasgupta et al., 2013, 2014a,b).

A number of other nanoparticle studies using molecular dynamics have been reported over the last 2 years. De Leo et al. (2013) simulated the nanoparticle interactions with monoclonal antibodies. This was the first reported all-atom explicit solvent simulation investigating the structural and dynamic properties of a noncovalent bioconjugate in which the antibody was adsorbed on a carbon nanotube surface. Wang et al. (2015) and Tavanti et al. (2015a,b) tackled the interaction dynamics of nanoparticles with proteins. Zhang (2014) investigated nanoparticle aggregation and Todorova et al. (2013) validated the autocatalytic self-assembly of amyloidogenic peptides triggered by the penetration of nanoparticles through the brain.

ELUCIDATING INTERACTIONS BETWEEN NANOMATERIALS AND PROTEINS

In complex physiological or natural environments, nanoparticles and other nanomaterials often do not remain in the pristine state. Proteins and other biomolecules bind rapidly to metal/metal oxide nanoparticles, for example, forming a protein/biomolecule corona that is the actual "biologically relevant entity" that interacts with cells in the first instance (Bhunia et al., 2013; Docter et al., 2015a,b). It is the corona that determines the fate of nanoparticles in biosystems. The corona formation is subject to a number of factors, such as the nanoparticle size, shape, and surface chemistry, or the nature of the environment in which the nanoparticle resides, particularly the relative concentrations and binding affinities of the biomacromolecules. This corona coating is dynamic, that is, can change over time or when the nanoparticle travels from one biological compartment to another. The ability to explain the corona formation and evolution is a crucial step in understanding the biological effects and toxicity of nanoparticles and other nanomaterials (see Chapter 1). Clearly there are also some types of nanoparticles that adventitiously or by design attract little or no protein corona; see, for instance, recent work by Rotello (Bhunia et al., 2013).

Gold nanoparticle–protein interactions have been the subject of many reported studies. Hoefling et al. (2010) laid the foundation by studying the interaction between gold (111) surface and individual amino acids. The potential of mean force between each of the 20 amino acids and the gold surface was calculated. A strong dependence of the binding affinities on the amino acid chemistry was found. The interaction free energy was also identified to correlate with the propensity of amino acids to form β-sheets. The interaction between gold nanoparticles and peptides has been studied by different groups. Verde et al. (2009) reported all atom MD simulations for 14 residue gold-binding and nongold-binding peptides. The dependence of adsorption on both the identity of the amino acids and the mobility of the peptides was examined. The results showed that adsorption onto the gold surface took place when peptides consisted of mostly random coils or when their secondary structural motifs (helices or sheets) were short and connected by flexible hinges. Braun et al. (2002) used MD simulations to predict the secondary structures of three polypeptides consisting of 84, 84, and 94 residues when bound to Au (111) and (211) surfaces. The adsorption energy was calculated and the binding pathways were assessed. Lee and Ytreberg (2012) performed MD simulations for six different peptides bound to gold nanoparticles in water. Results showed that conjugating the peptides to gold nanoparticles might increase or decrease the peptide conformation flexibility depending on the amino acid sequences. The peptide structures could become more extended or compact. MD methods were also employed by Lee and Schatz to study the conformation of four single-strand DNAs attached to the (111) faces of a 201 atom truncated octahedral gold particle (Lee and Schatz, 2009a), as well as six highly packed single- and double-strand DNAs on a flat gold surface (Lee and Schatz, 2009b).

A number of studies have focused on the interaction between gold nanoparticles and human proteins, such as ubiquitin, albumin, and insulin. Brancolini et al. (2012) reported ubiquitin–gold nanoparticle interactions studied using atomic simulations. Both bare gold and gold covered in citrate were taken into account. It was found that short-range, nonelectrostatic interactions and binding to citrate surfactant were the most significant factors. Some citrate molecules were found to interact with the adsorbed protein. Tavanti et al. (2015a) also studied

citrate-coated gold nanoparticles but looked at the interactions between these nanoparticles and insulin or fibrinogen as two of the most abundant proteins in the plasma. In this case the corona was composed of a single layer of proteins. A maximum of 20 insulin molecules could bind to the citrated-coated nanoparticles of 5-nm core diameter whereas only 3 fibrinogens were able to make persistent interactions with the nanoparticle. The binding site for insulin was specific and constituted mainly by the C-terminal residues of the two dimer chains regardless of the number of insulin molecules considered in the simulations. In contrast, fibrinogen had a different binding mode that depended on the protein concentration and composition. The authors also observed a competitive process for gold nanoparticle binding when both proteins were present in the simulations. The overall secondary structure of both proteins was maintained upon binding to the gold nanoparticles. However, small changes in helix and sheet percentages were found and a partial unfolding of the α-helix bundle was found for fibrinogen when bound to two nanoparticles. Apart from citrate coating, (16-mercaptohexadecyl)trimethylammonium bromide (MTAB) coating on 12-nm gold nanoparticles has also been studied by Lin et al. (2015). MD simulations provided insights on the orientation of α-synuclein protein on the nanoparticles. The N-terminus of the protein molecule bound to the citrate-coated gold nanoparticles whereas the C-terminus bound to the MTAB particles, as can be seen in Fig. 4.5. The interactions between gold nanoparticles and a set of small human proteins or a DNA were the subject of a study by Krupa et al. (2014). They showed the secondary structure of protein may either be maintained or destroyed on binding to gold

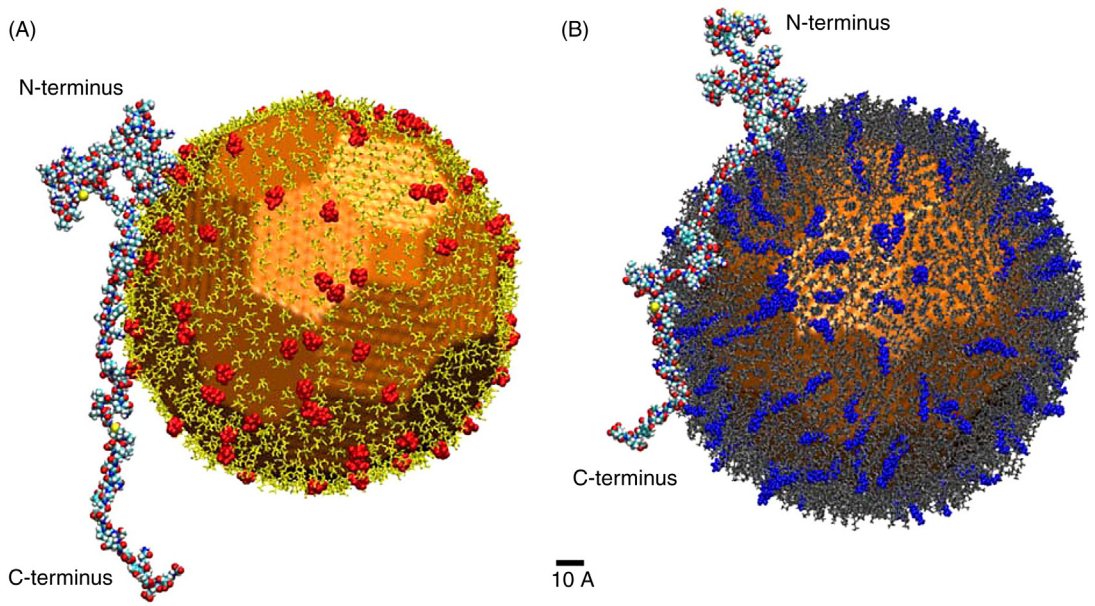


FIGURE 4.5 Binding of α-synuclein to (A) citrate coated and (B) MTAB-coated gold nanoparticles, where 10% of citrate and MTAB ligands are charged. *Source: Reprinted from Lin, W., Insley, T., Tuttle, M.D., Zhu, L.Y., Berthold, D.A., Kral, P., et al., 2015. Control of protein orientation on gold nanoparticles. J. Phys. Chem. C. 119, 21035–21043, with permission from ACS.*

nanoparticles. For a gold cluster of 64 atoms, the nanoparticles could bind to the DNA major or minor grooves and break the Watson–Crick interactions between the base pairs. Ramezani et al. (2014) studied the effect of gold nanoparticle shape on the albumin protein–nanoparticle interaction and observed an unfolding (denaturing) of the protein structure and a decrease in the helix formation in all cases. The authors found that cubic gold nanoparticles had stronger unfolding effects on the albumin than the spherical ones.

Studies on the interaction between silver nanoparticles and protein have also been reported. MD simulations were used by Ding et al. (2013) to characterize the silver nanoparticle–ubiquitin corona formation. Ubiquitin was observed to compete with citrate at the nanoparticle surface and bind to the particle in a specific manner. A multilayer corona of ubiquitins was formed on the surface of the nanoparticle when the protein/nanoparticle stoichiometry was high. Furthermore, the binding exhibited an unusual stretched–exponential behavior and destabilized the α-helices while increasing the β-sheets of the proteins. Interactions between branched polyethylenimine (BPEI) coated silver nanoparticles and lysozyme or alpha lactalbumin were studied by Wang et al. (2015). MD offered insights into the differential structure, dynamics, and thermodynamics of the coated silver nanoparticles binding with the two protein homologs. The binding had a minimal impact on the secondary structure of lysozyme but a strong effect on that of lactalbumin. The latter lost its structure as the temperature rose, but did not dissociate from the nanoparticles.

The binding of blood proteins including fibrinogen, immunoglobulin, albumin, transferrin, and ferritin to single wall carbon nanotubes was studied by Ge et al. (2011). These proteins were found to have different adsorption capacities and packing modes. The π–π stacking interactions between the nanotubes and aromatic residues (Trp, Phe, Tyr) were suggested to play a critical role in the adsorption capacity. The impact of hydroxylated fullerene and its derivatives with different surface chemistry on the structure of dynamics of ubiquitin was reported by Radic et al. (2014). All derivatives were observed to bind to the model protein. The highly hydrophilic nanoparticles had a larger number of hydroxyl groups bound to the protein surface and helped stabilize the protein structures. In contrast, fullerene derivatives with a small number of hydroxyl groups tucked their hydrophobic surface inside the protein and caused protein denaturation. Noon et al. (2002) also studied the fullerene–protein interaction using MD simulations. The fullerene buckyball was mainly surrounded by hydrophobic amino acid side chains. There was a rich set of π-interactions between the buckyball and the aromatic side chains of the antibody protein. A three-tiered π-stacking interaction was observed between the buckyball, Tyr-52 and Trp-33. Two distinct interaction modes for the side chains of Trp-47 and Arg-50 were observed.

Silica nanoparticles and cytochrome *c*, RNase A or lysozyme enzyme interactions have been studied by Sun et al. (2014). All enzymes were found to absorb onto the surfaces of 4-nm or 11-nm silica nanoparticles and the smaller nanoparticles induced greater structural stabilization. The active site of cytochrome *c* was observed to be far away from the surface of 4-nm nanoparticles but was adsorbed onto the surface of the 11 nm ones.

Overall, although there have been a number of studies on the interactions between different nanoparticles and proteins, a lot more work needs to be done to provide a comprehensive understanding. The development of new force fields and efficient computational sampling techniques will lead to a wider application of computational approaches and for larger simulation systems.

OTHER MODELING APPROACHES FOR NANOTOXICOLOGY

Other quantitative approaches exist for modeling the biodistribution of internalized nanomaterials and the consequential adverse responses. They are the Physiologically-Based-Pharmaco-Kinetics (PBPK) and Pharmaco-Dynamics (PBPD) models (MacCalman et al., 2009). The PBPK models describe the pathways from exposure (via inhalation, ingestion, or dermal exposure) to the internal dose and the distribution of this internal dose beyond the portal of entry—the lung, gut, and skin—to secondary organs. The PBPD models describe the pathways from the deposited dose, at the target tissues, to the responses at the cellular, molecular level. These models are based mainly on data from animal experiments. However, recent advances in in vitro techniques have allowed the estimation of some key model parameters (e.g., partition coefficients).

These models have captured the most important aspect of toxicology, namely—that dose causes the effect. The subsequent dosimetric question, "what metric(s) can best describe the dose?," will help in integrating QSAR and PBPK models. Indeed, since the kinetics of nanoparticles in the body are also dependent on their physicochemical characteristics, it is also possible to use QSAR techniques to determine the kinetics parameters of PBPK models. PBPK/PD models can be extrapolated from their basis in animal data to humans by means of allometric scaling (Feng et al., 2000). The resulting "human based" model can be used for risk assessment. The model can be further extended to capture the uncertainty and variations observed in the data. This is done by assigning different probabilistic distributions to the model parameters resulting in a probabilistic distribution for each model output instead of a point prediction as in the case of a deterministic model (Marino and Starr, 2007). PBPK models are also used to extrapolate to lower exposure levels and derive a no effect level of exposure. This Derived No Effect Level (DNEL) is compared to the measured exposure level (in the workplace or ambient air) to assess risk. The integration of QSAR and PBPK/PD will help to extend PBPK/PD models to a wider class of engineered nanomaterials.

CONCLUSIONS

The few publications summarized here provide substantial encouragement that computational modeling methods can provide a valuable and synergistic element to the understanding and prediction of biological effects, particularly adverse effects, of engineered nanomaterials. Even at this early stage, it has been shown that several of the computational tools that have been invaluable for design of biologically active molecules, or predicting their properties or toxicities, are useful for modeling properties of nanomaterials. Recent reviews have summarized the still relatively small amount of published work on the application of computational modeling to the prediction of the biological effects of nanomaterials (Winkler, 2015) or machine learning and statistical modeling tools to complex nonbiological materials more generally (Le et al., 2012). This reinforces the likelihood that these and the quantum chemical and molecular dynamics methods exemplified earlier will find a useful role in assessing the risk of nanomaterials in the workplace and environment.

There are a number of potential roadblocks and challenges that computational nanotoxicology needs to address. Statistical modeling and machine learning methods require

nanomaterials to be represented mathematically by a set of *descriptors*, mathematical objects that encapsulate the molecular or physicochemical properties of nanomaterials. Development of new *nano-specific* descriptors is a major research need. Unlike chemicals, the structures of nanomaterials change in an environment-specific manner. Nanoparticles may be coated with ions, proteins, or other molecules like humic substances. The physical form nanoparticles adopt in a given environment is poorly understood. Modeling biological effects of nanomaterials requires measurement or prediction of the actual biologically active entity. QSAR approaches can play a valuable role in modeling and predicting the in situ form of nanoparticles. Similar computational methods can help predict the in vivo effects of nanomaterials based on the results of relevant in vitro assays and knowledge of the transport of nanomaterials through various biological or environmental compartments, thus extending the limited experimental in vivo data collected.

The key requirement for using these computational methods is having sufficient relevant experimental data to allow understanding of mechanisms and transport to allow robust predictive computational models to be developed that extend these data and provide a useful framework for regulation of engineered nanomaterials.

TAKE-HOME MESSAGES

- Nanomaterials are reaching the marketplace in increasing numbers but we have insufficient knowledge of their biological effects.
- Nanoparticles are changed by the environment in which they exist, taking on a corona of ions and/or complex molecules that affect their physicochemical and biological properties.
- Machine learning methods show great promise in modeling the changes that occur in nanoparticle structure in different environments, and in relating these to in vitro and in vivo biological responses.
- However, lack of sufficient experimental data, and need for new descriptors for some types of nanomaterials, is holding back the field.
- Other computational approaches using quantum chemistry or molecular dynamics can yield valuable insight into mechanisms of biological interactions of nanoparticles.
- Questions of how nanoparticles enter the body, how they are distributed, metabolized, and excreted are still largely unanswered.
- If the existing resources on nanoparticle manufacture, characterization, biological screening, modeling, and analysis can be harnessed by international collaboration, there are good prospects for making useful predictions of risk profiles of nanomaterials within a decade. High-throughput technologies will be pivotal in driving this progress.
- The development and use of such predictive models will guide regulators on how to minimize adverse effects of nanomaterials on humans and the natural environment, while maximizing useful application of these novel materials.

References

Ahlers, J., Stock, F., Werschkun, B., 2008. Integrated testing and intelligent assessment-new challenges under REACH. Environ. Sci. Pollut. Res. 15, 565–572.

Bahrami, A.H., 2013. Orientational changes and impaired internalization of ellipsoidal nanoparticles by vesicle membranes. Soft Matter 9, 8642–8646.

Barnard, A.S., 2006. A thermodynamic model for the shape and stability of twinned nanostructures. J. Phys. Chem. B. 110, 24498–24504.
Barnard, A.S., 2009a. Computational strategies for predicting the potential risks associated with nanotechnology. Nanoscale 1, 89–95.
Barnard, A.S., 2009b. How can ab initio simulations address risks in nanotech? Nat. Nanotechnol. 4, 332–335.
Barnard, A.S., 2010a. One-to-one comparison of sunscreen efficacy, aesthetics and potential nanotoxicity. Nat. Nanotechnol. 5, 271–274.
Barnard, A.S., 2010b. Modelling of nanoparticles: approaches to morphology and evolution. Rep. Prog. Phys. 73, 86502.
Barnard, A.S., 2014a. Clarifying stability, probability and population in nanoparticle ensembles. Nanoscale 6, 9983–9990.
Barnard, A.S., 2014b. In silico veritas. ACS Nano 8, 6520–6525.
Barnard, A.S., Snook, I.K., 2011a. Ideality versus reality: predicting the effect of realistic environments on the electronic properties of nanographene. Nanosci. Nanotechnol. Lett. 3, 59–62.
Barnard, A.S., Snook, I.K., 2011b. Modelling the role of size, edge structure and terminations on the electronic properties of graphene nano-flakes. Model Simul. Mater. Sci., 19.
Barnard, A.S., Xu, H.F., 2008. An environmentally sensitive phase map of titania nanocrystals. ACS Nano 2, 2237–2242.
Bhunia, A.K., Samanta, P.K., Saha, S., Kamilya, T., 2013. ZnO nanoparticle-protein interaction: corona formation with associated unfolding. Appl. Phys. Lett., 103.
Brancolini, G., Kokh, D.B., Calzolai, L., Wade, R.C., Corni, S., 2012. Docking of ubiquitin to gold nanoparticles. ACS Nano 6, 9863–9878.
Braun, R., Sarikaya, M., Schulten, K., 2002. Genetically engineered gold-binding polypeptides: structure prediction and molecular dynamics. J. Biomater. Sci. Polym. E. 13, 747–757.
Brayner, R., 2008. The toxicological impact of nanoparticles. Nano Today 3, 48–55.
Brazma, A., 2009. Minimum information about a microarray experiment (miame)—successes, failures, challenges. Sci. World J. 9, 420–423.
Brazma, A., Hingamp, P., Quackenbush, J., Sherlock, G., Spellman, P., Stoeckert, C., et al., 2001. Minimum information about a microarray experiment (MIAME)—toward standards for microarray data. Nat. Genet. 29, 365–371.
Burden, F.R., Winkler, D.A., 1999. Robust QSAR models using Bayesian regularized neural networks. J. Med. Chem. 42, 3183–3187.
Burden, F.R., Winkler, D.A., 2009a. An optimal self-pruning neural network and nonlinear descriptor selection in QSAR. QSAR Comb. Sci. 28, 1092–1097.
Burden, F.R., Winkler, D.A., 2009b. Optimal sparse descriptor selection for QSAR using Bayesian methods. QSAR Comb. Sci. 28, 645–653.
Cappai, R., Barnham, K.J., 2008. Delineating the mechanism of Alzheimer's disease A beta peptide neurotoxicity. Neurochem. Res. 33, 526–532.
Chen, P.Y., Seabrook, S.A., Epa, V.C., Kurabayashi, K., Barnard, A.S., Winkler, D.A., et al., 2014. Contrasting effects of nanoparticle binding on protein denaturation. J. Phys. Chem. C. 118, 22069–22078.
Dasgupta, S., Auth, T., Gompper, G., 2013. Wrapping of ellipsoidal nano-particles by fluid membranes. Soft Matter 9, 5473–5482.
Dasgupta, S., Auth, T., Gompper, G., 2014a. Shape and orientation matter for the cellular uptake of nonspherical particles. Nano Lett. 14, 687–693.
Dasgupta, S., Auth, T., Gompper, G., 2014b. Towards a mechanistic understanding of cellular uptake. Biophys. J. 106, 576a.
De Leo, F., Sgrignani, J., Bonifazi, D., Magistrato, A., 2013. Structural and dynamic properties of monoclonal antibodies immobilized on CNTs: a computational study. Chem. Eur. J. 19, 12281–12293.
Ding, F., Radic, S., Chen, R., Chen, P.Y., Geitner, N.K., Brown, J.M., et al., 2013. Direct observation of a single nanoparticle-ubiquitin corona formation. Nanoscale 5, 9162–9169.
Ding, H.M., Ma, Y.Q., 2015. Theoretical and computational investigations of nanoparticle-biomembrane interactions in cellular delivery. Small 11, 1055–1071.
Dix, D.J., Houck, K.A., Martin, M.T., Richard, A.M., Setzer, R.W., Kavlock, R.J., 2007. The ToxCast program for prioritizing toxicity testing of environmental chemicals. Toxicol. Sci. 95, 5–12.
Docter, D., Strieth, S., Westmeier, D., Hayden, O., Gao, M., Knauer, S.K., et al., 2015a. No king without a crown—impact of the nanomaterial-protein corona on nanobiomedicine. Nanomedicine 10, 503–519.
Docter, D., Westmeier, D., Markiewicz, M., Stolte, S., Knauer, S.K., Stauber, R.H., 2015b. The nanoparticle biomolecule corona: lessons learned—challenge accepted? Chem. Soc. Rev. 44, 6094–6121.

Epa, V.C., Burden, F.R., Shaw, S., Tassa, C., Weissleder, R., Winkler, D.A., 2011. Modelling cellular uptake and apoptosis by nanoparticles. J. Am. Chem. Soc. 12, 5808–5812.
Feng, M.R., Lou, X.C., Brown, R.R., Hutchaleelaha, A., 2000. Allometric pharmacokinetic scaling: towards the prediction of human oral pharmacokinetics. Pharm. Res. 17, 410–418.
Figueiredo, M.A.T., 2003. Adaptive sparseness for supervised learning. IEEE Trans. Pattern Anal. Mach. Intell. 25, 1150–1159.
Fourches, D., Pu, D.Q.Y., Tassa, C., Weissleder, R., Shaw, S.Y., Mumper, R.J., et al., 2010. Quantitative nanostructure-activity relationship modeling. ACS Nano 4, 5703–5712.
Ge, C.C., Du, J.F., Zhao, L.N., Wang, L.M., Liu, Y., Li, D.H., et al., 2011. Binding of blood proteins to carbon nanotubes reduces cytotoxicity. Proc. Natl. Acad. Sci. USA 108, 16968–16973.
Goldberg, E., Scheringer, M., Bucheli, T.D., Hungerbuhler, K., 2015. Prediction of nanoparticle transport behavior from physicochemical properties: machine learning provides insights to guide the next generation of transport models. Environ. Sci. Nano 2, 352–360.
Gulson, B., Wong, H., McCall, M., Casey, P., Trotter, J., McCulloch, M., et al., 2008. Dermal absorption of ZnO nanoparticles in sunscreen using the stable isotope approach. Toxicol. Lett. 180, S222.
Hansen, S.F., Maynard, A., Baun, A., Tickner, J.A., 2008. Late lessons from early warnings for nanotechnology. Nat. Nanotechnol. 3, 444–447.
Hoefling, M., Iori, F., Corni, S., Gottschalk, K.E., 2010. Interaction of amino acids with the Au(111) surface: adsorption free energies from molecular dynamics simulations. Langmuir 26, 8347–8351.
Jaworska, J., Gabbert, S., Aldenberg, T., 2010. Towards optimization of chemical testing under REACH: a Bayesian network approach to integrated testing strategies. Regul. Toxicol. Pharm. 57, 157–167.
Judson, R., Richard, A., Dix, D., Houck, K., Elloumi, F., Martin, M., et al., 2008. ACTOR—aggregated computational toxicology resource. Toxicol. Appl. Pharmacol. 233, 7–13.
Kamath, P., Fernandez, A., Giralt, F., Rallo, R., 2015. Predicting cell association of surface-modified nanoparticles using protein corona structure—activity relationships (PCSAR). Curr. Top. Med. Chem. 15, 1930–1937.
Kar, S., Gajewicz, A., Puzyn, T., Roy, K., 2014b. Nano-quantitative structure-activity relationship modeling using easily computable and interpretable descriptors for uptake of magnetofluorescent engineered nanoparticles in pancreatic cancer cells. Toxicol. In Vitro 28, 600–606.
Kar, S., Gajewicz, A., Puzyn, T., Roy, K., Leszczynski, J., 2014a. Periodic table-based descriptors to encode cytotoxicity profile of metal oxide nanoparticles: a mechanistic QSTR approach. Ecotoxicol. Environ. Saf. 107, 162–169.
Kaweeteerawat, C., Ivask, A., Liu, R., Zhang, H.Y., Chang, C.H., Low-Kam, C., et al., 2015. Toxicity of metal oxide nanoparticles in *Escherichia coli* correlates with conduction band and hydration energies. Environ. Sci. Technol. 49, 1105–1112.
Krupa, P., Mozolewska, M.A., Rasulev, B., Czaplewski, C., Leszczynski, J., 2014. Towards Mechanisms of Nanotoxicity—Interaction of Gold Nanoparticles With Proteins and DNA. CBSB14. Task Quarterly, Gdansk, Poland, p. 337–341.
Le, T., Epa, V.C., Burden, F.R., Winkler, D.A., 2012. Quantitative structure-property relationship modeling of diverse materials properties. Chem. Rev. 112, 2889–2919.
Le, T.C., Yin, H., Chen, R., Chen, Y., Zhao, L., Casey, P.S., et al., 2015a. An experimental and computational approach to the development of ZnO nanoparticles that are safe by design. ACS Nano 12, 3568–3577.
Le, T.C., Yan, B., Winkler, D.A., 2015b. Robust Prediction of personalized cell recognition from a cancer population by a dual targeting nanoparticle library. Adv. Funct. Mater. 25, 6927–6935.
Lee, O.S., Schatz, G.C., 2009a. Molecular dynamics simulation of DNA-functionalized gold nanoparticles. J. Phys. Chem. C. 113, 2316–2321.
Lee, O.S., Schatz, G.C., 2009b. Interaction between DNAs on a gold surface. J. Phys. Chem. C. 113, 15941–15947.
Lee, K.H., Ytreberg, F.M., 2012. Effect of gold nanoparticle conjugation on peptide dynamics and structure. Entropy Switz. 14, 630–641.
Lin, W., Insley, T., Tuttle, M.D., Zhu, L.Y., Berthold, D.A., Kral, P., et al., 2015. Control of protein orientation on gold nanoparticles. J. Phys. Chem. C. 119, 21035–21043.
Liu, J.Z., Hopfinger, A.J., 2008. Identification of possible sources of nanotoxicity from carbon nanotubes inserted into membrane bilayers using membrane interaction quantitative structure-activity relationship analysis. Chem. Res. Toxicol. 21, 459–466.
Liu, R., Jiang, W., Walkey, C.D., Chan, W.C.W., Cohen, Y., 2015a. Prediction of nanoparticles-cell association based on corona proteins and physicochemical properties. Nanoscale 7, 9664–9675.

Liu, R., Rallo, R., George, S., Ji, Z., Nair, S., Nel, A.E., et al., 2011. Classification NanoSAR development for cytotoxicity of metal oxide nanoparticles. Small 7, 1118–1126.
Liu, Y., Winkler, D.A., Epa, V.C., Zhang, B., Yan, B., 2015b. Probing enzyme-nanoparticle interactions using combinatorial gold nanoparticle libraries. Nano Res. 8, 1293–1308.
Liu, Y.Y., Winkler, D.A., Epa, V.C., Zhang, B., Yan, B., 2015c. Probing enzyme-nanoparticle interactions using combinatorial gold nanoparticle libraries. Nano Res. 8, 1293–1308.
MacCalman, L., Tran, C.L., Kuempel, E., 2009. Development of a bio-mathematical model in rats to describe clearance, retention and translocation of inhaled nano particles throughout the body. J. Phys. Conf. Ser. 151, 677.
Mackay, D.J.C., 1992. A practical Bayesian framework for backpropagation networks. Neural Comput. 4, 448–472.
Marino, D.J., Starr, T.B., 2007. Probabilistic dose-response modeling: case study using dichloromethane PBPK model results. Regul. Toxicol. Pharm. 49, 285–300.
Maynard, A., Rejeski, D., 2009. Too small to overlook. Nature 460, 174.
Morris, J., Willis, J., De Martinis, D., Hansen, B., Laursen, H., Sintes, J.R., et al., 2011. Science policy considerations for responsible nanotechnology decisions. Nat. Nanotechnol. 6, 73–77.
Nabholz, J.V., 1991. Environmental-hazard and risk assessment under the United-States Toxic-Substances Control Act. Sci. Total Environ. 109, 649–665.
Nel, A., Xia, T., Madler, L., Li, N., 2006. Toxic potential of materials at the nanolevel. Science 311, 622–627.
Nicholson, A., Sandler, J., Seidle, T., 2004. An evaluation of the US high production volume (HPV) chemical-testing programme: a study in (Ir)relevance, redundancy and retro thinking. Atla Altern. Lab. Anim. 32, 335–341.
Noon, W.H., Kong, Y.F., Ma, J.P., 2002. Molecular dynamics analysis of a buckyball-antibody complex. Proc. Natl. Acad. Sci. USA 99, 6466–6470.
Oberdorster, G., Elder, A., Rinderknecht, A., 2009. Nanoparticles and the brain: cause for concern? J. Nanosci. Nanotechnol. 9, 4996–5007.
Oksel, C., Ma, C.Y., Liu, J.J., Wilkins, T., Wang, X.Z., 2015. (Q)SAR modelling of nanomaterial toxicity: a critical review. Particuology 21, 1–19.
Osmond, M.J., McCall, M.J., 2010. Zinc oxide nanoparticles in modern sunscreens: an analysis of potential exposure and hazard. Nanotoxicology 4, 15–41.
Puzyn, T., Rasulev, B., Gajewicz, A., Hu, X.K., Dasari, T.P., Michalkova, A., et al., 2011. Using nano-QSAR to predict the cytotoxicity of metal oxide nanoparticles. Nat. Nanotechnol. 6, 175–178.
Qu, Z.G., He, X.C., Lin, M., Sha, B.Y., Shi, X.H., Lu, T.J., et al., 2013. Advances in the understanding of nanomaterial-biomembrane interactions and their mathematical and numerical modeling. Nanomedicine 8, 995–1011.
Radic, S., Nedumpully-Govindan, P., Chen, R., Salonen, E., Brown, J.M., Ke, P.C., et al., 2014. Effect of fullerenol surface chemistry on nanoparticle binding-induced protein misfolding. Nanoscale 6, 8340–8349.
Ramezani, F., Amanlou, M., Rafii-Tabar, H., 2014. Gold nanoparticle shape effects on human serum albumin corona interface: a molecular dynamic study. J. Nanopart. Res. 16, 2512.
Rihn, B.H., Joubere, O., 2015. Comment on "Protein Corona Fingerprinting Predicts the Cellular Interaction of Gold and Silver Nanoparticles". ACS Nano 9, 5634–5635.
Sakamoto, Y., Nakae, D., Fukumori, N., Tayama, K., Maekawa, A., Imai, K., et al., 2009. Induction of mesothelioma by a single intrascrotal administration of multi-wall carbon nanotube in intact male Fischer 344 rats. J. Toxicol. Sci. 34, 65–76.
Salahinejad, M., 2015. Nano-QSPR modelling of carbon-based nanomaterials properties. Curr. Top. Med. Chem. 15, 1868–1886.
Shaw, S.Y., Westly, E.C., Pittet, M.J., Subramanian, A., Schreiber, S.L., Weissleder, R., 2008. Perturbational profiling of nanomaterial biologic activity. Proc. Natl. Acad. Sci. USA 105, 7387–7392.
Sun, X.T., Feng, Z.W., Zhang, L.L., Hou, T.J., Li, Y.Y., 2014. The selective interaction between silica nanoparticles and enzymes from molecular dynamics simulations. PLoS One 9, e107696.
Takagi, A., Hirose, A., Nishimura, T., Fukumori, N., Ogata, A., Ohashi, N., et al., 2008. Induction of mesothelioma in p53+/- mouse by intraperitoneal application of multi-wall carbon nanotube. J. Toxicol. Sci. 33, 105–116.
Tavanti, F., Pedone, A., Menziani, M.C., 2015a. Competitive binding of proteins to gold nanoparticles disclosed by molecular dynamics simulations. J. Phys. Chem. C. 119, 22172–22180.
Tavanti, F., Pedone, A., Menziani, M.C., 2015b. A closer look into the ubiquitin corona on gold nanoparticles by computational studies. New J. Chem. 39, 2474–2482.
Todorova, N., Makarucha, A.J., Hine, N.D.M., Mostofi, A.A., Yarovsky, I., 2013. Dimensionality of carbon nanomaterials determines the binding and dynamics of amyloidogenic peptides: multiscale theoretical simulations. PloS Comput. Biol. 9, e1003360.

Toropov, A.A., Rallo, R., Toropova, A.P., 2015a. Use of Quasi-SMILES and Monte Carlo optimization to develop quantitative feature property/activity relationships (QFPR/QFAR) for nanomaterials. Curr. Top. Med. Chem. 15, 1837–1844.
Toropov, A.A., Toropova, A.P., 2014. Optimal descriptor as a translator of eclectic data into endpoint prediction: mutagenicity of fullerene as a mathematical function of conditions. Chemosphere 104, 262–264.
Toropov, A.A., Toropova, A.P., 2015a. Quasi-SMILES and nano-QFAR: united model for mutagenicity of fullerene and MWCNT under different conditions. Chemosphere 139, 18–22.
Toropov, A.A., Toropova, A.P., 2015b. Quasi-QSAR for mutagenic potential of multi-walled carbon-nanotubes. Chemosphere 124, 40–46.
Toropova, A.P., Toropov, A.A., 2015. Mutagenicity: QSAR—quasi-QSAR—nano-QSAR. Mini Rev. Med. Chem. 15, 608–621.
Toropov, A.A., Toropova, A.P., Veselinovic, A.M., Veselinovic, J.B., Nesmerak, K., Raska, I., et al., 2015b. The Monte Carlo method based on eclectic data as an efficient tool for predictions of endpoints for nanomaterials—two examples of application. Comb. Chem. High T. Scr. 18, 376–386.
Toropova, A.P., Toropov, A.A., Benfenati, E., Korenstein, R., Leszczynska, D., Leszczynski, J., 2015b. Optimal nano-descriptors as translators of eclectic data into prediction of the cell membrane damage by means of nano metal-oxides. Environ. Sci. Pollut. R. 22, 745–757.
Toropova, A.P., Toropov, A.A., Benfenati, E., Puzyn, T., Leszczynska, D., Leszczynski, J., 2014. Optimal descriptor as a translator of eclectic information into the prediction of membrane damage: the case of a group of ZnO and TiO_2 nanoparticles. Ecotoxicol. Environ. Saf. 108, 203–209.
Toropova, A.P., Toropov, A.A., Rallo, R., Leszczynska, D., Leszczynski, J., 2015a. Optimal descriptor as a translator of eclectic data into prediction of cytotoxicity for metal oxide nanoparticles under different conditions. Ecotoxicol. Environ. Saf. 112, 39–45.
Vacha, R., Martinez-Veracoechea, F.J., Frenkel, D., 2011. Receptor-mediated endocytosis of nanoparticles of various shapes. Nano Lett. 11, 5391–5395.
Vance, M.E., Kuiken, T., Vejerano, E.P., McGinnis, S.P., Hochella, M.F., Rejeski, D., et al., 2015. Nanotechnology in the real world: redeveloping the nanomaterial consumer products inventory. Beilstein J. Nanotechnol. 6, 1769–1780.
Verde, A.V., Acres, J.M., Maranas, J.K., 2009. Investigating the specificity of peptide adsorption on gold using molecular dynamics simulations. Biomacromolecules 10, 2118–2128.
Veselinovic, J.B., Toropov, A.A., Toropova, A.P., Nikolic, G.M., Veselinovic, A.M., 2015. Monte Carlo method-based QSAR modeling of penicillins binding to human serum proteins. Arch Pharm. 348, 62–67.
Walkey, C.D., Olsen, J.B., Song, F.Y., Liu, R., Guo, H.B., Olsen, D.W.H., et al., 2014. Protein corona fingerprinting predicts the cellular interaction of gold and silver nanoparticles. ACS Nano 8, 2439–2455.
Wang, B., Seabrook, S.A., Nedumpully-Govindan, P., Chen, P.Y., Yin, H., Waddington, L., et al., 2015. Thermostability and reversibility of silver nanoparticle-protein binding. Phys. Chem. Chem. Phys. 17, 1728–1739.
Weissleder, R., Kelly, K., Sun, E.Y., Shtatland, T., Josephson, L., 2005. Cell-specific targeting of nanoparticles by multivalent attachment of small molecules. Nat. Biotechnol. 23, 1418–1423.
Winkler, D.A., 2015. Recent advances, and unresolved issues, in the application of computational modelling to the prediction of the biological effects of nanomaterials. Toxicol. Appl. Pharmacol. 299, 96–100.
Winkler, D.A., Burden, F.R., Yan, B., Weissleder, R., Tassa, C., Shaw, S., et al., 2014. Modelling and predicting the biological effects of nanomaterials. SAR QSAR Environ. Res. 25, 161–172.
Xia, X.R., Monteiro-Riviere, N.A., Riviere, J.E., 2010. An index for characterization of nanomaterials in biological systems. Nat. Nanotechnol. 5, 671–675.
Yang, K., Ma, Y.Q., 2010. Computer simulation of the translocation of nanoparticles with different shapes across a lipid bilayer. Nat. Nanotechnol. 5, 579–583.
Zeeman, M., Auer, C.M., Clements, R.G., Nabholz, J.V., Boethling, R.S., 1995. U.S. EPA regulatory perspectives on the use of QSAR for new and existing chemical evaluations. SAR QSAR Environ. Res. 3, 179–201.
Zhang, W., 2014. Nanoparticle aggregation: principles and modeling. Adv. Exp. Med. Biol. 811, 19–43.

CHAPTER

5

Exposure Assessment

*Kai Savolainen**, *Antonio Pietroiusti*****

***Nanosafety Research Centre, Finnish Institute of Occupational Health, Helsinki, Finland;**
****University of Rome 'Tor Vergata', Rome, Italy**

OUTLINE

Background 103

How can Workers be Exposed to ENMs? 104

Exposure Routes 105

Characterization of Behavior of ENMs 106

Challenges to Assess Exposure to ENMs 108

Exposure Assessment of ENMs in Workplaces 111

Evidence for Release of Engineered Nanoparticles in the Workplace 111

Harmful Effects on Workers 112

How to Measure Nanoparticles: Metrics 113

How to Measure Nanoparticles: Measurement Strategies 113

Instruments Measuring Number Concentration 114

Instruments Measuring Surface Concentration 115

Instruments Measuring Mass Concentration 116

Instruments Giving Indirect Estimates 116

Sample Collection and Characterization 117

ENM Modifications After Their Release 117

Workers' Protection 118

Health Surveillance 119

Take-Home Messages 120

References 120

BACKGROUND

Nanotechnology is recognized as a cross-cutting technology, whose products, engineered nanomaterials (ENMs), possess different physical and chemical properties than their bulk counterparts. For this reason, they are widely used for a variety of applications (Schmid et al., 2010). However, during their production and use there is the chance of exposure for

Adverse Effects of Engineered Nanomaterials. http://dx.doi.org/10.1016/B978-0-12-809199-9.00005-7
Copyright © 2017 Elsevier Inc. All rights reserved.

workers, and the effects of such exposure cannot be predicted, given the fact that at the nanoscale level the material has different physico-chemical properties than in the bulk form. More than 1600 nanoenabled products are currently on the market (http://www.nanotechproject.org/cpi/), and, according to recent estimates, 6 million workers will be potentially exposed to engineered nanoparticles in 2020 (Roco, 2011). According to the definition of European Commission (EC), ENMs are "natural, incidental, or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1–100 nm" (European Commission, 2011). This definition implies that the number concentration of ENMs should be known in the process of risk assessment (indirectly suggesting the need for the evaluation of this parameter). Of note, the EC definition has legislative purposes, which means that adverse health effects may occur even at a lower level of exposure (i.e., an exposure to material having less than 50% of the particles in the nanoscale range).

Using the EC definition in the context of assessment of exposure to ENMs one needs to take into account that there are very few data on health effects subsequent to assessment of exposure by using number concentrations (Van Broekhuizen et al., 2012). All health-based values proposed for occupational or other exposure limits are based on the mass of nanoparticles (NIOSH, 2013). All proposed control values regarding exposure assessment in the occupational and based on particle number concentrations are precautionary, not health-based in nature (Van Broekhuizen et al., 2012). Hence, in assessing the usefulness of various proposals, their nature needs to be carefully considered. So far, most studies dealing with exposure to and effects of ENMs have used mass as the default for exposure (Guseva Canu et al., 2016; NIOSH, 2013; Van Broekhuizen et al., 2012).

HOW CAN WORKERS BE EXPOSED TO ENMs?

Exposure in the workplace may occur in case ENMs are released from a powder, a liquid, or a solid matrix during manufacturing, use or recycling (ISO, 2008). The handling of dry powders poses the highest risk of exposure, whereas in the case of ENMs embedded in a liquid or solid matrix, may be related to the amount of energy applied to these matrices during the industrial or laboratory process. Some processes typically associated with the delivery of higher amount of energy (e.g., production, spraying, and machining) seem to release more ENMs than lower energy processes, such as packing and bagging or cleaning. Interestingly, the physico-chemical characteristics of the ENM may have an influence on the rate of release. For example, Johnson et al. (2010) reported a threefold increase in the release of functionalized multiwalled carbon nanotubes (MWCNTs) from the same medium during the same process (sonication), in comparison to pristine MWCNTs.

There are a number of manufactured ENMs whose assessment is considered a high priority. The organization for economic cooperation and development (OECD)'s Working Party on Manufactured Nanomaterials has agreed upon a prioritized list of nanomaterials which are to be addressed (OECD, 2010). The list includes: single-walled carbon nanotubes (SWCNTs), MWCNTs, polystyrene, fullerene (C60), aluminum oxide, dendrimers,

titanium dioxide, zinc oxide, cerium oxide, iron, silver, gold, layered silicates (nanoclays), and silicon dioxide. Data on exposure are currently available for the vast majority of them (Pietroiusti and Magrini, 2014). Classification of one of the carbon nanotubes (CNT) as a possible carcinogen for humans by the International Agency for Research on Cancer (IARC) emphasizes the importance of evaluation of safety of this class of materials (Grosse et al., 2014).

EXPOSURE ROUTES

There is little doubt that the respiratory system is the prominent exposure route for ENM in the occupational environment (Borm et al., 2006; Shvedova et al., 2005). Due to their size, the primary region for the deposition of ENMs is the alveolar region of the human lung. Many ENMs are characterized by a high surface reactivity and may therefore induce inflammation and generation of reactive oxygen species at the site of deposition causing local injury (Donaldson et al., 2010; Ryman-Rasmussen et al., 2009). A very small fraction can translocate to internal organs and organ systems via lymphatics and blood, and may cause damage through direct and indirect mechanisms (Kreyling et al., 2014). In addition, even in the absence of ENM translocation, systemic damage may arise from the release of inflammatory mediators into the systemic circulation (Erdely et al., 2011; Saber et al., 2013). Donaldson et al. (2013) also introduce the Biologically Active Dose (BED) paradigm to be used in exposure, hazard, and risk assessment. BED is the fraction of the total dose that actually drives any toxic effect. In particulate inhalation toxicology, Donaldson et al. (2013) define BED as the entity within any dose of particles in tissue that drives a critical pathophysiologically relevant form of toxicity, such as oxidative stress, genotoxicity, or inflammation. To use the BED approach, one may have to use either mass, particle number, or surface area. BED may, in fact, be relevant for different routes of exposure, but most relevant for inhalational exposure to particles.

The gastrointestinal route is potentially important for consumers (Pietroiusti, 2012); however, it is considered less relevant for workers, at least in comparison to the pulmonary route. It should be considered, however, that a substantial percentage of inhaled nanoparticles are cleared by the muco-ciliary escalator cells into the oral cavity and thereafter into the gastrointestinal tract (Geiser and Kreyling, 2010), and that ENMs deposited in the skin may reach the gut lumen through hand–mouth contact. Once they reach the gut, ENMs may exert local toxic effects (Bergin and Witzman, 2013; Nguyen et al., 2015). An interesting new perspective is represented by the possible effects mediated by the interaction of ENMs with the gut microbiota (the community of organisms living within the gastrointestinal tract). There is, in fact, recent evidence that the gut microbiota may have a relevant role in modulating both local and systemic biological effects of ENMs (Pietroiusti et al., 2015).

The skin is the largest organ in the human body, and hence the potential contribution of dermal exposure to ENMs is hard to ignore. Estimates of possible dermal exposure to manufactured ENMs in the workplace have been reported (Van Duuren-Stuurman et al., 2010). However, no clear evidence of skin damage or penetration through the intact skin is currently available.

For a thorough discussion of the potential impact of ENMs on the lungs, the skin, and the gastrointestinal tract, refer to Chapters 9, 10, 15, and 16, respectively.

CHARACTERIZATION OF BEHAVIOR OF ENMs

One major uncertainty in the safety and risk evaluation of ENMs arises from a lack of systematic knowledge about the physico-chemical characteristics of the material arriving at the receptor (in this context the receiving organ or tissue), be it the nose, the skin, or the mouth of an exposed human. This is true for all forms of ENMs, whether they exist in the form of macroscopic solid objects, as powders, emulsions or suspensions, or as aerosols, that is, in the form mixture of air and the particles. All these ENMs are essentially constituted of nanoparticles, or at least nanostructured building blocks, such as agglomerates, with the potential of being released by some kind of mechanism into a transport chain from the "source," meaning an industrial process or handling of nanomaterials, to the receptor meaning in this context the exposed individual, whether a worker or a consumer (Nel et al., 2009).

An issue requiring special and thorough consideration is possible exposure to products containing ENM during the entire life cycle of the products. Many products are not likely to cause exposure of workers or consumers as long as the ENM are embedded in the polymers or other matrices in a given product (Savolainen et al., 2010). The situation may change, when a given material needs further processing, becomes waste, is weared during use, or is recycled and in that context, for example, crushed or, when dealing with surfaces painted with nanoparticle containing paints, sanded with a sanding machine that generate dust containing ENMs. A detailed discussion of this issue is beyond the scope of this chapter, but will require a thorough life cycle analysis as the number and volume of products containing ENMs rapidly increases.

The general release and transport mechanisms are perhaps best researched for aerosols, highly dynamic systems, and this information is also relevant to exposure. Nanoaerosols often consist of strongly agglomerated primary particles, already close to the source. Depending on their origins and transport history, these nanostructured agglomerates have a potential for subsequent break-up (Rothenbacher et al., 2008).

The tendency of airborne ENMs to agglomerate or become attached to the ubiquitous background aerosol is of special importance because this may very rapidly change their specific characteristics that depend on the size of the particles. Such transformations can be modeled for given scenarios provided enough information is available about the system (Seipenbusch et al., 2008). The changes in airborne ENMs characteristics during transport have several consequences. Indeed, a change in airborne size could alter the deposition mechanisms of ENMs and hence the effective dose of exposure. For the time being, it is not understood whether the agglomeration process affects the toxicological mechanisms as well. Additionally, growth and/or attachment of airborne ENMs to other particles challenge the existing methods for their adequate characterization. Separation and identification of ENMs against the submicron background aerosol originating from different sources is therefore a difficult or impossible task facing ENM monitoring and characterization in workplaces and other environments (Peters et al., 2009). The influence of environmental factors on the dynamic equilibrium

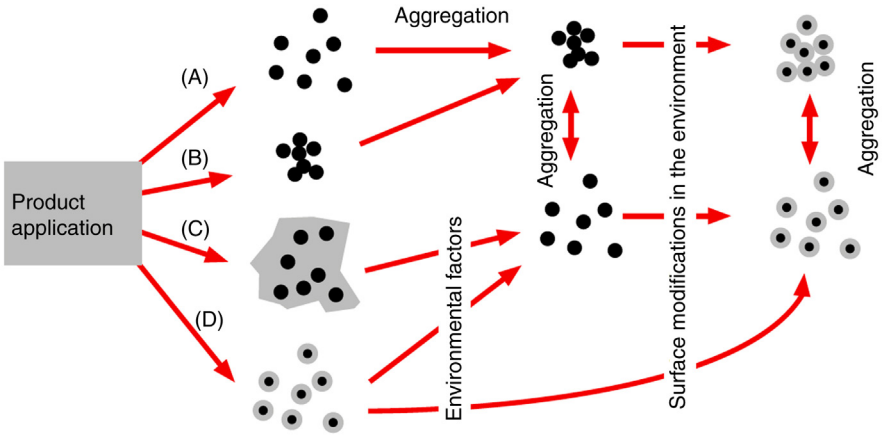


FIGURE 5.1 Release of ENMs. (A) free, (B) aggregated, (C) bound in a matrix, (D) functionalized. *Source: From Nowack and Bucheli, 2007; reprinted with permission from Savolainen, K. (Ed.) Nanosafety in Europe 2015–2025: towards safe and sustainable nanomaterials and nanotechnology innovations. Finnish Institute of Occupational Health, Helsinki.*

between agglomerated and deagglomerated ENMs released from different sources is schematically represented in Fig. 5.1.

At present, there are no rapid techniques available that would allow online distinction between background nanoparticles and ENMs. Instead, one has to collect the aerosol and do off-line image or compositional analysis, for example, by transmission electron microscopy (TEM). Therefore, TEM remains the gold standard for this type of analysis (Kuhlbusch et al., 2009; Peters et al., 2009).

Novel materials introduce entirely new particle shapes, such as CNT which, upon release into the air, can further form larger fiber-like structures resembling, for example, crocidolite fibers with potentially serious health consequences (Ryman-Rasmussen et al., 2009; Sakamoto et al., 2009). The addition of fullerene structures to the backbone of SWCNT produces nanobuds (Nasibulin et al., 2006) which may be associated with unexpected biological effects. The effects of functionalization of ENM add to the complexity of their risk assessment, and are beyond this discussion, and for the most part, are not done so far.

The characterization of airborne ENMs is thus complicated by the dynamic behavior of ENMs as an aerosol as well as the structural complexity of the individual particles. The large set of parameters required for their complete characterization, the range of ENMs already in use, and the multitude of biological responses create a special challenge for this undertaking. At present, there is no robust set of devices which could be used for monitoring, measuring, and characterizing engineered nanoparticles in workplace environments. A publication of European Agengy for Safety and Health at Work (EU-OSHA) (European Agency for Safety and Health at Work, 2009) provides a hierarchical set of terms for ENMs. Nanoobject is the highest term divided into nanoparticles, nanorods, and nanoplates from which nanorods can be further subdivided into nanowires, nanotubes, and nanofibers (Fig. 5.2).

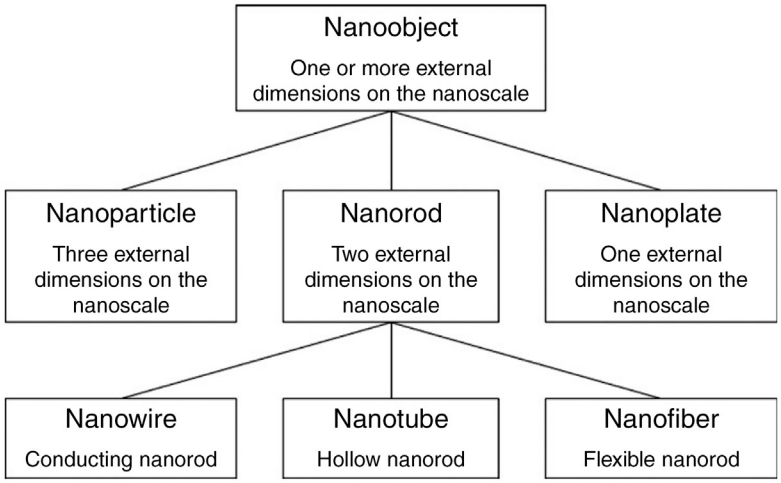


FIGURE 5.2 Hierarchical relationship between terms. *Source: From European Agency for Safety and Health at Work (EU-OSHA).*

CHALLENGES TO ASSESS EXPOSURE TO ENMs

Measurement and monitoring of ENM present in the air of workers' breathing zones means capturing all relevant information about the amount, that is, number, surface area or mass concentration and size distribution, as well as shape, composition and chemical reactivity of airborne ENMs in a given size class or a broad size range (Nel et al., 2009; Savolainen et al., 2010). Selection of the most relevant metric(s) for health-related sampling of ENM is an important component in the development of the concepts, methods, and technology for ENM monitoring at workplaces (Maynard, 2006; Maynard and Aitken, 2007; Schulte et al., 2008; SCENIHR, 2010; Seipenbusch et al., 2008). For this purpose, understanding the relationship between ENM metrics and toxicological effects of ENMs is necessary, but as yet unavailable. This is because a consensus on the correct metrics to be measured to assess exposure to ENMs has not yet been reached internationally (OECD, 2010; SCENIHR, 2007, 2009). There are other approaches developed especially for inhalation toxicology, such as the BED, but with this approach one faces challenges similar to those with other approaches to assess exposure and effects of engineered nanoparticles.

The multitude of relevant metrics, in combination with the different possible release mechanisms for ENM into workplace air as well as the poorly defined transport pathways between source an receptor, that is, the exposed individual, make it imperative to establish and define typical ENM exposure scenarios. The complexity of ENM metrics is further compounded by the fact that all of the factors listed earlier have an impact on particle characteristics. Regarding different exposure scenarios for ENM, one may need to assess exposure to "fresh ENM" emitted from different production processes, as well as for settings in which prevalent exposure to "aged" and "attached" ENM takes place (Seipenbusch et al., 2008). These work processes may include, for example, handling and packing of partly

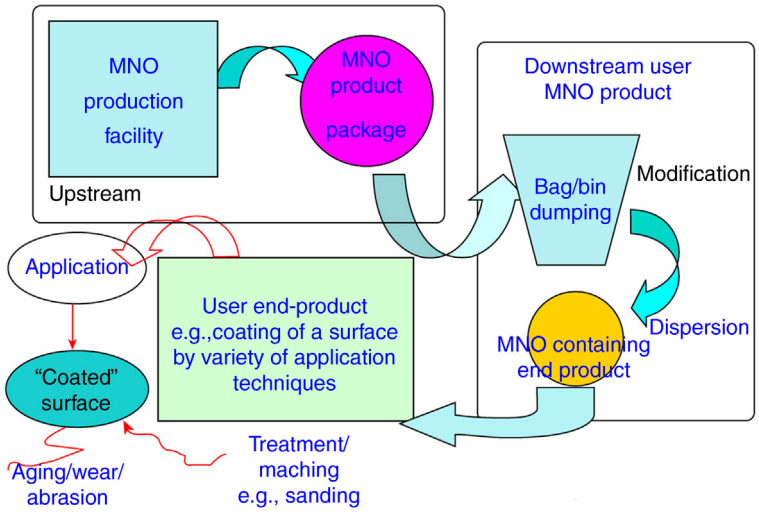


FIGURE 5.3 Schematic diagram of the potential for exposure to MNO in different exposure situations. A "MNO embedded" coating product as an example. *Source: Reprinted from Brouwer, D., 2010. Exposure to manufactured nanoparticles in different workplaces, Toxicology, 269, 120–127, with permission from Elsevier.*

agglomerated and aggregated ENMs. It is important to obtain data from these types of exposure settings to be able to assess true exposure levels of ENMs in workplaces (Peters et al., 2009; Savolainen et al., 2010; Schulte et al., 2008, 2009). Fig. 5.3 depicts different exposure scenarios and the path of ENMs through the different stages of the life cycle of these materials whether as primary particles in aerosols or as incorporated in products, or when recycled (Brouwer, 2010).

Currently, aerosol technology disposes of a range of methods for monitoring of ENM-containing aerosols. Broadly speaking, they include on-line as well as off-line methods to capture a number of different metrics. The array of techniques and concepts for obtaining physical aerosol information related to concentration or size is relatively large, with a general trend away from mass toward number or surface area-based techniques (Kuhlbusch and Fissan, 2006).

Some devices are stationary and capable of providing detailed information about size or composition, while others are "personal" at the expense of detailed information. The benefit of acquiring detailed size distribution data on line (especially of number size distributions) is that such information can easily and accurately be converted to almost any other physical metric (except shape, which requires additional parameters). Keeping in mind the open-ended discussion about appropriate metrics, number size distribution data are thus the most valuable record which leaves the door open to a posteriori tests of new hypotheses concerning health risks of ENM.

Over the past two decades, methods providing direct chemical, compositional, or biological information of airborne particles, usually on the basis of optical or mass spectroscopy, have received a major push in the field of atmospheric sciences and most recently also in response to global security threats (Noble and Prather, 2000; for an excellent summary).

However, these devices are often quite expensive and complex and the technology has not yet filtered down to affordable levels for health hazard evaluation.

Generally, one of the real challenges ahead for ENM monitoring and health risk assessment is (1) to redesign "ENM-capable" instruments already in laboratory use into portable or personal and affordable devices, (2) to expand the sensing technology available for ENM detection by adopting new options with realistic potential for real-time measurement and compact design; and (3) to extend the metrics into new areas, such as CNT shape identification as well as surface chemical or catalytic properties. These latter concepts are especially interesting, since they have the potential of distinguishing ENMs against background particles on the basis of special morphological features or functions (not composition).

The current inability to separate ENM from the background nanoparticles by straightforward concentration, and size distribution measurements makes it highly problematic to set occupational exposure limits (OEL) for ENMs. A mandatory prerequisite for such background distinction of nanosized particles from ENMs would require the ability for online chemical characterization of particles, a feature that the currently available measuring technologies enable, at least in miniaturized and affordable form. Harmful health effects, such as increased cardiac and pulmonary mortality, of ambient fine particles have been emphasized (Dockery et al., 1993). Wide utilization of nanotechnologies globally is a novel phenomenon, and is likely to have an impact on human health on a global scale in future. Besides, exposure to particle aerosols outdoors or indoors is in most cases exposure to a mixture of particles with a wide range of diameters, and hence the importance of assessing the impact the particle size range on human health is a special challenge. There are, however, data which suggest that exposure to ultrafine particles, the ubiquitous background nanosized particles, may be especially harmful in inducing health effects, such as pulmonary inflammation, effects on circulation, and even increased cardiac mortality (Borm et al., 2006; Duffin et al., 2007; Rossi et al., 2010).

When assessing the health impact of ENMs in occupational and other environments, the distinction between background ultrafine or nanoparticles from ENMs becomes especially important, because the ability of dissect these effects is the prerequisite of setting of OEL. In spite of these limitations, unofficial OELs for different ENMs have been released by different institutions, however there is a lack of uniformity both in metrics to be used to express the OELs and in the OELs themselves. As an example, the National Institute of Occupational Health and Safety (NIOSH) suggests a mass-based OEL of 1 $\mu g/m^3$ CNTs (NIOSH, 2013). This proposed inhouse value for a proposed OEL is based on the inflammatory effects of various types of CNT. It stands for a life time exposure of 45 years with 8-h working days during the working career. The Japanese New Energy and Industrial Technology Development Organization, in turn, proposes an OEL of 30 $\mu g/m^3$ 0.03 mg/m^3 (i.e., about 30 times higher) (AIST, 2011). Other organizations, such as the British Standard Institute (BSI), Safe Work Australia (SWA), the German Social Accident Insurance (IFA), and the Dutch Minister of Social Affairs and Employement (DMSAE), express their OELs in number concentration (BSI, 2007; IFA, 2009; Morawska et al., 2012; Van Broekhuizen et al., 2012). Once again different limits are proposed: IFA, BSI, and DMSA fix the upper limit to 10^4 fibers/m^3, whereas a higher exposure (10^5 fibers/m^3) is considered still acceptable by SWA. Furthermore, the IFA and DMSA make a distinction between rigid, biopersistent CNTs, which the earlier reported OELs are referred to, and other CNTs, to which higher OELs should be applied, whereas the

other organizations do not discriminate between different types of CNTs. Similar consideration may be applied for the proposed ENMs regarding other ENMs. This issue has been recently extensively reviewed by Pietroiusti and Magrini (2014).

Each of the earlier avenues addresses an important demand: (1) making current ENM monitoring technology more compact, more affordable and more versatile will provide imminent short-term solutions required by toxicologists and the inhalation exposure community; (2) new sensing technology will have a mid-term effect by providing sophisticated measurement options for very small particles which can be adapted to the needs of aerosol monitoring technology; (3) finally, the need of devices capable of capturing entirely new properties will provide new tools to characterize airborne ENM. It will be important for these new devices to provide real-time and on-line data. However, the discussion earlier also makes it clear than an ideal, all-purpose monitoring method will only become available (if it ever becomes available), once a clear link between health effects and ENM characteristics is well-established for a majority of exposure scenarios; and this will only happen after sufficient data have been collected and analyzed.

EXPOSURE ASSESSMENT OF ENMs IN WORKPLACES

The considerations presented earlier suggest the needs to carefully characterize exposure to ENM at workplaces. In fact, they may be released in the workplace and consequently exposure to them may involve several activities and hence differently exposed workers. Furthermore, their chemical, optical, magnetic, biological, and structural properties (size, shape, surface chemistry, reactivity and area, number concentration, mass concentration, state of agglomeration) make them potentially dangerous, indeed harmful effects to workers have been reported.

Regarding the assessment of ENMs in the occupational setting there are several still unresolved or partially resolved issues as the metrics and instruments that should be used to measure nanoparticles, the ENM modifications after their release and what ENMs in the workplace may imply in terms of workers protection. The possible links between the ENM physico-chemical characteristics and their detailed biological activity and toxicity are discussed in detail in other chapters of this book. However, issues related to ENM characteristics and associated health effects have been briefly discussed also in the earlier section.

Evidence for Release of Engineered Nanoparticles in the Workplace

Several workplace air monitoring studies for manufactured nanoobjects are now available, many of them published during the last 3 years (these studies are summarized in Pietroiusti and Magrini, 2014). They regard both commercial and research scale, and several ENMs were investigated, including those prioritized by OECD. A number of activities including handling of engineered nanoparticles have been investigated, such as collecting and sorting during ENM production, physical/chemical synthesis, weighing/mixing, machining/abrasion, cleaning/maintenance, others (e.g., spraying and sonication). In some surveys, personal breathing devices were used. Interestingly, quite different values than those given by static

devices were reported in one study (Birch et al., 2011). The metric used in most evaluations was number concentration, followed by mass concentration. Surface area concentration was rarely assessed and, albeit being probably a good estimator of a toxicological effect, its use is still very limited to the research field.

ENMs released were mainly in an agglomerated state. This fact would imply that workers' exposure to ENMs occurs mainly in the form of particles that consists of particles beyond the nanosize. This, in turn, may be exempt from the peculiar nanosize-linked toxic effects. On the other hand, it is possible that these agglomerates are later broken up into smaller or primary nanoparticles. Deagglomeration for nanoparticle agglomerates in the human respiratory system has been indeed reported (Creutzenberg et al., 2012). Another relevant feature, highlighted in several studies, is that exposure is characterized by transient high peaks, a finding suggesting the need for the development of short-term OELs.

Harmful Effects on Workers

Some case reports suggest that serious adverse effects may be observed in workers exposed to ENM. The first report regards seven workers that worked in the same room of a plant where they sprayed paste containing nanosized polycyclic ester particles onto polystyrene boards. They were admitted to the hospital with clinical findings of pleural effusion and pericardial effusion. The same nanoparticles to which they were exposed at work were found in the chest fluid and lung biopsies of the workers, two of whom subsequently died from respiratory failure (Song et al., 2009). The results of this paper have been challenged, and the contribution of poor occupational hygiene at that workplace in general has been claimed as the reason for the illnesses and deaths. The fact though remains that the exposure to ENM in this occasion was high. However, further studies are required which levels of these or other ENM as aerosols may endanger the health of workers, and hence OELs and their reliable monitoring will be increasingly important in the future.

In the second report, a worker that inhaled an estimated gram of nickel nanoparticles over an approximate 90 min period, and who died from adult respiratory distress syndrome is described (Phillips et al., 2010). However, when compared to the OSHA permissible exposure limit for nickel of 1 mg/m^3 over an 8-h period, the worker's exposure was large regardless of if the nickel was composed of nanoparticles.

Furthermore, bronchiolitis obliterans organizing pneumonia was detected in a 58-year-old man who had been working for 3 months with polyester powder paint containing nanosized TiO_2. The ENM was detected in the pulmonary cells of this worker (Cheng et al., 2012).

CNTs were detected in the lungs of seven subjects who developed lung disease consistent with small airways disease, bronchiolocentric parenchymal disease, and nonnecrotizing granulomatous condition, after being exposed to dust and smoke at the World Trade Center in New York City during the tragic events of 9/11 (Wu et al., 2010). The similarity between the histopathological findings in World Trade Center survivors and those detected in in vivo studies in animals pulmonary exposure to CNTs suggests a causal relationship between exposure and pulmonary disease in humans. However, the small number of cases in the context of the World Trade Centre incident renders drawing firm conclusions on the role of nanoparticles on the deaths problematic.

How to Measure Nanoparticles: Metrics

It should first of all be emphasized that exposure assessment is not just concentration measurement. Information on the following parameters is in fact needed: magnitude, physical form (e.g., dry powder or slurry formulation), route of absorption, frequency duration of exposure, and distribution (spatial, within worker-between workers) of control measures. In addition, observational walkthrough to make oneself familiar with processes and work practices is also recommended. These factors can be used to identify lower or higher risk situations. Current methods of measurement of nanoparticles are far from being considered optimal. There are theoretical and practical limitations to a reliable workplace evaluation. As far as metrics are concerned, the three most widely used metrics (mass concentration, number concentration, and surface concentration) have weak points: The mass of nanoparticles is too small. To collect a sample that could be detected gravimetrically, the sample would most likely require a longer sampling time than an 8-h work sample. If larger particles were also collected, the mass of the larger particles would mask any increase from the nanoparticles. Number concentration may have large spatial and temporal variations, making difficult discrimination with background nanoparticle number concentration. Finally, surface concentration alone cannot in general be used to demonstrate the presence or absence of nanoparticles in the breathing zone of workers, due to the coagulation/aggregation phenomena (see later). On the other hand, the concomitant testing of mass, number, and surface concentration is impractical, because no single instrument giving reliable concomitant information on the three parameters currently exists, and the concomitant routine use of several instruments is too cumbersome (among the others, for economic reasons) to be applied in workplaces.

How to Measure Nanoparticles: Measurement Strategies

There are currently several sampling strategies to assess the exposure to engineered nanoparticles. A rather similar approach is used around the world with the aim of separating the exposure to engineered nanoparticles to the ubiquitous background nanosized or ultrafine particles traffic exhausts or energy production as the main sources. This approach has been developed simultaneously by a number of research organizations in the world. A good description of one typical approach has been provided by NIOSH (NIOSH, 2009).

In workplaces, exposure to engineered nanoparticles is carried out by using a direct-reading hand held device, such as condensation particle counters (CPC) or optical particle counters (OPC) which provide information on the particle numbers and size distribution but not the source or other characteristics of the particles. The particles can also be collected on filter for electron microscopic (EM) more accurate analysis (Maynard et al., 2004). Both background particles number concentrations in the surrounding will be measured and compared with the particle concentrations near the source to assess whether the occupational levels exceed the background levels suggesting occupational exposure. Similar measurements can be made in the workers' breathing zone, and these measurements should be repeated before and at the end of each workshift, where engineered nanoparticles are being used. In addition to CPC or OPC, also fast mobility particle sizer (FMPS), scanning mobility particle sizer (SMPS), and electric low pressure impactor (ELPI) can be used to measure the nanoparticles number

concentrations (Methner et al., 2010a). If there is a clear indication that the nanoparticle number concentration exceeds that of the number concentration of background nanoparticles one can assume that the workers are exposed to process derived engineered nanoparticles (Methner et al., 2010b). A good example of this approach has been provided by Peters et al. (2009) in which they measured nanoparticles concentrations before and after the workday and throughout the day, and overlaid the particle number concentration on daily work activities trying to find associations between those activities and alterations in nanoparticle number concentrations in the workplace air. The association was not particularly good, but some association was found.

If there is a marked difference between the two measurements, one prior to and the other during a workshift with engineered nanoparticles, a release of engineered nanoparticles from the handling or the process is considered to be present; otherwise, no further evaluation is necessary.

Particles can also be collected on filters: background sampling, source specific sampling, and personal sampling are performed. The collected material is thereafter transferred for scanning electron microscopy (SEM), or TEM, which may be coupled with energy dispersive X-ray analyzer (EDS) for a complete structural and chemical analysis. Elemental analysis may also be performed by means of inductively coupled plasma-mass spectrometry (ICP-MS). This, however, is laborious, time consuming, and expensive, and not suitable for routine work place exposure assessment. In order to validate the procedure, the background particle number concentration need to be measured again.

The appeal of the described procedures is that it is relatively simple and may be performed on the field with portable instruments of relatively low cost. This process cannot be considered a definitive response to the issue of workplace evaluation of nanoparticles. Furthermore, at some time, in the not too distant future, it is likely that workers exposure standard will be promulgated, and therefore a quick, simple repeatable and inexpensive exposure assessment will have to be developed, in order to compare it with a permissible (PEL) or occupational exposure limit (OEL). In the meantime, however, assessments in occupational settings should be performed, adopting uniform, standardized strategies. A challenge with the procedure based on the measurement of nanoparticle number concentrations is that our knowledge on the physical–chemical parameters of ENMs does not yet enable us to conclude which measurements are justified to assess potential health hazards to the exposed workers. Rather than number concentrations, mass or particle surface area may also be relevant in many cases, and the suitable metric for different engineered nanoparticles may vary (Juric et al., 2015; Savolainen et al., 2010).

Instruments Measuring Number Concentration

1. CPCs provide real time number concentration measurements between their particle diameter detection limits. Without a nanoparticle preseparator they are not specific to the nanometer size range. Some models have diffusion screen to limit top size to 1 μm. Consequently, they can provide real time measurements in the range 10 nm to 1 μm. To count the particles, the CPC increases the size of submicron particles by vapor (alcohol/water) supersaturation so the enlarged particles can then be detected by a laser. Depending on the model, particle counts can be logged every second.

2. OPCs provide real time number concentration measurements between their particle diameter detection limits. Particle size diameters begin at 300 nm and may go up to 10 μm. The optical particle counters alone are not appropriate for nanoparticles, since they are insensitive to particles smaller than approximately 300 nm in diameter. However, they may give useful information if used in combination with CPC. In fact, OPC provides a count concentration in the size range of 300–20,000 nm and allows for the determination of both number concentration and particle size distribution. CPC counts particles >10 nm with an upper limit of about 1000 nm. CPC is useful for detecting particles in the nanosize range, but it does not provide a size distribution by separating particles into size range. When OPC and CPC are used together, there is an overlap of the two instruments in the range 300–1000 nm, making possible an indirect evaluation of particles <300 nm by subtracting all OPC counts in the size bins ranging from 300 to 1000 nm from the counts between 10 and 1000 nm made by CPC. Comparisons have been made to evaluate the performance of different CPCs and OPCs. Both CPS and OPC can be useful for measuring nanoparticle exposures but the results from an individual monitor should be interpreted based upon the instruments technical parameters (Liu et al., 2014).
3. SMPS is composed of a differential mobility analyzer (DMA) and a CPC. It works by detecting real time size-selective (mobility diameter) number concentration, giving aerosol size distribution. It measures the size of the particles by using positive electrostatic charges on the particles to attract them to a negatively charge rod. The possible detectable particle size range is from 3 to 800 nm (although during monitoring this must be narrowed down to a smaller range). The SMPS utilizes a radioactive source (Kr 85). Depending on the model, it may require up to 2 min to conduct a measurement which may be too slow to identify nanoparticle sources. Even if the recently introduced FMPS allows a much better time resolution, the size, expense, and radioactivity emission of both SMPS and FMPS limit their application in the workplace.

Instruments Measuring Surface Concentration

1. Diffusion Chargers (DCs) give real-time measurement of aerosol active surface-area. Active surface-area does not scale directly with geometric surface-area above 100 nm. It is important to note that not all commercially available DCs have a response that scales with particle active surface-area below 100 nm. Therefore, DCs are only specific to nanoparticles if used with appropriate inlet preseparator. For particles > 100 nm DCs underestimates the surface. Depending on the model, measurements can be logged every 10 s. DCs vary in size with some small enough to be carried by a worker. Results are reported as micro square meter per cubic centimeter or similar. Although they have the advantages of having no working fluids, no radioactive sources, and the possibility of any orientation, they have limitations (e.g., signal interpretation is a bit confusing) that need to be evaluated with respect to measuring workplace aerosols. A recent improvement in DCs is represented by Diffusion Size Classifiers (DiSCs) which allow obtaining both total number concentration and average particle size. The time resolution is about 2 s, and are battery-powered and portable. The efficiency of DCs is still a matter of debate (Vosburg et al., 2014).

2. Electrical Low Pressure Impactor (ELPI). It gives real time size-selective (aerodynamic diameter) detection of active surface area concentration of particles between 7 nm and 10 μm, combining electrical charging, inertial collection, and electrical detection. In the ELPI, a real time cascade impactor, particles first pass through a diffusion charger, are positively charged, and then they enter a Berner-type low pressure impactor made up of 13 stages where the particles are collected onto electrically isolated stages by aerodynamic diameter. The current on each stage, measured by electrometers is further converted into a particle number concentration. Depending on the model, measurements can be logged every five seconds. It is also possible the off-line characterization (TEM, ICP-MS, etc.) of samples. Widespread application in the workplace is limited by size, expense and complexity of operation, and sensitivity to harsh field conditions. Technical improvements in precision and resolution power of ELPI have been recently suggested (Yli-Ojanperä et al., 2010).
3. Nanoparticle surface area monitor (NSAM) is a recently launched instrument aimed to determine lung-deposited particle surface area concentrations, based on the widely accepted model developed by the International Commission on Radiological Protection (ICRP). Limitation and perspective of this kind of measurement have been extensively discussed by Asbach et al. (2009).
4. Modified electrical aerosol detector (MEAD). The same principles as NSAM, but less expensive, less bulky, and easy to use. Validation is at the initial stage (Wang et al., 2010).

Instruments Measuring Mass Concentration

1. Size selective sampler (SSS). Cascade impactors are currently the only devices giving a direct estimation of mass concentration with a cut-off point around 100 nm, that is, can measure a total mass above 100 nm and also below 100 nm (down to approximately 20 nm). They are easy to use and provide a mass concentration for a defined range of particle sizes. The only disadvantage is that they are not too portable but if a trolley is provided they can be used in an industrial setting.
2. Tapered element oscillating microbalance (TEOM) measures nanoaerosol mass concentration on line with a suitable size selective inlet. The main disadvantage is that the TEOM does not provide size information. There is a version of the TEOM that can be used as a personal monitor.

Instruments Giving Indirect Estimates

Some of the earlier listed instruments may give, in addition to the measured metric, an indirect estimate of other parameters. The reliability of the assumptions and of the calculations needed to perform such estimations is questionable. Some of the estimations are as follows:

1. indirect estimation of mass concentration is given by ELPI and SMPS;
2. indirect estimation of number concentration: ELPI and DiSC;
3. indirect estimation of surface area: combined use of SMPS and ELPI may be performed to infer particle fractal dimension.

Sample Collection and Characterization

Several relevant parameters can only be determined through off-line analytical techniques (electron microscopy, ICP-MS, etc). Each technique requires specific methods for sample collection and preparation. For electron microscopy (EM), uniform deposit, minimum overlapping, and good collection efficiency are required. SEM has a 10–50 nm resolution, whereas TEM has a resolution <10 nm. TEM analysis provides direct information on the projected area of collected particles which may be related to geometric area for some particles shapes. TEM can provide an estimate of the particle size distribution and, if equipped with an EDS, a determination of elemental composition with the examination of one sample. Samples are collected onto filters or other substrates (e.g., TEM grid), for subsequent off-line analysis. Recent developments in sampling techniques for EM have been recently performed: Handled TEM sampler (Miller et al., 2010), and sample with automatic determination of sampling time (Azong-Wara et al., 2010) will probably be available in the near future. In this context it is important to note that using particle counters in combination with sample collection for chemical analysis allows a good evaluation of worker exposure to ENMs but if this assessment strategy will continue to rely only on static or area sampling some uncertainty will always exist in estimating worker exposures. A specific problem is posed by the high aspect ratio nanoparticles (HARN) (e.g., CNTs). Owing to their very small diameter, high magnifications are necessary for their detection, decreasing the likelihood of finding countable fibers (i.e., with both ends counted). The highly agglomerated nature of some types of HARN (e.g., SWCNT) adds further problems to the identification of single fibers.

ENM Modifications After Their Release

In the workplace, workers may come in contact with ENMs present in aerosols (generation of nanoparticles in nonenclosed systems, incidental leakage from gas-phase reactor), powder milling, pouring or mixing, handling (e.g., weighing, spraying), liquid (pouring or mixing operations with suspensions of ENMs, any agitation with some energy, cleaning operations), materials (machining, cutting, grinding, sanding, drilling, or any other mechanical operation on materials containing ENMs or nanostructured material).

A potentially relevant property of ENM in workplace is represented by their dustiness (nanodustiness); this is especially true for workers handling nanopowders. Tsai et al. (2008) determined the dustiness of two nanoscale TiO_2 and ZnO, in standard 1 min and 30-min tests and found that very few particles below 100 nm were generated. Thus, nanodustiness does not seem to be of major concern in workplace exposure. However, this work, along with other reports and conceptual models (Brouwer, 2010; Schneider and Jensen, 2009; Schneider et al., 2011) confirms the dominance of nanoparticle agglomerates or nanoparticles attached to background particles in the air during production and handling of nanopowders. These larger particles should be taken into account when measuring the presence of nanoparticles in the workplace, because all particles in the respirable size range can enter and deposit in the deep lung, including aggregates and agglomerates. As an example, Cena and Peters (2011) found negligible release of nanoparticles during the processing of CNT-epoxy nanocomposites materials; nevertheless, there was a substantial release of micron-sized CNT containing particles, which were poorly controlled by the fume hood.

Indeed, most "field" studies reveal the formation of larger agglomerates after the release of ENM. However, methodological issues may render difficult the interpretation of the data in the light of workers exposure (Brouwer, 2010). A consensus on the optimal methods of assessing the dynamics of nanoparticles/agglomerates equilibrium in the workplaces is urgently needed.

Workers' Protection

Until exposure limits have been established, protective measures should be based on the precautionary principle (i.e., any exposure to nanoparticles in the workplace should be reduced to background levels). In general, there are three main complementary approaches to risk and exposure control: engineering techniques, administrative means, and personal protective equipment. Although it is frequently assumed that the usual protective measures would not be effective, this assumption is not consistent with physical principles: particles larger than 300 nm are most effectively separated by impaction on the filter material, entrapment by the filter material, or sedimentation owing to gravity. For particles smaller than 300 nm, separation by diffusion (Brownian movement) and electrostatic forces becomes increasing relevant with decreasing size. It can therefore be asserted as a rule that technical measures which are effective against micrometric particles are also suitable for the elimination of nanoparticles. Of course, the engineering control systems, such as enclosure and ventilation, must be designed according to the gaseous and particulate properties of the nanoparticles and considering several parameters as the quantity of the bulk ENM that is synthesized or handled in the manufacture of a product, the physical form of the ENMs and task duration and frequency. Moreover, all ventilation systems should be evaluated, approved and maintained by the organization (company, research institute, university) health and safety officers. It is therefore not surprising that available data seem to indicate that well-designed engineered protective measures are effective for nanoparticles (NIOSH, 2009). In particular, airborne exposure to nanomaterials can most likely be controlled at most processes and job tasks using a wide variety of engineering control techniques similar to those used in reducing exposures to general aerosols (Burton, 1997; Ratherman, 1996). The most widely used engineering control of nanoparticle exposure is represented by fume hoods and it has been recently reported that the air-curtain hood, a new design with significantly different airflow pattern from traditional hoods, may effectively control the release of nanoparticles under all operating conditions (Tsai et al., 2010).

Administrative means of control constitute an additional approach when the other methods have not achieved the expected control levels. In these situations, reduction of work periods, modification of work practices, personal hygiene measures, housekeeping, and preventive maintenance constitute other ways of limiting the occupational exposure risks. As examples of good practices, hand washing, showering, changing, and cleaning clothes facilities should be provided to prevent the inadvertent contamination of other areas (including take-home) caused by the transfer of nanoparticles on clothing and skin. The storage and consumption of food or beverages in workplaces should be prevented where nanomaterials are handled. Dry mopping, sweeping, dusting, cleaning using compressed air or portable blowers or fans should be prohibited. Work areas should be cleaned at the end of each work shift (at a minimum) using either a high-efficiency particulate air (HEPA)-filtered vacuum cleaner or wet

wiping methods. Furthermore, it should be forbidden for workers to handle ENM in the open air and they should store dispersible ENM, whether suspended in liquids or in a dry particle form, in closed containers whenever possible. There are also some good practices for management, the most important of which are educating workers on the safe handling of ENM to minimize the likelihood of inhalation exposure and skin contact, providing information on the hazardous properties of ENM and providing additional control measures to ensure that ENM are not transported outside the work area.

Nanoparticle exposure may often be attributable to the wearing of inadequate personal protective equipment (PPE). Protective clothing that would typically be required for a wet-chemistry laboratory would be appropriate and could include, but should not be limited to:

- gauntlet-type gloves or nitrile gloves with extended sleeves;
- chemical splash goggle and laboratory coats.

Standard tests showed that nonwoven fabrics, like high density polyethylene textile (Tyvek type), seems to be much more efficient against nanoparticles penetrations. Indeed, recent data indicate that their efficiency in inhibiting the penetration of nanoparticles may be even higher than for larger material (Gao et al., 2011).

The use of respirators is often required when engineering and administrative controls do not adequately keep worker exposures to an airborne contaminant below a regulatory limit or an internal control target. Rengasamy et al. (2009) used silver nanoparticles (4–30 nm) and sodium chloride particles (20–400 nm) in order to test NIOSH-tested (N95, P100) and CE-compliant (FFP2, FFP3) respiratory filter masks. The filter materials employed complied with the requirements stated in the standards.

Health Surveillance

At the current level of knowledge, the initial step of health surveillance programs for ENM exposure should include a detailed information on the type of ENMs involved and on the workers having a chance of being exposed in specific workplaces. The implementation of exposure registries is the logical consequence of this initial step. These registries may represent the basis for performing research studies, for timely and targeted risk communication, and for intervention and advice. Initiatives having this background have been taken in US (Dahm et al., 2015) and France (Haut Conseil de la Santé Publique, 2009).

It is not clear whether, in addition to traditional evaluations administered to workers exposed to dust and chemicals, additional testing may be needed for workers exposed to ENMs, although probably further evaluation should be provided, in relation to the specific risk posed by different ENMs. Efforts in research are directed toward the identification of specific biological markers of exposure or response to ENMs. A promising approach may be represented by omics approaches, consisting in the mapping of expression data of proteins, lipids, or other biomolecules involved in vital cellular functions. A potential use of global lipidomics using mass spectrometry was recently provided by Shvedova et al. (2012) who reported that pulmonary exposure of mice to SWCNTs was followed by the selective peroxidation of two phospholipids (phosphatidylcholine and phosphatidylethanolamine) while sparing the other two most abundant phospholipids, cardiolipin, and phosphatidylinositol. However, confirmation of the suitability of such or other similar approaches is needed.

TAKE-HOME MESSAGES

- Nanoscale particles are ubiquitous in many workplaces; hence, exposure of humans is not limited only to scientists dealing with ENMs, manufacturers of nanoobjects, or nanoproduct users, but involves also other categories of workers exposed to ultrafine or incidental nanoparticles.
- Currently, there is no single device that can be used to characterize or evaluate the magnitude of exposure to nanoparticles; instead an array of devices is required to cover the needs of assessment of exposure to engineered and other nanoparticles.
- A strategy for the assessment of exposure to engineered nanoparticles is emerging but this approach is for the time being unable to identify—at least online—process derived nanoparticles from background nanoparticles, and it requires, therefore continuous development
- At present, there are not yet OEL for engineered nanoparticles, and until then protective measures shall be based on precautionary approaches.
- Current control measures provide in most cases appropriate protection against excessive exposure to engineered nanoparticles.

References

Asbach, C., Fissan, H., Stahlmecke, B., Kulbush, T.A.J., Pui, D.Y.H., 2009. Conceptual limitations and extentions of lung-deposited nanoparticle surface area monitor [NSAM). J. Nanopart. Res. 11, 101–109.

Azong-Wara, N., Asbach, C., Stahlmecke, B., Fissan, H., Kaminski, H., Plitzko, S., Kulbush, T.A.J., 2010. Optimization of a thermophoretic personal sampler for nanoparticle exposure studies. J. Nanopart. Res. 12, 1611–1624.

Bergin, I.L., Witzman, F.A., 2013. Nanoparticle toxicity by the gastrointestinal route: evidence and knowledge gaps. Int. J. Biomed. Nanosci. Nanotechnol. 3, 1–2.

Birch, M.E., Ku, B.K., Evans, D.E., Ruda Eberenz, T.A., 2011. Exposure and emissions monitoring during carbon nanofiber production-part i: elemental carbon and iron-soot aerosols. Ann. Occup. Hyg. 55, 1016–1036.

Borm, P.J., Robbins, D., Haubold, S., Kuhlbusch, T., Fissan, H., Donaldson, K., Schins, R., Stone, V., Kreyling, W., Lademann, J., Krutmann, J., Warheit, D., Oberdorster, E., 2006. The potential risks of nanomaterials: a review carried out for ECETOC. Part. Fibre. Toxicol. 3, 11.

British Standard Institute (BSI), 2007. Nanotechnologies—part 1. Good practice guide for specifying manufactured nanomaterials. London, UK, British Standard Institute, PD 6699-1, 2007.

Brouwer, D., 2010. Exposure to manufactured nanoparticles in different workplaces. Toxicology 269, 120–127.

Burton, J., 1997. General methods for the control of airborne hazards. In: DiNardi, S.R. (Ed.), The occupational environment—its evaluation and control. American Industrial Hygiene Association, Fairfax, VA.

Cena, G., Peters, T.M., 2011. Characterization and control of airborne particles emitted during production of epoxy/carbon nanotube nanocomposites. J. Occup. Environ. Hyg. 8, 86–92.

Cheng, T.-H., Ko, F.-C., Chang, J.-L., Wu, K.-A., 2012. Bronchiolitis obliterans organizing pneumonia due to titanium nanoparticles in paint. Ann. Thor. Surg. 93, 663–669.

Creutzenberg, O., Bellmann, B., Korolewitz, R., Koch, W., Mangelsdorf, I., Tillmann, T., Schaudien, D., 2012. Change in agglomeration status and toxicokinetic fate of various nanoparticles in vivo following lung exposure in rats. Inhal. Toxicol. 24, 821–830.

Dahm, M.M., Schubauer-Berigan, M.K., Evans, D.E., Birch, M.E., Fernback, J.E., Deddens, J.A., 2015. Carbon nanotube and nanofiber exposure assessments: an analysis of 14 on site visits. Ann. Occup. Hyg. 6, 705–723.

Dockery, D.W., Pope, III, C.A., Xu, X., Spengler, J.D., Ware, J.H., Fay, M.E., Ferris, Jr., B.G., Speizer, F.E., 1993. An association between air pollution and mortality in six U.S. cities. N. Engl. J. Med. 329, 1753–1759.

Donaldson, K., Murphy, F.A., Duffin, R., Poland, C.A., 2010. Asbestos, carbon nanotubes and the pleural mesothelium: a review of the hypothesis regarding the role of long fibre retention in the parietal pleura, inflammation and mesothelioma. Part. Fibre. Toxicol. 7, 5.

Donaldson, K., Schinwald, A., Murphy, F., Cho, W.S., Duffin, R., Tran, L., Poland, C., 2013. The biologically effective dose in inhalation nanotoxicology. Acc. Chem. Res. 46, 723–732.

Duffin, R., Mills, N.L., Donaldson, K., 2007. Nanoparticles-a thoracic toxicology perspective. Yonsei. Med. J. 48, 561–572.

Erdely, A., Liston, A., Salmen-Muniz, R., Hulderman, T., Young, S.H., Zeidler-Erdely, P.C., Castranova, V., Simeonova, P.P., 2011. Identification of systemic markers from a pulmonary carbon nanotube exposure. J. Occup. Environ. Med. 53 (6 Suppl.), S80–S86.

European Agency for Safety and Health at Work (EU-OSHA), 2009. Workplace exposure to nanoparticles. Kosk-Bienko, J. (Ed.).

European Commission, 2011. Commission recommendation of 18 October 2011 on the definition of nanomaterial [2011/696/EU). Off. J. Eur. Union. L275, 38–40. Available from: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2011:275:0038:0040:EN:PDF.

Gao, P., Jacques, P.A., Hsiao, T.-C., Shepherd, A., Eimer, B.C., Yang, M., Miller, A., Gupta, B., Shaffer, R., 2011. Evaluation of nano and submicron particle penetration through ten non woven fabrics using a wind-driven approach. J. Occup. Environ. Health 8, 13–22.

Geiser, M., Kreyling, W.G., 2010. Deposition and biokinetics of inhaled nanoparticles. Part. Fibre. Toxicol. 7, 2.

Grosse, Y., Loomis, D., Guyton, K.Z., Lauby-Secretan, B., El Ghissassi, F., Bouvard, V., Benbrahim-Tallaa, L., Guha, N., Scoccianti, C., Mattock, H., Straif, K., Kane, A.B., Debia, M., Dion, C., Møller, P., Savolainen, K., Canu, I.G., Jaurand, M.C., Comba, P., Fubini, B., Kobayashi, N., Morimoto, Y., Tsuda, H., Yu, I.J., Vermeulen, R., Bugge, M.D., Bateson, T.F., Kuempel, E.D., Morgan, D.L., Pinkerton, K.E., Sargent, L.M., Stayner, L., 2014. Carcinogenicity of fluoro-edenite, silicon carbide fibres and whiskers, and carbon nanotubes. Lancet Oncol. 15, 1427–1428.

Guseva Canu, I.G., Bateson, T.F., Bouvard, V., Debia, M., Dion, C., Savolainen, K., Yu, I.-J., 2016. Human exposure to carbon-based fibrous nanomaterials, a review. Int. J. Hyg. Environ. Health 219, 166–175.

Haut Conseil de la Santé Publique (HCSP), 2009. La securitè de travailleurs lors de l'exposition aux nanotubes de carbon. Available from: http://www.hcsp.fr/docspdf/avisrapports/hcspa20090107_Exp_Nano_Carbone.pdf.

IFA, 2009. Criteria for the assessment of protective measures. Institut fur Arbeitsschutz der Deutschen Gesetzlichen Unfallversicherung. Available from: http://www.dguv.de/ifa/en/fac/nanopartikel/beurteilungmasstaebe/index.jsp.2009.

International Organization for Standardization, (ISO), 2008. Health and Safety Practices in Occupational Settings Relevant to Nanotechnologies, ISO Document No. ISO/ TR ISO/TR 12885:2008(E). Geneva, Switzerland.

Japan National Institute of Advanced Industrial Science and Technology (AIST), 2011. Final reports on risk assessment of three manufactured nanomaterials. Available from: http://www.aist-riss.jp/main/modules/product/nano_rad.html.

Johnson, D.R., Methner, M.M., Kennedy, A.J., Steevens, J.A., 2010. Potential for occupational exposure to engineered carbon-based nanomaterials in environmental laboratory studies. Environ. Health Perspect. 118, 49–54.

Juric, A., Meldrum, R., Liberda, E.N., 2015. Achieving control of occupational exposures to engineered nanomaterials. J. Occup. Environ. Hyg. 12, 501–508.

Kreyling, W.G., Hirn, S., Moller, W., Schleh, C., Wenk, A., Celik, G., Lipka, J., Schäffler, M., Haberl, N., Johnston, B.D., Sperling, R., Schmid, G., Simon, U., Parak, W.J., Semmler-Behnke, M., 2014. Air-blood barrier translocation of tracheally instilled gold nanoparticles inversely depends on particle size. ACS Nano 8, 222–233.

Kuhlbusch, T.A., Fissan, H., 2006. Particle characteristics in the reactor and pelletizing areas of carbon black production. J. Occup. Environ. Hyg. 3, 558–567.

Kuhlbusch, T.A., John, A.C., Quass, U., 2009. Sources and source contributions to fine particles. Biomarkers 14, 23–28.

Liu, Y., Beaucham, C.C., Pearce, T.A., Zhuang, Z., 2014. Assessment of two portable real-time particle monitors used in nanomaterial workplace exposure evaluations. Plos One 9, 1–8.

Maynard, A.D., 2006. Nanotechnology: A Research Strategy for Addressing Risk. Woodrow Wilson International Center for Scholars, Washington, DC.

Maynard, A.D., Aitken, R.J., 2007. Assessing exposure to airborne nanomaterials: current abilities and future requirements. Nanotoxicology 1, 26–41.

Maynard, A.D., Baron, P.A., Foley, M., Shvedova, A.A., Kisin, E.R., Castranova, V., 2004. Exposure to carbon nanotube material: aerosol release during the handling of unrefined single-walled carbon nanotube material. J. Toxicol. Environ. Health A 67, 87–107.

Methner, M., Hodson, L., Geraci, C., 2010a. Nanoparticle emission assessment technique (NEAT) for the identification and measurement of potential inhalation exposure to engineered nanomaterials-Part A. J. Occup. Environ. Hyg. 7, 127–132.

Methner, M., Hodson, L., Dames, A., Geraci, C., 2010b. Nanoparticle emission assessment technique (NEAT) for the identification and measurement of potential inhalation exposure to engineered nanomaterials-Part B: results from 12 field studies. J. Occup. Environ. Hyg. 7, 163–176.

Miller, A., Frey, G., King, G., Sunderman, C., 2010. A handling electrostatic precipitator for sampling airborne particles and nanoparticles. Aerosol. Sci. Technol. 44, 417–427.

Morawska, L., McGarry, P., Morris, H., Knibbs, L., Bostrom, T., Capasso, A., 2012. Measurements from particle emissions from nanotechnology processes with assessment of measuring techniques and workplace controls. Safe Work Australia.

Nasibulin, A.G., Queipo, P., Shandakov, S.D., Brown, D.P., Jiang, H., Pikhitsa, P.V., Tolochko, O.V., Kauppinen, E.I., 2006. Studies on mechanism of single-walled carbon nanotube formation. J. Nanosci. Nanotechnol. 6, 233–246.

National Institute for Occupational Safety and Health, (NIOSH), 2009. Approaches to Safe Nanotechnology. Managing the Health and Safety Concerns Associated with Engineered Nanomaterials. DHHS (NIOSH) Publication No. 2009–125.

National Institute for Occupational Safety and Health (NIOSH), 2013. Current Intelligence Bulletin 65: Occupational Exposure to Carbon Nanotubes and Nanofibers. DHHS (NIOSH) Publication No. 2013–145.

Nel, A.E., Mädler, L., Velegol, D., Xia, T., Hoek, E.M., Somasundaran, P., Klaessig, F., Castranova, V., Thompson, M., 2009. Understanding biophysicochemical interactions at the nano-bio interface. Nat. Mater. 8, 543–557.

Nguyen, T.H., Lin, M., Mustapha, A., 2015. Toxicity of graphene oxide on intestinal bacteria and Caco-2 cells. J. Food. Prot. 78, 996–1002.

Noble, C.A., Prather, K.A., 2000. Real-time single particle mass spectrometry: a historical review of a quarter century of the chemical analysis of aerosols. Mass Spectrom. Rev. 19, 248–274.

Organisation for Economic Co-operation and Development., 2010. Environment Directorate Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology, Series on the Safety of Manufactured Nanomaterials No. 27: List of Manufactured Nanomaterials and List of Endpoints for Phase one of the Sponsorship Programme for the Testing of Manufactured Nanomaterial: Revision 2010.

Peters, T.M., Elzey, S., Johnson, R., Park, H., Grassian, V.H., Maher, T., O'Shaughnessy, P., 2009. Airborne monitoring to distinguish engineered nanomaterials from incidental particles for environmental health and safety. J. Occup. Environ. Hyg. 6, 73–81.

Phillips, J.I., Green, F.Y., Davies, J.C., Murray, J., 2010. Pulmonary and systemic toxicity following exposure to nickel nanoparticles. Am. J. Ind. Med. 53, 763–767.

Pietroiusti, A., 2012. Health implications of engineered nanomaterials. Nanoscale 4, 1231–1247.

Pietroiusti, A., Magrini, A., 2014. Engineered nanoparticles at the workplace: current knowledge about workers' risk. Occ. Med. 64, 319–330.

Pietroiusti, A., Magrini, A., Campagnolo, L., 2015. New frontiers in nanotoxicology: gut microbiota/microbiome-mediated effects of engineered nanomaterials. Toxicol. Appl. Pharmacol. 299, 90–95.

Ratherman, S., 1996. Methods of control. In: Plog, B. (Ed.), Fundamentals of industrial hygiene. National Safety Council, Itasca, IL.

Rengasamy, S., Eimer, B.C., Shaffer, R.E., 2009. Comparison of nanoparticle filtration performance of NIOSH-approved and CE-marked particulate filtering facepiece respirators. Ann. Occup. Hyg. 53, 117–128.

Roco, M.C., 2011. The long view of nanotechnology development: the National Nanotechnology Initiative at 10 years. J. Nanopart. Res. 13, 427–445.

Rossi, E.M., Pylkkänen, L., Koivisto, A.J., Vippola, M., Jensen, K.A., Miettinen, M., Sirola, K., Nykäsenoja, H., Karisola, P., Stjernvall, T., Vanhala, E., Kiilunen, M., Pasanen, P., Mäkinen, M., Hämeri, K., Joutsensaari, J., Tuomi, T., Jokiniemi, J., Wolff, H., Savolainen, K., Matikainen, S., Alenius, H., 2010. Airway exposure to silica-coated TiO_2 nanoparticles induces pulmonary neutrophilia in mice. Toxicol. Sci. 113, 422–433.

Rothenbacher, S., Messerer, A., Kasper, G., 2008. Fragmentation and bond strength of airborne diesel soot agglomerates. Part. Fibre. Toxicol. 5, 9.

Ryman-Rasmussen, J.P., Cesta, M.F., Brody, A.R., Shipley-Phillips, J.K., Everitt, J.I., Tewksbury, E.W., Moss, O.R., Wong, B.A., Dodd, D.E., Andersen, M.E., Bonner, J.C., 2009. Inhaled carbon nanotubes reach the subpleural tissue in mice. Nat. Nanotechnol. 4, 747–751.

Saber, A.T., Lamson, J.S., Jacobsen, N.R., Ravn-Haren, G., Hougaard, K.S., Nyendi, A.N., Wahlberg, P., Madsen, A.M., Jackson, P., Wallin, H., Vogel, U., 2013. Particle induced pulmonary acute phase response correlates with neutrophil influx linking inhaled particles and cardiovascular risk. PLoS One 8, e69020.

Sakamoto, Y., Nakae, D., Fukumori, N., Tayama, K., Maekawa, A., Imai, K., Hirose, A., Nishimura, T., Ohashi, N., Ogata, A., 2009. Induction of mesothelioma by a single intrascrotal administration of multi-wall carbon nanotube in intact male Fischer 344 rats. J. Toxicol. Sci. 34, 65–76.

Savolainen, K., Alenius, H., Norppa, H., Pylkkänen, L., Tuomi, T., Kasper, G., 2010. Risk assessment of engineered nanomaterials and nanotechnologies—a review. Toxicology 269, 92–104.

Schmid, K., Danuser, B., Riediker, M., 2010. Nanoparticle usage and protection measures in the manufacturing industry—a representative survey. J. Occup. Environ. Hyg. 7, 224–232.

Schneider, T., Jensen, K.A., 2009. Relevance of aerosol dynamics and dustiness for personal exposure to manufactured nanoparticles. J. Nanopart. Res. 11, 1637–1650.

Schneider, T., Brouwer, D.H., Koponen, I.K., Jensen, K.A., Fransman, W., van Duuren-Stuurman, B., van Tongeren, M., Tielemans, E., 2011. Conceptual model for assessment of inhalation exposure to manufactured nanoparticles. J. Expo. Sci. Environ. Epidemiol. 21, 450–463.

Schulte, P., Geraci, C., Zumwalde, R., Hoover, M., Castranova, V., Kuempel, E., Murashov, V., Vainio, H., Savolainen, K., 2008. Sharpening the focus on occupational safety and health in nanotechnology. Scand. J. Work Environ. Health 34, 471–478.

Schulte, P.A., Schubauer-Berigan, M.K., Mayweather, C., Geraci, C.L., Zumwalde, R., McKernan, J.L., 2009. Issues in the development of epidemiologic studies of workers exposed to engineered nanoparticles. J. Occup. Environ. Med. 51, 323–335.

Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2007. The appropriateness of the risk assessment methodology in accordance with the Technical Guidance Documents for new and existing substances for assessing the risks of nanomaterials. Brussels, Belgium.

Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2009. Risk Assessment of Products of Nanotechnologies. Brussels, Belgium.

Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2010. Scientific Basis for the Definition of the Term "nanomaterial." Brussels, Belgium.

Seipenbusch, M., Binder, A., Kasper, G., 2008. Temporal evolution of nanoparticle aerosols in workplace exposure. Ann. Occup. Hyg. 52, 707–716.

Shvedova, A.A., Kisin, E.R., Mercer, R., Murray, A.R., Johnson, V.J., Potapovich, A.I., Tyurina, Y.Y., Gorelik, O., Arepalli, S., Schwegler-Berry, D., Hubbs, A.F., Antonini, J., Evans, D.E., Ku, B.K., Ramsey, D., Maynard, A., Kagan, V.E., Castranova, V., Baron, P., 2005. Unusual inflammatory and fibrogenic pulmonary responses to single-walled carbon nanotubes in mice. Am. J. Physiol. Lung Cell. Mol. Physiol. 289, L698–L708.

Shvedova, A.A., Pietroiusti, A., Fadeel, B., Kagan, V.E., 2012. Mechanisms of carbon nanotube-induced toxicity: focus on oxidative stress. Toxicol. Appl. Pharmacol. 261, 121–133.

Song, Y., Li, X., Du, X., 2009. Exposure to nanoparticles is related to pleural effusion, pulmonary fibrosis and granuloma. Eur. Resp. J. 34, 559–567.

Tsai, C.-J., Wu, Ch.-H., Leu, M.-L., Chen, S.-Ch., Huang, Ch.-Y., Tsai, P.-J., Ko, F.-H., 2008. Dustiness test of nanopowders using a standard rotating drum with a modified sampling train. J. Nanopart. Res. 10, 121–131.

Tsai, S.J., Huang, R.F., Ellenbecker, M.J., 2010. Airborne nanoparticle exposures while using constant-flow, constant-velocity, and air-curtain-isolated fume hoods. Ann. Occup. Hyg. 54, 78–87.

Van Broekhuizen, van Veelen, W., Streekstra, W.-H., Schulte, P., Reijnders, L., 2012. Exposure limits for nanoparticles: report of an international workshop on nano reference values. Ann. Occup. Hyg. 56, 515–524.

Van Duuren-Stuurman, B., Pelzer, J., Moehlmann, C., Berges, M., Bard, D., Wake, D., Mark, D., Jankowska, E., Brouwer, D., 2010. A structured observational method to assess dermal exposure to manufactured nanoparticles [MNPs): DREAM as an initial assessment tool. Int. J. Occup. Environ. Health 16, 397–403.

Vosburg, D.J.H., Ku, B.K., Peters, T.M., 2014. Evaluation of a diffusion charger for measuring aerosols in a workplace. Ann. Occup. Hyg. 58, 424–436.

Wang, Y.F., Tsai, P.J., Chen, C.W., Chen, D.R., Hsu, D.J., 2010. Using a modified electrical aerosol detector to predict nanoparticle exposures to different regions of the respiratory tract for workers in a carbon black manufacturing industry. Environ. Sci. Technol. 44, 6767–6774.

Wu, M., Gordon, R.E., Herbert, R., Padilla, M., Moline, J., Mendelson, D., Litle, V., Travis, D.W., Gil, J., 2010. Case report: lung disease in World Trade Center Responders exposed to dust and smoke: carbon nanotubes found in the lungs of World Trade Center patients and dust samples. Environ. Health Perspect. 118, 499–504.

Yli-Ojanperä, J., Kannosto, J., Marjamäki, M., Keskinen, J., 2010. Improving the nanoparticle resolution of ELPI. Aerosol. Air Qual. Res. 10, 360–366.

CHAPTER

6

Biomonitoring

Enrico Bergamaschi[*], *Irina Guseva Canu*[**], *Adriele Prina-Mello*[†], *Andrea Magrini*[‡]

[*]University of Turin, Torino, Italy; [**]Public Health France, Direction of Noncommunicable Diseases and Traumatisms, Saint Maurice, France; [†]School of Medicine and CRANN Institute of Molecular Medicine, Trinity Centre of Health Sciences, Dublin, Ireland; [‡]University of Rome 'Tor Vergata', Rome, Italy

OUTLINE

Introduction 125

Issues in the Development of Biomarkers for Engineered Nanomaterials 128

Factors Affecting Biomarkers of Exposure 128

Biomarkers of Effects 131
Inflammation and Oxidative Stress 132
Systemic Biomarkers 134

Genetic and Epigenetic Biomarkers 135
Epigenetic Mechanisms 136
Intercellular Communication 137

Toward Nanospecific and Predictive Biomarkers 138

Biomonitoring in Health Surveillance and Epidemiology 141

Biomonitoring Programs: Practical and Ethical Considerations 146

Conclusions 149

Take-Home Messages 150

References 150

INTRODUCTION

The increasing introduction on the commercial market of first-generation nanoobjects, including their aggregates and agglomerates (NOAA) is undoubtedly a key to industrial innovation in many fields. This increased availability and use brings with it innovative applications but can also lead to personal exposure with the potential for unwanted health effects.

Adverse Effects of Engineered Nanomaterials. http://dx.doi.org/10.1016/B978-0-12-809199-9.00006-9
Copyright © 2017 Elsevier Inc. All rights reserved.

To sustain nanotechnology development in a responsible way, scientists and regulatory bodies should enable reliable prediction and mitigation of potential health effects of manufactured nanomaterials (NMs) and their components. Although no overt health effects in humans caused by NM have been reported yet, and many good studies have demonstrated, though the careful analysis of available dose–response relationships, that we are operating in a safe area so that no significant impact on human health or the environment is expected (Krug, 2014), ensuring the safety of workers and consumers is mandatory for the responsible development and the long-term sustainability of nanotechnology-enabled industry (Bergamaschi et al., 2015).

Risk is a function of the level of exposure to a substance and how hazardous it is; in many cases, occupational exposure to NM may be of concern at all stages of the material life cycle from research through manufacture to use and disposal, whereas consumer and environmental exposures are only estimated, in silico, by modeled data. The available information of the final steps of the life cycle of these materials remains incomplete, so far regarding amounts of handled materials and levels of exposure. To truly understand risks of adverse effects resulting from exposure we must at least understand the hazards a material poses. For NM, there is considerable debate surrounding the accuracy and appropriateness of reading across from other materials (e.g., their bulk counterpart); as a result, gaining accurate knowledge of the hazard status of NM requires in many cases in vitro and/or in vivo toxicological testing.

The diversity and complexity of an ever-increasing number of NM with varying physicochemical properties, and the complex and changeable nature of nano–bio interactions, make the biological behavior of NM not yet predictable on the basis of their inherent properties, because these are subjected to changes arising from the biological environment (Monopoli et al., 2012). Recent findings indicate that the physicochemical properties and the integrity of nanoparticles can change dramatically following internalization by cells in vitro and in vivo, even for nanoparticles with high colloidal stability such as polymer-coated gold nanoparticles (AuNPs) (Kreyling et al., 2015). Whereas the material-intrinsic properties determine the "synthetic identity," the context-dependent properties of the NM influence its "biological identity," which is shaped, in part, by the adsorption of biomolecules that form a "corona" on the surface of nanoparticles; the composition of this "biocorona" depends on the particular biofluid and may exhibit dynamic changes as the nanoparticle crosses from one biological compartment to another (Fadeel et al., 2013). Moreover, particles of similar size and composition can raise qualitatively different effects in relation to their methods of synthesis, as for amorphous silica nanoparticles (Di Cristo et al., 2015).

Besides intrinsic characteristics of particles, the presence of contaminants can cause significant changes in their chemical identity, leading to increase in biological activity. This issue is clearly described by synergistic effect of nanoparticle and endotoxins on cytokine production and immune cells activation (Bianchi et al., 2015; Li and Boraschi, 2016). When considering the harmful effects from particles of concern in occupational/environmental settings, these are consistently associated with a multiplicity of factors, including changes occurring at the surface of nanoparticles from the early stages of their life, including the capacity of NM to act as vectors for the transport of other toxic chemicals. Such alterations in surface characteristics and changes in chemical identity have important experimental implications for biomarker research, because experimental setting may not be truly representative of particle–cell

interactions in a real-life scenario. As a result, our current testing strategy is actualized by using simplified models, different dosing regimens, and exposure routes and so represents a compromise to understand what could happen in more complex systems, in humans, which requires more caution in the interpretation of findings with consideration of the weight of evidence (Bergamaschi et al., 2015).

Insufficiency and ambiguity of the existing in vitro and in vivo toxicological data on NM are hampering the risk assessment process required for scientifically sound regulatory and policy decision-making. Accurate assessment of health risk requires information on critical target organs and knowledge of toxic endpoints from studies in which relevant exposure routes and exposure levels have been used. Such aspect is attenuated where the NM is deployed in applications/products where a risk/benefit ratio can be clearly assessed, such as nanodrugs for instance (Bregoli et al., 2016).

To overcome uncertainties due to the inherent limitations of such simplified models for predicting long-term health effects, search for biomarkers as tools to protect workers has recently been emphasized (Schulte and Hauser, 2012) and a complementary approach based on the application of biological monitoring (BM) strategy has been proposed (Bergamaschi, 2012; Bergamaschi et al., 2015; Iavicoli et al., 2014). BM has been used for decades as a fundamental tool in environmental and occupational health risk assessment to identify potential hazards of new and emerging chemicals and in health practice to monitor groups at risk of health outcomes through the periodic detection of early, preferably reversible, biological indicators which are indicative, if compared with adequate reference values, of an actual or potential condition of exposure, effect or susceptibility possibly resulting in health damage or disease (Manno et al., 2010). These indicators are referred to as biomarkers, which are used as surrogate indicators of designated events in a biological system due to the inaccessibility of target organs.

Biomarkers are recommended for use in assessing the effects or exposure to harmful substances, specifically where there are low or intermittent levels of exposure, mixtures of toxicants that may act synergistically, or exposure resulting in disease with long latency period. Since biomarkers are directly related to the adverse effects which one attempts to prevent, the use of BM represents an essential tool in health risk assessment (IPCS, 1993; Manno et al., 2010; Smolders et al., 2010) with twofold benefit: (1) identifying groups at higher risk of health outcomes and, ideally, (2) validate the "observed" exposure limits (should they exist). Fulfilment of these aims would provide preventive tools for diseases.

Although biomarkers are typically divided into three functional categories (biomarkers of exposure, effect, and susceptibility), from a pragmatic point of view, the use of a biomarker rather than its intrinsic properties may define its classification and biological meaning. For instance, from a toxicological perspective, a biomarker of exposure should predict the likelihood of adverse effects or biological responses, whereas a biomarker of effect should be consistently correlated with the dose. From an epidemiological perspective, a biomarker of exposure should be consistently correlated with exposure (considering both background and interfering factors), whereas a biomarker of effect should be correlated with the risk of disease. To become useful tools in risk assessment, biomarkers must be relevant, that is, appropriate to provide information on important issues concerning health risks, and valid from both analytical and epidemiological aspects. In particular, external validity is required to obtain results which could be generalized to other populations (Mutti, 2001).

ISSUES IN THE DEVELOPMENT OF BIOMARKERS FOR ENGINEERED NANOMATERIALS

Although a causal relationship between exposure to mineral dusts and fibers and pleuropulmonary diseases is clear (Donaldson and Seaton, 2012), the identification of reliable biomarkers of exposure, effect, and susceptibility for particles—including combustion-derived ultrafine particles UFP—become possible once the molecular mechanisms involved in particle-induced diseases have been elucidated (Bergamaschi et al., 2014). The current body of information on the human health effects potentially related to NM comes from the studies on incidental nanoparticles, air pollution epidemiology, and studies on occupational exposures with similarities to nanoparticles such as welding fumes, ultrafine carbon black (CB), diesel exhausts (Madl and Pinkerton, 2009), fumed silica (Merget et al., 2002), metal oxides (Antonini et al., 2003; Luo et al., 2009) and fibers of concern. The particle paradigm is based on the assumption that NM may pose similar, albeit greater, hazards due to their size and other inherent physicochemical properties. With the huge amount of particles investigated, it has been possible to confirm that the main mechanisms involved in their toxicity has been associated with their ability to generate free radicals with ensuing oxidative stress and inflammation as an effect of surface reactivity and physicochemical composition (Donaldson and Poland, 2012; Fubini et al., 2010; Møller et al., 2010). As a result, biomarkers of oxidative stress and inflammation would provide the means of assessing exposure and damage and hence may serve as appropriate predictive tool to detect early changes and for the possibility of preventive intervention (Gulumian et al., 2006). Compared to larger particles, three exceptions should be taken into account when in search for biomarkers of exposure for NM. The first relies on their ability to translocate from the route of entry due to their small size and hence the possibility for the assessment of their concentrations in blood and urine (Cho et al., 2013). The second is the ability of some nanoparticles to release ions due to their dissolution (Cho et al., 2012; Donaldson and Seaton, 2012) and hence the possibility for the measurement of the concentration of these ions in biological matrices (Simpson et al., 2013). If we look for the specific biomarker of NMs in toxicology and safety research, what is different is the biopersistence; conversely, if the NM dissolves, it becomes a normal chemical toxicology issues, meaning the chemistry contained in it and the chemical causing toxicity. Consideration over the composition and what consequence the chemical identity has on its toxicity should be brought forward as risk potential by dissolution pathway which falls under the non-nanospecific issue. As a result, the main issues in the development of biomarkers and biomonitoring practice for NM should also deal with toxicokinetic considerations, including the definition of the dose which can reach target organs and the changes the NM undergo in contact with the biological environment, and also toxicodynamic, that is, what consequence the chemical identity has on “nanospecific” toxic effects.

FACTORS AFFECTING BIOMARKERS OF EXPOSURE

Exposure, that is, the contact between a foreign substance and the body surface to a given chemical, usually results in uptake and leads to an internal dose. For traditional chemicals, the internal dose is usually assessed by both the amount of the substance and/or its metabolites

or as a product of interaction with biomolecules in biological fluids. However, a suitable biomarker of exposure should be not only specific for the exposure of interest, but also detectable in small quantities, measurable by noninvasive techniques and provided of a positive predictive value to a specific health status (Mutti, 2001). For particles, the likelihood of being taken up can vary dramatically among exposure conditions and by the behavior of particle aerosols in which nanoobjects are commonly present and taken up as agglomerates and/or aggregates of various aerodynamic diameter (Kuhlbusch et al., 2011). After their production, NM may be transformed by agglomeration or deagglomeration, or by loss, change, or development of coatings, which may have impact on the uptake and biological effect after uptake. Particles whose aerodynamic diameter is between 0.1 and 1.0 μm may reach the lower respiratory tract, but their deposition is low compared with particles with lower and higher aerodynamic diameter due to symmetric human lung morphology (Broday and Agnon, 2007). It has been estimated that the daily dose of inhaled NP in humans is limited to a maximal NP aerosol concentration of $10^{12}/m^3$ (beyond that, coagulation rapidly occurs under ambient conditions), and the daily volume of air inhaled by an adult is 15 m^3. Assuming a particle deposition of 0.3, the daily deposition amounts to about $5 \cdot 10^{12}$ nanoparticles (Oberdörster, 2010). Although it may take years to reach an appreciable internal dose, with repeated exposures and the low particle fraction showing migration, with years, a considerable internal dose might be achieved. Conversely, the biologically effective dose (i.e., the entity within any dose of particles in tissue that drives a critical pathophysiologically relevant form of toxicity (e.g., oxidative stress, inflammation, genotoxicity, or cell proliferation) may be achieved independently from the mass of inhaled particles (Donaldson et al., 2013).

Whereas primary nanoparticles exist before the contact with the body, at the site of absorption they usually appear as aggregated nanoparticles and, at later stages nanoparticles are firmly attached to the cellular matrix, thus affecting the final fate of the NM (Bergamaschi et al., 2014). Once inhaled, nanoparticles can deposit in lung cells and translocate through epithelial and endothelial cells into the blood and lymph circulation, potentially reaching sensitive target sites including bone marrow, lymph nodes, spleen, heart, and central nervous system (Cho et al., 2013; Choi et al., 2010; Geiser and Kreyling, 2010; Holgate, 2010; Oberdörster et al., 2004; Sung et al., 2009; Yokel et al., 2014). Available biokinetic data suggest that translocation rates from the portal-of-entry—the respiratory tract—to secondary organs, is size- and charge-dependent (Choi et al., 2010), but the amount of particles reaching the systemic circulation is very low (Kreyling et al., 2002, 2009). For instance, nanoceria distribution from the lung to the remaining body is less than 1% of the deposited dose in rodents (Yokel et al., 2014). Cho et al. (2013) studied the absorption, distribution, and excretion patterns of TiO_2 and ZnO nanoparticles following oral administration. Zinc concentrations in blood, organs, and urine were higher than concentrations of titanium when nanoparticles were administered orally for 13 weeks. The urine concentration of Ti in the TiO_2-treatment groups showed no significant differences compared with the control group; in contrast, the concentration of Zn in the urine of ZnO-treatment groups was significantly increased in the middle- and high-dose groups and showed positive trend dose-responses. Similarly, the translocated fraction of AuNPs across the lung epithelial tissue barrier was inversely proportional to the particle size, but independent of the applied dose (up to 100 ng/cm^2) (Bachler et al., 2015). Recent experiments using radio-labeled Au cores and polymer shells, have shown that the polymer shells of polymer-coated Au nanoparticles are partially removed in vitro and in vivo.

In vivo, the nanoparticles are mostly retained in the liver, and fragments of the organic shell are excreted through the kidneys, probably due to proteolytic digestion (Kreyling et al., 2015).

Where agglomeration/deagglomeration might affect the biokinetics of NM and results in local effects at the deposition sites, solubility is important in considering uptake since large or agglomerated NM cannot leave the systemic circulation. Low-soluble NM (e.g., CNT, TiO_2, or CeO_2)—even though they can partially break down in contact with phagocytic cells—show a slow clearance leading to accumulation over time with exposure. For many particles, size and agglomeration state are critical in dictating their toxicity; thus, particle dissolution and the release of ions—which would be expected to be greater for smaller particles—may affect organ distribution. For instance, following liver accumulation, metal nanoparticles show a protracted elimination of particles and slow release from target organs into systemic circulation (Johnston et al., 2010), giving rise to detectable trace amounts in body fluids. However, if a material dissolves before or during migration through a tissue barrier, then the impact of this from the "nano" property perspective is negligible. In this case, the toxicological process must be measured against different standards than those applicable to nanotoxicology, for example, chemical standard indicated in the RipOn document by ECHA (Aitken et al., 2011). These concepts may have a limited impact for biopersistent NM, such as CNTs, for which local toxic or functional effects are mostly driven by agglomeration and direct contact with target cells (Rotoli et al., 2015).

To date, the main biological matrices used to assess exposure are blood serum, urine, and the exhaled breath. Similar to metal species in welding fumes—a reliable occupational model of ultrafine and nanoparticle exposure—metallic elements are measurable in blood and urine with appropriate analytical methods currently used in occupational health surveillance. However, data on humans are very preliminary and fragmented; pilot field studies in occupational settings have determined the urinary excretion of indium, silver, and titanium dioxide in workers occupationally exposed to metal nanoparticles. Workers manufacturing indium tin oxide in Taiwan showed mean S-In level and U-In level in high exposed group of 8.01 and 3.45 ppb, respectively, and were significantly higher than that of low exposed and reference group (Liu et al., 2012). Two male workers who had worked for 7 years in the business of manufacturing silver NM, exposed to silver concentrations of 0.35 and 1.35 $\mu g/m^3$ showed silver concentrations of 0.034 and 0.0135 μg/dL for blood and 0.043 μg/dL and not detected level for urine (Lee et al., 2012). A most comprehensive study has been carried out by Pelclova et al. (2015a) in a cohort of workers ($n = 20$, employed in production or research) exposed to fine and nano-TiO_2 particles. The average airborne TiO_2 number concentration was 1.98×10^4 particles/cm^{-3} and the median mass concentration was 0.65 mg/m^3 with about 80% of the particles smaller than 100 nm in diameter. Titanium levels in urine was analyzed using the inductively coupled plasma–mass spectrometry (ICP-MS) method and Raman microspectroscopic analysis was performed in urine to identify the phase composition of TiO_2 particles observed. In the urine of workers, TiO_2 particles were detected in 2/20 postshift urine samples only, whereas titanium was under the limit of detection (LOD = 1.2 μg/L). Taken together, these data seem to question the usefulness of the blood and urine as biological matrices to detect amount of excreted NM (or chemical species) in occupationally exposed workers.

A more pragmatic approach to estimate exposure is to assess the uptake and deposited dose of inhaled particles. Sampling of exhaled breath condensate (EBC) in occupational

research provides a matrix for the simultaneous monitoring of metal exposure and effects on target organ. As EBC mainly consists of water that is practically free of potentially interfering solutes, it is an ideal biological fluid for elemental determinations based on relatively common techniques, such as electrothermal atomic absorption spectroscopy (ETAAS), or less frequently available reference instruments such as ICP-MS. This novel approach has represented a significant advance over the analysis of alternative media (blood, serum, urine, hair), which are not as reliable (owing to interfering substances in the complex matrix) and reflect systemic rather than lung (target tissue) levels of metallic elements.

Metal content in EBC has been assessed in workers exposed to cobalt and tungsten (Goldoni et al., 2004), in chrome plating workers (Goldoni et al., 2006), in gas metal arc welders in relation to welding fume exposure (Hoffmeyer et al., 2012). Studies involving workers exposed to (nano)TiO_2 pigment in Czech Republic have been carried out (Pelclova et al., 2015b, 2016). The concentration of titanium in the EBC of production workers in the first study was very stable; the preshift (24.1 ± 1.8 μg/L) and postshift samples (24.1 ± 1.9 μg/L) did not differ significantly. Particles of rutile and/or anatase were found in the EBC of exposed workers in 8/20 (40%) of the preshift and 14/20 (70%) of the postshift samples. Based on these results, only workers from the production sector of the plant with higher exposure were further examined, and only postshift samples were collected. The mean concentration of titanium in the EBC in production workers was 24.1 ± 1.8 μg/L. In the research workers the values were below the limit of quantitation, for example, 4.0 ± 0.2 μg/L, as well as in the controls. The concentration of titanium originating from TiO_2 in EBC might serve as a direct exposure marker in workers producing TiO_2 pigment; however, the stability of its concentrations in EBC not being influenced by current exposure, support the use as a biomarkers of deposited dose following long-term exposure.

Although assessing target organ dose or internal dose is problematic, obtaining quantitative data is essential for dosimetry model validation, for building dose–response relationships for the most sensitive organs and, ultimately, for ascertaining the range of exposure level with no observed adverse effects (NOAEL). When dose–effect and dose–response relationships are known, a biomarker of dose may predict an adverse effect rather than only exposure, making it appropriate for preventive purposes, such as the removal of people from excessive exposure before the onset of adverse or irreversible health effects.

BIOMARKERS OF EFFECTS

A biomarker of effect has been defined as "a measurable biochemical, physiological, or other alteration within an organism that, depending on magnitude, can be recognized as an established or potential health impairment or disease" (IPCS, 1993). Biomarkers of effect can be used to evaluate whether a well-characterized exposure is associated with a shift in the distribution of relevant biochemical or functional endpoints indicative of early changes in the target or critical organs/tissues. To assess such early events associated with exposure to NM, potential biomarkers can include biomolecules indicative of local and systemic oxidative stress, inflammatory response in target organs—like in respiratory and cardiovascular systems—and systemic inflammation (Bergamaschi, 2012; Bergamaschi et al., 2014).

Inflammation and Oxidative Stress

Since airway inflammation is the main outcome investigated following NM exposure, inflammatory biomarkers deserve greater importance for biomonitoring purposes. Quantification of lung inflammation in both clinical setting and health monitoring practice is currently based on noninvasive methods including EBC analysis. EBC contains 99.9% water and a minor proportion of water-soluble and insoluble compounds. These nonvolatile compounds released from the airway lining fluid in the form of aerosolized particles can range from small inorganic ions, through larger organic molecules (urea, organic acids, amino acids), to proteins and macromolecules (nucleic acids) as well as cytokines. EBC can be collected during tidal breathing using a specially designed condenser (e.g., EcoScreen, Jaeger, Würzburg, GE; ECoScreen2, FILT, Berlin, GE; TURBODECCS, Italchill, Parma, Italy). Being collected in a totally noninvasive way, EBC is particularly suitable to be applied in field studies and for longitudinal assessments of pulmonary biology. Despite some (still) unresolved problems in standardization (Horvath et al., 2005), the identification of selective profiles of biomarkers might be useful for relative measures of several biomarkers, including inflammatory mediators (Montuschi, 2007). EBC analysis allows the detection of several biomarkers of oxidative stress and inflammation, including thiobarbituric acid reactive substances (TBARS), such as malondialdehyde (MDA)—produced by lipoperoxidation of membranes—and 8-isoprostane, a peroxidation products of prostaglandin metabolism (Montuschi, 2007). Changes in pH, proteins, and proinflammatory cytokines, such as leukotrienes B_4 (LTB_4) can also be determined. Inflammatory response in the airways is characterized by an influx of neutrophils, whose activation is associated with a respiratory burst resulting in overproduction of hydrogen peroxide (H_2O_2), measurable in EBC. Glutathione (GSH), which is present in high concentration in epithelial lining fluid and at tissue level of the lung, represents one of the major biological antioxidant defense mechanisms against reactive oxygen species (ROS) and the secondary products ROS reacting with thiol groups can severely deplete the GSH pool. The simultaneous assessment of these biomarkers, help understanding the pathophysiological mechanisms underlying the inflammatory effect; for instance, if no changes are seen in 8-isoprostane levels, while a marked increase in H_2O_2 is observed immediately after exposure, such finding is consistent with the neutrophil influx and activation with subsequent generation of superoxide anion, which is rapidly dismutated to H_2O_2.

Various classes of volatile organic compounds (VOCs) can also be measured in exhaled breath, including the saturated hydrocarbons and oxygen-containing substances formed during the fatty acid lipid peroxidation of cell membranes during respiratory burst. Saturated aldehydes such as hexanal, heptanal and nonanal are formed by the peroxidation of omega-3 and -6 fatty acids (PUFAs), the basic components of cell membrane phospholipids. Volatile aldehydes are poorly soluble in blood and are therefore excreted into the breath within minutes of their formation in tissues, making it possible the study of pulmonary diseases associated with inflammatory status or lung cancer (Poli et al., 2010). EBC concentrations of MDA, 4-hydroxy-*trans*-nonenale (HNE) and 4-hydroxy-*trans*-hexenale (HHE) were significantly different between unexposed workers and exposed workers with all three being elevated in the exposed group in two studies (Lee et al., 2015; Pelclova et al., 2015b).

Breath can be analyzed also in the gaseous phase (as exhaled breath). Among the broad spectrum of gaseous compounds detectable in exhaled air, nitric oxide (NO) is produced

by all cellular components of pulmonary inflammation (macrophages, epithelial cells, mast cells, lymphocytes, and granulocytes). Studying NO exhaled from the lower airways offers a unique possibility to study features of pulmonary NO metabolism noninvasively in all states characterized by lung inflammation (Pacher et al., 2007). In a follow-up study, Wu et al. (2014) found an increase in fractional exhaled nitric oxide (FENO) among workers handling nano-TiO_2 powders, but not in all of the NM exposed categories investigated; the values recorded were over the threshold of 35 ppb, suggesting a chronic airways inflammation.

Under physiologic conditions, NO is unstable, reacting with oxygen to form oxides of nitrogen such as nitrite (NO_2^-) and nitrate (NO_3^-), and with superoxide anion to form the potent oxidant peroxynitrite ($ONOO^-$), which reacts with a wide variety of molecules, including DNA, lipids, and sulfhydryl groups on proteins thus promoting nitrosative stress. Nitrosothiols (RS-NOs), which are formed by interaction of nitric oxide (NO) with GSH, can be quantified in EBC of humans and reliably used as biomarkers of airway inflammation (Bergamaschi, 2012).

Clara cells are nonciliated secretory epithelial cells lining the pulmonary airways, distinct from mucous and serous secretory cells in morphology and secretory products. Among these, the Clara cell protein (CC16) plays an immunomodulatory activity, it can inhibit phospholipase A2 activity, while inflammatory cytokines like TNF-α can modulate CC16 production (Hermans and Bernard, 1999). CC16 and surfactant-associated protein B (SP-B) in the serum have been validated as markers of alveolocapillary barrier integrity/permeability in human studies on gaseous pollutants (Broeckaert et al., 2000) or mineral particles, such as silica (Gulumian et al., 2006; Wang et al., 2007). There is evidence that acute exposures to certain pulmonary irritants can cause a transient increase in serum CC16 levels, and limited evidence also suggests that a transient increase in serum CC16 levels can be caused by a localized pulmonary inflammation without impairment of pulmonary function. Serum CC16 levels have shown significant increase after ultrafine particle exposure to wood smoke in healthy volunteers (Barregard et al., 2008), and workers exposed to elemental carbon ultrafine particles (Alessandrini et al., 2010) while ambiguity in levels were detected in workers exposed to mixtures of engineered NMs (Liou et al., 2012). In workers in indium tin oxide (ITO) production plants, significant positive relationships were found between S-In and surfactant protein A (SP-A), and surfactant protein D (SP-D) levels, sensitive markers of interstitial lung disease. SP-A and SP-D levels were elevated significantly in the workers with moderately high indium exposure (Liu et al., 2012). The great advantage of pneumoproteins is their great sensitivity in detecting epithelial effects at levels of pollutants below current air standards. However, the biological interpretation of chronic changes in serum CC16 is less clear, owing to chronobiological variability and the evidence that chronic exposure to pneumotoxicants leads to a decrease in the Clara cell population (Lakind et al., 2007).

Oxidative stress is a central mechanisms of action for both pulmonary and extrapulmonary health effects of particulate matter (PM) (Mills et al., 2009) and a number of studies of exposure to particles in humans have applied biomarkers of oxidative damage in the blood compartment or in terms of products excreted in urine or EBC. Metaanalysis of panel studies and cross-sectional investigations considering the application of such biomarkers have shown a consistent association between exposure to combustion particles with oxidative damage of DNA and lipids, suggesting that it is possible to use these measurements as biomarkers of biologically effective dose (Loft et al., 2008; Møller and Loft, 2010). Among the DNA oxidation

products, such as 8-oxo-7,8-dihydroguanine (8-oxoGua) or the corresponding deoxynucleoside 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) measured in DNA and urine, the exocyclic M1 adduct to guanine (M1dG) were the most studied. Base excision repair of oxidative damage to DNA measured in urine, such as 8-hydroxy-2′-deoxyguanosine (8OHdG), seems to originate mostly from the oxidation of the deoxynucleotide pool and does not represent solely repairing/excretion of the oxidized-DNA guanine, but biomarkers of effective dose. Once produced, 8OHdG is very stable and is not further metabolized in the systemic circulation. After exposure to oxidants, the repair and final 8OHdG excretion in urine is rapid, that is, within at least 24 h (Cooke et al., 2009). Urinary 8-OH-dG concentration has been investigated as a potential biomarker of oxidative stress in response to exposure to incidental NPs emitted from photocopiers, and it was found significantly increased as compared with background levels (Khatri et al., 2013). Biomarkers of oxidative stress to proteins [such as *o*-tyrosine (*o*-Tyr), 3-chlorotyrosine (3-ClTyr) and 3-nitrotyrosine (3-NOTyr)] were found elevated in EBC of workers occupationally exposed to TiO_2 aerosols as compared to not exposed people (Pelclova et al., 2015b). The same study found an increase in markers of oxidation of nucleic acids [including 8-hydroxy-2-deoxyguanosine (8-OHdG), 8-hydroxyguanosine (8-OHG), 5-hydroxymethyl uracil (5-OHMeU)] in EBC of production workers (Pelclova et al., 2015b). Conversely, in workers handling NMs in 14 plants in Taiwan, 8-OH-dG urinary and plasma levels did not show significant differences compared with controls (Liou et al., 2012).

Systemic Biomarkers

Early events at vascular level can be assessed by a panel of circulating biomarkers reflecting inflammation endpoints, platelet activation, antioxidant capacity [e.g., by assessing the activity of Cu/Zn-superoxide dismutase (SOD) and glutathione peroxidase-1 (GPx-1)] (Delfino et al., 2010). Inactivation of antioxidant enzymes within erythrocytes, plasma interleukin-6 (IL-6), and soluble tumor necrosis factor-receptor II (sTNF-RII), investigated during a longitudinal study, showed a positive association with vehicle emissions tracers.

Exposure to NM could enhance the adhesion of endothelial cells and modify the membrane structure of vascular endothelium which plays an important role in the regulation of fibrinolysis. Radomski et al. (2005) showed that both urban dusts and engineered carbon particles, such as CNT and CB—except C_{60}CS—stimulated platelet aggregation and accelerated the rate of vascular thrombosis in rat carotid arteries with a similar rank order of efficacy. All particles resulted in upregulation of GPIIb/IIIa in platelets. In contrast, particles differentially affected the release of platelet granules, as well as the activity of thromboxane-, ADP-, matrix metalloproteinase-, and protein kinase C-dependent pathways of aggregation. Consistent with the in vitro results, exposure to nano-Ag (0.05–0.1 mg/kg i.v. or 5–10 mg/kg intratracheal instillation) in vivo enhanced platelet aggregation and promoted venous thrombus formation in rats (Jun et al., 2011). Therefore, assessment of platelet aggregation and expression of GPIIb/IIIa in platelets may be useful to evaluate possible prothrombotic effects induced by NM. Early systemic prothrombotic effects induced by fine particle (<2.5 μm) exposure were detected by the quantification of tissue factor (TF) activity in plasma—which is expressed in subendothelial cells upon injury or on the surface of circulating monocytes, macrophages, and neutrophils—and the levels of the plasminogen activator inhibitor-1 (PAI-1) (Kilinç et al., 2011). Vesterdal et al. (2010) showed that i.t. instillation of Printex 90 in apolipoprotein E knockout

mice (apoE−/−) resulted in modest vasomotor impairment without increases in the expression of vascular adhesion molecule (VCAM-1) and intercellular adhesion molecule (ICAM-1) on endothelial cells. Workers exposed to a mixture of NM showed statistically significant changes across exposure risk classes for high sensitive C reactive protein levels and inflammatory cell activation (increased ICAM-1 in macrophages), IL-6, and fibrinogen. Moreover, the depression of antioxidant enzymes, namely SOD and GPx, were associated with NM handling (Liou et al., 2012).

Cytokines perform pleiotropic functions to mediate and regulate the immune response and are generally recognized as biomarkers of immunotoxicity. While the specificity and validity of certain cytokines as markers of adverse immune response has been established for chemicals and drugs, research on their applicability for predicting and monitoring the immunotoxicity of NM is still ongoing; in general, IL-3, Il-4, IL-5, IL-6, IL-10, IL-12, TNF-α, and IFN-γ are pointed out in the immunotoxicity of nanoparticles (Elsabahy and Wooley, 2013).

Erdely et al. (2011) demonstrated that the pulmonary exposure to CNT results in an elevated series of measurable potential biomarkers in the blood, including genes expressed in the circulating blood cells and/or soluble proteins not unique to the type of particles, but representing the unity of local and systemic pathophysiological responses induced by the exposure. In particular, MWCNT-induced gene upregulation of more than half of the tested genes in the lung related to inflammation, oxidative stress, coagulation, and tissue remodeling and in a significant increase in the circulating blood gene expression of several biomarkers of neutrophil response, such as CXCL2 (MIP-2), S100a8, IL-8rβ, and Mac-1. Furthermore, several genes were activated in the circulating blood cells but not in the lung at least 4 h after pulmonary exposure to MWCNT, for example, osteopontin, colony stimulating factor-1 (CSF-1), and insulin growth factor receptor 1 (IGF-1R). Pulmonary exposure to CNT also triggered the induction of primary cytokines such as IL-6 and IL-1β, which regulate multiple pathways of the inflammatory cascade as well as secondary inflammatory mediators, chemokines (CCL2, 4, 19, 22; CXCL1, 2), which directly regulate leukocyte recruitment to the inflammatory site.

GENETIC AND EPIGENETIC BIOMARKERS

The DNA damaging potential of nanoscale particles is known (see Chapter 18), as well as and the appropriate methodologies to be used for investigation of genotoxic effects of NPs, in vitro and in vivo (Magdolenova et al., 2013; Singh et al., 2009). NM of different composition (metal/metal-oxide nanoparticles, silica, quantum dots, fullerenes, nanofibres) can affect DNA also by direct mechanisms, including a mechanical interference with cellular and nuclear components, like microtubules of the mitotic spindle (Gonzalez et al., 2008). The majority of the NM tested both in vitro and in vivo are able to cause DNA strand breaks or oxidative DNA lesions. DNA damage in peripheral blood leukocytes can be quantified by single cell gel electrophoresis (SCGE; Comet assay) a simple and sensitive method for detecting DNA strand breaks and alkali-labile sites, while oxidatively damaged DNA can be analyzed using the enzyme formamidopyrimidine glycosylase (FPG). Although interactions between the particles and the assay cannot be totally excluded, the use of Comet assay in human biomonitoring studies could provide valuable information for hazard identification of NM (Karlsson, 2010; Karlsson et al., 2015). Modified SCGE provides parallel information

on oxidative DNA damage caused by NM, such as CNT, detecting oxidized/damaged pyrimidines and purines (Migliore et al., 2010; Muller et al., 2008), and revealing dose–effect and dose–response relationships between the mass concentration of NM and the frequency of micronucleated lymphocytes. Both tests have been applied in a cohort of NM workers in Taiwan, but there were no significant differences in changes between the exposed and control workers between baseline and the 6-month follow up (Liao et al., 2014b).

There is growing evidence that *epigenetic* modifications, such as changes in DNA methylation, histone modifications, and microRNA (miRNA) expression, may be caused by environmental factors. DNA methylation—a major genomic mechanism of gene expression control can be affected by ROS, which are considered one of the main cellular stressors generated by PM exposure as well as by some metals. Conditions associated with reduced global DNA methylation content have been shown to interact with ambient PM exposure to produce health-related outcomes. For instance, gene expression and the associated epigenetic changes have been demonstrated following exposure to metal-rich PM with aerodynamic diameters <10 μm (PM10) in workers in an electric furnace (Bollati et al., 2010; Tarantini et al., 2009).

Epigenetic Mechanisms

Epigenetic mechanisms are involved in the induction of toxicity of engineered NM (Stoccoro et al., 2013), although the transgenerational impact of such changes is not fully understood (Shyamasundar et al., 2015). Stoccoro et al. (2013) highlighted the ability of certain nanoparticles to induce an impaired expression of genes involved in DNA methylation reactions leading to global DNA methylation changes, as well as changes of gene-specific methylation of tumor suppressor genes, inflammatory genes, and DNA repair genes, all potentially involved in cancer development. Brown et al. (2016) assessed the promoter methylation of inflammatory genes (IFN-γ and TNF-α) after MWCNT exposure and found a correlation between these changes and initial cytokine production. In addition, methylation of a gene involved in tissue fibrosis (Thy-1) was also altered in a way that matched collagen deposition. These authors also demonstrated that DNA hypomethylation in the lung and blood coincided with disease development (i.e., fibrosis).

Micro (mi)RNAs are highly conserved, noncoding small RNAs that regulate the expression of broad gene networks at the posttranscriptional level, for example, interacting with several mRNA targets, and their use as possible biomarkers of the effects of acute and chronic environmental exposure has been suggested (Vrijens et al., 2015). Some nanosized compounds are able to induce selected miRNAs deregulated expression, and this may help in finding specific fingerprints. Ng et al. (2011) demonstrated that AuNPs altered the expression of 19 genes in human fetal lung fibroblasts, upregulating the miRNA-155 (miR-155) and downregulating the *PROS1* gene—a gene encoding a vitamin K-dependent plasma protein that functions as a cofactor for the anticoagulant protease, activated protein C (APC) to inhibit blood coagulation. Silencing of miR-155 established *PROS1* as its possible target gene. DNA methylation profiling analysis of the *PROS1* gene revealed no changes in the methylation status of this gene in AuNP-treated fibroblasts, whereas chromatin condensation and reorganization was observed in the nucleus of fibroblasts exposed to AuNPs. A miRNA microarray analysis carried out on blood of rats at 1 week and 2 months after injection with AuNPs showed an increase in rno-miR-298 [which regulates β-amyloid (Aβ) precursor protein-converting

enzyme-1 (BACE1)] at 1-week postinjection (Chew et al., 2012). Nagano et al. (2013) compared the effectiveness of serum levels of liver-specific or enriched miRNAs (miR-122, miR-192, and miR-194) with that of conventional hepatic biomarkers (alanine aminotransferase and aspartate aminotransferase) as biomarkers for silica nanoparticles of 70 nm diameter (nSP70)-induced liver damage in mice. Computationally predicted mRNA targets of the significantly differentially expressed miRNAs in response to silver (Ag) nanoparticles in human Jurkat cell line as a model were identified by Eom et al. (2014).

Intercellular Communication

A very promising field of research for biomarkers development is represented by the study of modes of intercellular communication, since this could also explain how exposure to NM can result in effects not limited to the portal of entry, but involves a systemic response in nonprimary target organs (Raposo and Stoorvogel, 2013). Cells release into the extracellular environment diverse types of membrane vesicles of endosomal and plasma membrane origin called exosomes and microvesicles (MVe), respectively. Exosomes are small cell–derived vesicles of 30–120 nm that are present in many and perhaps all biological fluids. Now we know that exosomes carry various molecular constituents of their cell of origin, including proteins, lipids, mRNAs, and miRNAs (Lin et al., 2015). They are released from many cell types, such as dendritic cells, lymphocytes, platelets, mast cells, epithelial cells, endothelial cells, and neurons, and can be found in most bodily fluids including blood, urine, saliva, amniotic fluid, breast milk, hydrothoracic fluid, and ascitic fluid, as well as in culture medium of most cell types (Théry et al., 2002). As a consequence of their origin, exosomes from different cell types contain endosome-associated proteins, some of which are involved in MVe biogenesis (van Niel et al., 2006). The cargo of MVe include both mRNA and miRNA; thus EV-associated mRNAs could be translated into proteins by target cells (Montecalvo et al., 2012; Valadi et al., 2007). Recently, analysis of RNA from vescicles by unbiased deep sequencing approaches demonstrated that, in addition to mRNA and miRNA, MVe also contain a large variety of other small noncoding RNA species enriched relative to the RNA profiles of the originating cells (Valadi et al., 2007) that can be characterized by high resolution flow cytometry–based methods (Nolte-'t Hoen et al., 2012). Different in vitro studies have revealed that cellular uptake of nanoparticles provoked MVe formation intracellularly; however, few studies have studied the biological consequences once the exosomes have been secreted extracellularly in vivo. Upon i.t. instillation (4 or 20 μg) with 43-nm diameter magnetic iron oxide nanoparticles (MIONs), Zhu et al. (2012) observed a dose-dependent generation of exosomes in the alveolar region of BALB/c mice. These exosomes were quickly eliminated from alveoli into systemic circulation and largely transferred their signals to the immune system, for example, leading to maturation of dendritic cells and activation of splenic T cells. Furthermore, exosome-induced T-cell activation was more efficient toward sensitized T cells and in ovalbumin (OVA)-sensitized mice than in the unsensitized counterparts. Activation of systemic T cells revealed a Th-1 polarization and aggravated inflammation, which might worsen allergic diseases in OVA-sensitized mice. Interestingly, the maximum dose used in this study is equal to a half-workday deposition mass in human lung tissue at the permissible exposure limit (PEL) of iron oxide fume (at a concentration of 10 mg/m^3) suggested by the Occupational Safety and Health Standards (OSHA, USA). These findings suggest that respiratory exposure to

MIONs can activate systemic T cells in susceptible individuals, through exosome-mediated signaling pathways and this may have implications for nanosafety evaluation, especially considering susceptible workers or consumers.

TOWARD NANOSPECIFIC AND PREDICTIVE BIOMARKERS

Research of biomarkers reflecting exposure to certain particles and fibers of concern have already generated a large amount of data supporting the validity of intermediate endpoints to assess changes before clinically apparent disease occurs (Gulumian et al., 2006) and several biomarkers can reliably help in assessing exposure and effects of NM (Bergamaschi et al., 2014; Iavicoli et al., 2014). The challenge that remains for the use of biomarkers of exposure for NM is the lack of specificity of all the biomarkers developed so far that could hamper the applicability of biomonitoring to nanoobjects (Bergamaschi et al., 2014). Since NM can interact with multiple organs and affect different cell functions, it is difficult to determine with conventional assay what changes and adverse effects may occur, and whether biochemical and/or functional changes can be material-specific or exposure-route specific. However, it should be recognized that the existence of nanospecific (size-dependent) effects is an arbitrary assumption, since the threshold of 100 nm do not infer, per se, new properties and instead there is a gradual change in reactivity associated with increasing surface area (Bergamaschi et al., 2015).

Using a macrophage cell model, Waters et al. (2008) demonstrated that the ability of unopsonized amorphous silica particles to stimulate inflammatory protein secretion and induce macrophage cytotoxicity scales closely with the total administered particle surface area across a wide range of particle diameters (7–500 nm). Whole genome microarray analysis of the early gene expression changes induced by 10- and 500-nm particles showed that the magnitude of change for the majority of genes affected correlated more tightly with particle surface area than either particle mass or number, and gene expression changes were particle size-specific. However, the overall biological processes represented by all gene expression changes were nearly identical, irrespective of particle diameter, in the 10–500 nm range of particles. These findings suggest that on an equivalent nominal surface area basis, common biological modes of action are expected for nano- and suprananosized silica particles. The most robust transcriptional change observed was an induction of the Cxcl2 chemokine (MIP-2a), implicated in crystalline silica-induced lung neutrophil recruitment. More definitive changes such as surface reactivity could actually modulate biological interactions and toxicity independently from size, as is also the case in classical particle and fiber toxicology. This does not mean that there are no changes specifically induced by some particles at certain size thresholds, but these changes should be interpreted as mode of actions instead as "new" toxicological properties (Donaldson and Poland, 2013; Lynch et al., 2014). For instance, the more toxicity and DNA damaging potential of nanosized than microsized CuO particles in A549 cells has been attributed to the ability to deliver higher amount of Cu^{++} inside cells (Trojan horse effect). In contrast, the micrometer particles of TiO_2 caused more DNA damage compared to the nanoparticles, which is likely explained by the crystal structures. In conclusion, nanoparticles are not always more toxic than micrometer particles, although the nanolevel gives rise to specific concern (Karlsson et al., 2009).

Advances in system toxicology and "-omic" techniques applied to nanotoxicology should help to identify nanospecific endpoints. systems toxicology is a recognized mean for the identification and application of biomarkers for improved safety assessments (Costa and Fadeel, 2016). system toxicology is expected to provide information on the dynamic interaction between molecular components of biological systems and about the possible mechanisms, by assessing whether specific biological pathways are activated/perturbed by specific NM, thus identifying fingerprints and nanospecific endpoints useful for hazard identification and, ultimately, for RA (Costa and Fadeel, 2016; Sturla et al., 2014). In particular, "omics" technologies could provide tools to measure biological responses to exposures, as reflected by profiles or signatures of transcripts, proteins, or metabolites. Despite the multitude of possible key markers, small biological metabolites (metabonome) and proteins that are likely to be activated when cells come into contact with nanoparticles, there are attempts seeking to identify potential biomarkers of nanoparticle exposure and their mechanistic relevance to provide a holistic view. High-content genomics tools used to compare in vivo pulmonary responses of MWCNT to those in cultured lung epithelial cells (FE1) in vitro assessing global transcriptomics, revealed a strong dose-dependent activation of acute phase and inflammation response in mouse lungs reflective mainly of an inflammatory response as observed in BALF (Søs Poulsen et al., 2013). In vitro, a wide variety of core cellular functions—including transcription, cell cycle, and cellular growth and proliferation—were affected. Oxidative stress, fibrosis, and inflammation processes were altered in both models. Despite similarities observed between the two models at the pathway level, the specific genes altered under these pathways were different, suggesting that the underlying mechanisms of responses are different in cells cultures and lung tissue suggesting the identification of biomarkers using in vitro models need to be assessed with care and validated in vivo as is often the case.

Omics have mainly been used in effects-oriented nanosafety research for the purpose of hazard identification, but have not been widely applied in biomonitoring, or identification of biomarkers. Apart from increasing our knowledge on how the cellular processes and pathways are affected by NMs, these findings could be used to identify specific biomarkers of toxicity. Higashisaka et al. (2011) employed a SDS-PAGE-based proteomics approach to identify candidate biomarkers of exposure and toxicity of silica nanoparticles following intravenous administration in mice. 70 nm Silica nanoparticles (nSP70) induced a higher level of acute phase proteins such as haptoglobin, C-reactive protein (CRP), and serum amyloid A (SAA) than larger silica particles (diameter >100 nm). In addition, the level of these acute phase proteins was elevated in the plasma of mice after intranasal treatment with nSP30. The same authors identified hemopexin (another acute phase protein) as a potential biomarker for predicting the effects of silica nanoparticles (Higashisaka et al., 2012). Interestingly, surface modification of silica particles with amino or carboxyl group suppressed the inflammatory response. Acute phase proteins, such as CRP, are commonly used as biomarkers of (inflammatory) disease, and associations have also been shown between inflammatory biomarkers including CRP and exposure to PM air pollution (for a review, see Bergamaschi et al., 2014). To address the safety assessment of AuNPs Gioria et al. (2015) developed an integrated approach combining the two techniques to study the effects of two sizes, 5 and 30 nm, of AuNPs in Caco-2 cells. After 72 h exposure, 61 responsive (up/downregulated) proteins were identified and 35 metabolites in the cell extract tentatively annotated. Several altered biological pathways were highlighted by integrating the obtained multiomics data

with bioinformatic tools, thus providing a unique set of molecular information on the effects of NMs at cellular level. The secretome (secreted proteome) analysis of A549 cells exposed to fumed silica nanoparticles lead the authors (Pisani et al., 2015) to derive a "no observed transcriptomic adverse effect level" (NOTEL) that showed a higher sensitivity as compared to conventional cell viability tests. With a similar approach, Palomäki et al. (2015) performed proteomics analyses of human macrophages exposed to tangled or rigid, long MWCNTs, or crocidolite asbestos, using hyphenated techniques and concluded that not all types of CNTs are as hazardous as asbestos fibers.

Despite known paradigms, particles of different size and composition can raise qualitatively similar—even though quantitatively different—biological responses. Comparative proteomic studies have shown strong similarities in the pulmonary response to different ENM with known hazardous particles and fibers. One study performed repeated aspiration of single-walled carbon nanotubes (SWCNT) and crocidolite asbestos (4 microgram/mouse, twice a week, for 3 weeks) allowed the identification of a pattern of 109 proteins representing cellular processes affected by SWCNT and crocidolite asbestos. A high-sensitivity marker of inflammation, S100a9, which can be found in asbestos workers was highly expressed after SWCNT exposure potentially suggesting a common inflammatory effect (Teeguarden et al., 2011). Palomäki et al. (2011) demonstrated that long, needle-like CNT and asbestos activated secretion of IL-1β from LPS-primed macrophages but only long, needle-like CNT-induced IL-1α secretion. SiRNA experiments demonstrated that the NLRP3 inflammasome was essential for long, needle-like CNT and asbestos-induced IL-1β secretion. Similarities and differences in molecular pathways of toxicity can be found, for example, after CB and titanium dioxide (TiO_2) nanoparticle exposures; TiO_2 and CB nanoparticles could induce a large variety of cellular mechanisms leading to toxicity, some of which are similar between these two NMs and represent maybe general pathways of nanotoxicity, whereas others are specific to each particle due to their different physicochemical characteristics, that are also indicative for the mode of action of other insoluble NMs (Boland et al., 2014). Following intra tracheal instillation of titanium dioxide (TiO_2), CB, diesel exhaust particles (DEP), and carbon nanotubes (CNTs), tissue mRNA expression of acute phase protein and plasma levels of serum amyloid 3 (SAA3) were increased. Interestingly, these inflammatory biomarkers significantly correlated with the magnitude of neutrophilic influx in bronchoalveolar lavage fluid (BALF), thus suggesting that lung inflammation is associated with the expression of biomarkers predictive of cardiovascular outcomes (Saber et al., 2013). The results of a metaanalysis involving seven studies reporting transcriptomic responses (i.e., mRNA profiles) following intratracheal exposure to increasing doses of CNTs, CB, and TiO_2 nanoparticles of varying properties, were compared with those observed in lung diseases caused by various agents to find similarities among involved pathways (Nikota et al., 2016). Two distinct clusters of gene expression changes were identified for TiO_2 and CNTs, the latter being consistent with lung fibrosis pathogenesis (for a comprehensive list of genes see Nikota et al., 2016).

Posttranslational mechanisms are also implicated in protein modification upon ENM exposure both in vitro and in vivo. For instance, accelerated citrullination of proteins has been recently recognized as a common posttranslational modification pathway specifically induced by exposure to SiO_2 nanoparticles, carbon black nanoparticles (CBNP) and SWCNTs via the Ca^{2+}-dependent enzyme peptidylarginine deiminase (PAD) both in cultured human cells and in the lungs of mice exposed to respirable SWCNT (Mohamed et al., 2012, 2013).

These studies indicate that increased protein citrullination represents an early response to SWCNTs and can be a potential robust indicator for a novel inflammation-independent paradigm defining the SWCNTs lung toxicity. C57BL/6 mice exposed to increasing doses of Printex 90 CBNP showed perturbation of pathways, networks, and transcription factors of predicted phenotypes (e.g., pulmonary inflammation and fibrosis), that correlated with dose and time. Comparison to inflammatory lung disease models and human disease profiles revealed that induced gene expression changes in CBNP-exposed mice were similar to those typical for pulmonary injury and fibrosis in humans, thus supporting the use of toxicogenomic in human health RA of NM (Bourdon et al., 2013).

Unlike genomics, transcriptomics, and proteomics, metabolomics provide a tool for measuring biochemical activity directly by monitoring the substrates and products transformed during cellular metabolism. Untargeted profiling of these chemical transformations at a global level serves as a phenotypic readout that can be used effectively in diagnosing pathologies, identifying therapeutic targets of disease and investigating the mechanisms of fundamental biological processes underlying toxicology (Patti et al., 2012; Roux et al., 2011). However, application of this approach to nanotoxicology is at its earlier stages, albeit these applications can be quite diverse, ranging from measurements made of metabolites differentially expressed and excreted in urine, found in serum, identified in organs such as the liver, or assessed intracellularly or extracellularly in in vitro studies (Lv et al., 2015). The current literature mainly addresses exposure to TiO_2 and SiO_2NP, but there are also examples of studies on silver, zinc, copper, CNTs. The main advantage of assessing cells' whole metabolome is represented by the possibility to assign specific markers for known toxicity paradigms, and, furthermore, give insights into novel intracellular events. This can allow a platform for both high-throughput screening, but also for toxicity hypothesis generation.

Collectively, the broad responses found across experiments indicates that although many studies focus on oxidative stress to predict toxicity, organisms respond with more than an oxidative stress response (Shvedova et al., 2010). Given the impacts of sublethal chronic exposures that will most likely occur, including a diversity of pathways become necessary to ultimately determine how NM may impact organisms in more realistic exposure scenarios (for a comprehensive review, see Klaper et al., 2014).

BIOMONITORING IN HEALTH SURVEILLANCE AND EPIDEMIOLOGY

BM is an important component of the occupational and environmental health surveillance, along with clinical monitoring that corresponds to the continuous systematic collection, analysis, and interpretation of specific health effects (Nasterlack, 2011). On one hand, BM based on the effects biomarkers can be helpful in providing the correct interpretation of doubtful clinical tests and a better objectivity of the clinical judgment. On the other hand, BM based on the exposure biomarkers can be helpful especially when occupational and/or environmental exposure monitoring data are unavailable or difficult to obtain. In Europe, the Framework Directive 89/391/EEC (1989) on safety and health at work aims to ensure that essential preventive measures are taken, including the surveillance of worker's health for risks assessed (not presumed) at work, while the article 10 of the Chemical Agents

Directive 98/24/EEC (Council Directive, 1998) lays down a more detailed framework for health surveillance, including requirements for individual health and exposure records to be introduced at national level. Any decision to include BM in medical surveillance should be taken after careful assessment of exposure to a health hazard and the availability of tests with a known sensitivity and specificity to detect health effects in an early, preferably reversible, stage. Thus, biochemical tests or functional parameters should be supported by consistent pathophysiological mechanisms. It should be recognized that potentially relevant health endpoints ascribed to NM (cardiovascular, pulmonary, and inflammatory) are often unspecific, show high prevalence in the general population, and share multiple nonoccupational risk factors (Bergamaschi, 2009; Nasterlack et al., 2008). Thus, to monitor short-term health effects, it is necessary to identify potential biomarkers, assess their relationship with exposure, and differentiate subclinical effects and the related biomarkers as well as relevant health endpoints potentially ascribed to NM (Riediker et al., 2012). In addition to oxidative stress and inflammation, it is important to consider that some of the NM interactions may also result in other forms of injury, such as protein denaturation, membrane damage, DNA damage, and immune reactivity.

The literature on short-term effects of air pollutants (Andreoli et al., 2015; Barath et al., 2013; Delfino et al., 2010; Møller and Loft, 2010; Patel et al., 2013; Roy et al., 2014; Sarnat et al., 2011; Steenhof et al., 2013) and the available literature on NM, suggest identifying multiple biomarkers—a biomarker profile—to assess both effects at the "portal of entry" (e.g., inflammatory changes, short-term respiratory changes, respiratory, eye, or skin irritation) and systemic effects [e.g., heart-rate variability (HRV), platelet aggregation and prothrombotic changes, acute phase proteins] in susceptible subgroups of the general population exposed to incidental nanoparticles or to a mixture of pollutants.

Despite the lack of validation of "nanospecific" biomarkers, it is proposed that the sensitivity instead of the specificity of biomarkers should be privileged to identify potentially predictive biomarkers suggestive of the biological pathway and mechanistic changes underlying the causality of exposure conditions and association with hazards (e.g., at the workplace) and also to identify predictive biomarkers (Bergamaschi et al., 2015).

Epidemiological data from human populations specifically exposed to NM are currently very limited. There are at least two explanations for this. On one hand, it is due to the relatively recent appearance of NM as a field of research (~10 years) and the still limited penetration of nanotechnology in industry. On the other hand, there is still the lack of consensus among toxicologists on the exposure metric to use and on the specific NM physicochemical characteristics which can be deemed dangerous or which can potentially cause biological damage (Guseva Canu et al., 2013, 2016). Consequently, owing to the short latency since the introduction of ENM on the market, possible target people or groups not have had long and consistent exposure leading to appreciable effects (Liou et al., 2015).

The heterogeneity of NM, making difficult identifying and recruiting enough workers with the same exposure, could be partially addressed by the exposure biomarkers. In epidemiological research, when occupational and/or environmental exposure data are limited, the estimation of individual exposure is often reduced to the qualitative classification of workers/subjects across exposure levels, according to their job titles (using a so-called job-exposure matrix approach), presence in an exposure registry (available for only limited number of occupational hazards) or point estimates of airborne pollution.

However, for NM, there are still neither precise recommendations on what to measure, nor coordinated strategy for exposure measurement which can be easily implemented and generalized in the workplace. Moreover, NOAA exposure assessment is also hampered by a shortage of equipment and methodologies to analyze their physicochemical characteristics and their large heterogeneity. In this context of very limited NM exposure assessment data, BM presents an excellent alternative. Also, BM could help to circumvent an important issue of exposure to a mixture of NM or a mixture of NM and background (incidental) NP by measuring mass concentration of the NM of interest or the products of interaction with biological systems in biological matrices. For instance, Pelclova et al. (2015b) measured TiO_2 concentration in urine and EBC in Czech workers, Lee et al. (2012) measured silver concentration in blood and urine in Korean workers exposed to silver nanoparticles, while in the study of Lee et al. (2015) molybdenum blood concentration was measured as a candidate biomarker for MWCNT exposure.

Under assumption that different nanoparticles may lead to the same pathway for disease, or share common mechanisms (e.g., inflammation) the issue of NM heterogeneity could be considered less critical in the health surveillance based on the effect biomarkers. Indeed, this assumption does not take into account any consideration of the cost-effectiveness of such surveillance. Thus, workers could be monitored and tested for ROS or inflammation markers by using noninvasive sampling methods (e.g., EBC collection) and exposure assessment could be further performed based on distribution of the magnitude of these biomarkers. Biomarkers of effect can be used in health surveillance programs aimed at the early diagnosis of exposure-related or associated diseases, but the application of effect monitoring is most often used to evaluate whether a well-characterized exposure can be associated with a shift in the distribution of relevant biochemical or functional endpoints indicative of early changes in the target or critical organs/tissue but completely reversible with the removal from the exposure.

The studies of Taiwanese workers exposed to a mixture of metal, metal oxide, and carbon-based NM illustrate such an approach (Liao et al., 2014a,b; Liou et al., 2012; Wu et al., 2014). These researchers carried out several cross-sectional and one-longitudinal studies with the aim of developing and identifying biomarkers of health effects among workers manufacturing NM. The CC16 and serum nuclear transcription activation factor-κB (NF-kB) levels (inflammation markers), the activity of antioxidant enzymes SOD and glutathione peroxidase (GPx), vascular cell adhesion molecule (VCAM), markers of oxidative damage (urine 8-hyroxydeoxyguanosine; methyl guanosine; plasma 8-hyroxydeoxyguanosine, and isoprostane) in EBC and HRV parameters were assessed. Among these biomarkers, CC16 and cardiovascular biomarkers, such as hsCRP and fibrinogen, and inflammatory cell activation [increased intracellular adhesion molecule-1 (ICAM-1) in macrophages] showed statistically significant changes among NM workers. The antioxidant enzymes SOD and GPx activity were both significantly decreased in the exposed group (Liou et al., 2012). In more recent studies, based on effect biomarkers, more attention has been paid on the exposure measurement (of MWCNTs) and more accurate selection of workers (Fatkhutdinova et al., 2016; Lee et al., 2015; Shvedova et al., 2016).

In a small cohort of workers occupationally exposed to MWCNTs, Shvedova et al. (2016) found significant changes in the ncRNA and mRNA expression profiles between exposed (inhalable concentration 14.42 + 3.8 $\mu g/m^3$; respirable concentration 2.83 + 0.6 $\mu g/m^3$ as

elemental carbon concentrations in breathing zone samples from workers) and nonexposed workers. A set of miRNAs and their target genes with roles in cell cycle regulation/progression/control, apoptosis, cell proliferation, and carcinogenetic pathways was characterized in eight workers having direct contact with MWCNT containing aerosols in the previous 6 months; the observed pattern confirmed a dysregulation of the gene profiles (Shvedova et al., 2016). In a concomitant study on the same workers, biological fluids of nasal lavage, induced sputum and blood serum were obtained from MWCNT-exposed and nonexposed workers for assessment of inflammatory and fibrotic markers (Fatkhutdinova et al., 2016). It was found that exposure to MWCNTs in the order of 3 times above the recommended exposure level (REL) proposed by the NIOSH (1 $\mu g/m^3$) caused significant increase in IL-1β, IL-4, IL-5, IL6, TNF-α, inflammatory cytokines, and KL-6 in sputum samples. Moreover, the level of TGF-β1 was increased in serum obtained from young (<30-years old) exposed workers. In the serum samples, levels of IL-1β, IL-4, IL-10, and TNF-α were significantly elevated in the MWCNT exposed group.

Table 6.1 summarizes currently available studies of workers exposed to ENMs. For the moment, all these studies use cross-sectional design and consequently do not allow confirming the observed effects and understanding the dynamic of their occurrence and duration. Consequently, this kind of study could only help to identify some "intermediate" biological changes of effects. Only the study of Lee et al. (2015) showed a reduction, though not clinically significant, in lung function parameters in a cohort of workers occupationally exposed to nanoscale CB (Lee et al., 2015). Longitudinal panel studies with repeated exposure and effect biomarker measurement are necessary to investigate whether this unspecific "intermediate" biological changes could be indicative or predictive of clinical effects and apply them in health surveillance programs. Moreover, to provide a coherent approach and make future epidemiological research a reality, a well-defined framework is needed for the careful choice of materials, exposure characterization, identification of study populations, definition of health endpoints, and evaluation of the appropriateness of study designs, data collection and analysis, and interpretation of the results (Riediker et al., 2012). However, despite the use of similar tools, it should be kept in mind that epidemiological and health surveillance have different although complementary goals, the former being often a prerequisite for the latter. The analytical epidemiological studies (case-control or cohort studies) are aimed at exploring the presence and the strength of an association between health outcomes and exposure of concerns, which can lead to the etiological or causal conclusions if corroborated by further interventional studies. These interventional studies might be undertaken as part of health surveillance programs in occupational or environmental settings and reinforce the preventive purpose of the health surveillance. For example, by identifying early signs in susceptible individuals, the occupational physician will remove them from harmful exposures or introduce an exposure control measures for reducing or avoiding exposure. By reassessing the biological/health status based on early effect biomarkers measurement, it could be possible to confirm the intervention effectiveness and generalize it elsewhere.

Thus despite apparently different goals of epidemiological and health surveillance, BM seems to bring a promising solution for predicting and preventing health damage in populations exposed to NM.

TABLE 6.1 Summary of Available Studies of Nanomaterial (NM) Workers

References	ENM	*N* (exposed/unexposed)	Main findings
Liou et al. (2012)	CNTs, SiO_2, TiO_2, Ag, composites	227/137	↓ Superoxide dismutase (SOD), glutathione peroxidase (GPx), ↑ CV fibrinogen, intercellular adhesion molecule (ICAM), and interleukin-6 (IL-6) in exposed workers
Liao et al. (2014a)		258/200	↑ Sneezing and aggravation of allergic dermatitis in exposed workers
Wu et al. (2014)		241/196	Association with Risk Level 2 and FENO. After adjustment, only the exposure to TiO_2 was associated with ↑ risk of FENO > 35 ppb
Liao et al. (2014b)		124/77	↓ SOD, GPX, vascular adhesion molecule (VCAM), ↓ paraxonase, ↓ Clara cell protein 16 (CC16) and lung function tests ($FVC_{25\%–75\%}$, FEV_1, and $FVC_{25\%}$) in exposed workers
Zhang et al. (2014)	Carbon black	81/104	↓ Lung function parameters, ↑ IL-1β, IL-6, IL-8, MIP1-β, TNF-α in serum of exposed workers
Lee et al. (2015)	CNTs	9/4	↑ Malondialdehyde (MDA), 4-hydroxy-2-hexenal (4-HHE) and *n*-hexanal in EBC of exposed workers
Pelclova et al. (2015a,b)	TiO_2	20/19	↑ C6–C13 *n*-alkanes in exposed workers was correlated with most biomarkers of lipid, proteins, and nucleic acids oxidation and with Ti concentration in the EBC. ↑ C6, C7, C10, C12, HHE, 8-OHG, and 3-Cl-Tyr in urine before and after shift, correlated with MDA, HNE, 8-isoprostane, 8-OHdG, 5-OHMeU, *o*-Tyr, and 3-Cl-Tyr
Pelclova et al. (2016)	Iron oxide aerosol	14/14	↑ MDA, HNE, HHE, C6-C10, 8-isoprostane, 8-OHdG, 8-OHG, 5-OHMeU, 3-ClTyr, 3-NOTyr, *o*-Tyr, and C11 ($P < 0.05$) in exposed workers compared with unexposed workers. Urine oxidative stress biomarkers were not elevated.
Shvedova et al. (2016)	MWCNTs	8/7	Dysregulated ncRNA and mRNA expression profiles (miRNAs and their target genes with roles in cell cycle regulation/progression/control, apoptosis, cell proliferation, and carcinogenetic pathways) in exposed workers
Fatkhutdinova et al. (2016)	MWCNTs	10/12	In exposed workers: ↑ IL-1β, IL-4, IL-5, IL-6, IL-8, and TNF-α in sputum; ↑ KL-6 (sputum); ↑ IL-1β, IL-4, IL-10, and TNF-α in serum; ↑ TGF-β1 in serum (only in workers <30-years old);

BIOMONITORING PROGRAMS: PRACTICAL AND ETHICAL CONSIDERATIONS

Individual BM results are medical data, thus should be handled accordingly. Interpretation of the results is the task of a physician trained in the field of occupational health and informed on the entire situation (workplace exposures and worker's health) although they can be shared among health professionals. Data handling, storage, and communication are specified in Chemical Agents Directive 98/24/EEC (CAD) and in further national legislations. Communication (and interpretation) of individual results should be made only toward the worker concerned. Group data can be communicated toward the employer and workers' representatives in an anonymous form. The ethical considerations must be kept during the entire process of a biomonitoring study. The International Commission on Occupational Health's code of ethics (ICOH, 2014) states that "Biomarkers must be chosen for their validity and relevance for protection of the health of the worker concerned, with due regard to their sensitivity, their specificity, and their predictive value." Principles of this internationally agreed document are: (1) BM should not be used as screening tests or for insurance purposes; (2) current knowledge in BM of susceptibility does not justify job opportunity discrimination of affected workers; (3) priority should be given to noninvasive and easily collected sampling, as is the case of using EBC or urine. In these cases, informed consent is usually not required for routine procedures using validated biomarkers; (4) invasive tests or tests posing a health risk calls for a risk–benefit analysis, and require the informed consent of the worker. For instance, considering the pulmonary (and cardiovascular) hazard for workers occupationally exposed to carbon NMs (CNTs and carbon nanofibers), the National Institute for Occupational Safety and Health (NIOSH) in 2013 recommended to implement a surveillance program to verify the efficacy of the recommended exposure limit (REL) of 1 $\mu g/m^3$ elemental carbon as a respirable mass 8-h time-weighted average (TWA) concentration in protecting the health of the workers. Unspecific biomarkers of effects, validated in animal studies, can be used; these includes serum cytokines (IL-6), acute phase proteins (e.g., CRP, SAA-1, SAP) and acute phase proteins related to immune activation (apolipoproteins A-1 and A-II, alpha-2-macroglobulin, transferrin) or of allergic airway inflammation (IL-5 and CCL11) (Erdely et al., 2011). Table 6.2 summarizes a panel of biomarkers of exposure and effect potentially available for human biomonitoring studies aimed at assessing early effects and health outcomes. To assess the quality of biomarkers, it is mandatory to consider: (1) temporal trends in exposure levels; (2) the quality of biomarkers able to reflect internal exposure or early/reversible biological changes; (3) analytical methods kept under control by quality assurance; (4) establishing reference ranges and limit values which enable the interpretation of eventual changes; (5) the health significance and predictive value of observed changes (Bergamaschi et al., 2014).

Questions remain about the appropriateness of biomarkers in assessing the continuum between NM exposure and possible health outcomes. Whereas transforming exposure into dose may take seconds to hours, the transformation of dose into biologically effective dose may require years to cumulative toxic chemicals. Subsequent steps show an even greater variability and are often reversible, early effects being repaired or compensated for, making temporal relationships with preceding events uncertain. However, questions about lowered resistance against NM-induced effects in susceptible populations, such as those with underlying disease, genetic susceptibility, previous coronary events, and those at an early developmental stage should be considered.

TABLE 6.2 Biomarkers of Exposure or Effects

Quality of biomarker	Biomarker	Physiological/pathological meaning
Exposure	Exhaled particles and/or elements in EBC Metallic elements analysis in biological fluids	Estimate of the "deposited dose" or "target tissue dose," excretion, body burden
Effective dose/early effect	Lipid peroxidation products in EBC or blood (**MDA**, **T-BARS**, LTB4, F2- and **8-isoprostane**); saturated aldehydes: 4-hydroxy-*trans*-nonenale (**HNE**) and 4-hydroxy-*trans*-hexenale (HHE)	Oxidative stress and airways inflammation (EBC) versus systemic (blood)
	IL-6, **IL-8**, sTNF-RII, **TNF-α**; high-sensitivity C-reactive protein (**hsCRP**) in plasma	Systemic inflammation
	DNA excision base products in plasma/urine: 8-hydroxy-2-deoxyguanosine (**8-OHdG**), 8-hydroxy-guanosine (8-OHG)	Systemic oxidative stress; oxidation of nucleic acids
	Blood/plasma antioxidant enzyme activity: **Gpx1**, glutathione peroxidase 1; **SOD**, copper-zinc superoxide dismutase; **MPO**, myeloperoxidase	Antioxidant capacity; systemic inflammation
	Exhaled breath Nitric Oxide (**FeNO**); **Nitrosothiols (RSNOs)**; 3-nitrotyrosine in serum	Nitrosative stress; airways inflammation. RSNOs are formed by interaction of NO with GSH
	Serum Clara Cell pneumoprotein (**CC16**)	Increased lung permeability, lung (distal airways) inflammation
	Platelet activation/aggregation; coagulation factors: fibrinogen, plasminogen activator inhibitor-1 (**PAI-1**), intercellular adhesion molecule (**ICAM-1**)	Closely associated with enhanced thrombosis by impairing fibrinolysis; prothrombotic risk
	Vascular adhesion molecules (**VCAM-1**)	Vascular endothelial activation
	Acute phase proteins: hsCRP, Serum Amyloid A (**SAA**), **Haptoglobin**, **Hemopexin**	Stimulation of hepatic production by proinflammatory cytokines; causal link between particle inhalation and risk of cardiovascular disease
	NRLP3 inflammasome as cascade IL1-beta, IL-18, as caspase 1–11	Aberrant inflammasome signaling contributes to pathology in a large number of infectious and autoimmune diseases

(*Continued*)

TABLE 6.2 Biomarkers of Exposure or Effects (*cont.*)

Quality of biomarker	Biomarker	Physiological/pathological meaning
Alterations in cell structure/function	**KL-6 glycoprotein**; **MMP-1**, **MMP-7**, **MMP-9**	Early marker of lung fibrosis. Degradation of extracellular matrix
	Osteopontin	Early mesothelioma development
	Calcium-related proteins (S100-A8, **S100-A9**)	Cell cycle progression and differentiation fibrogenic marker (in asbestos/CNT)
	DNA strand breaks (Comet assay + FPG-ENDO III)	Oxidatively damaged DNA
	DNA (hypo/hyper)methylation	NM ability to induce an impaired expression of genes involved in DNA methylation reactions leading to global DNA methylation changes, gene-specific methylation of tumor suppressor genes, inflammatory genes, and DNA repair genes, potential cancer development
	microRNA (miRNAs)	DNA hypermethylation might represent biomarker of both exposure and disease development (e.g., fibrosis, cancer)
	Chromatin histone (methyltransferases, demethylases, acetyltransferases)	Genomic profiling studies have provided clear genetic evidence that dysregulation of chromatin modifiers drives many types of cancer. Recurrent mutations were found in genes encoding enzymes that add, subtract, or interpret posttranslational modifications to histone H3. These enzymes include histone (lysine) methyltransferases (KMTs) and histone (lysine) demethylases (KDM); histone acetyltransferases (HATs) and histone readers
	Extracellular micro- and nanovescicles, exosomes (EMVs' cargo characterization)	Identification of unequivocal correlations between critical disease states and specific EMV signatures. Potential multidimensional and interdependent patterns of genetic, epigenetic, and phenotypic markers with predictive value, provided they can be followed routinely along the complete individual disease pathway with sufficient precision

Appraisal of biomarkers of exposure or effects investigated and validated (in bold) in in vivo or in studies on people exposed to different ultrafine or fine particulates or known fractions, and biomarkers specifically investigated in relation to ENM.

CONCLUSIONS

Although there is an increasing awareness that nanotechnology workers need to be protected in their everyday handling and dealing with NOAA, the effectiveness of preventive measures put in place should impose a periodical assessment of the potential hazards they are exposed to. Implementation of BM studies integrating different levels of hazard surveillance would improve the chances for detecting early adverse effects from exposure to a multiplicity of manufactured or engineered nanoparticles (Li and Nel, 2011).

There are still a number of obvious gaps that have been identified and that need to be filled to get a more complete understanding of NM-induced effects and a meaningful implementation of BM for NM exposure. BM should fulfill the following criteria: (1) the use of accessible biological matrices, (2) suitable parameters, able to reflect internal exposure, biochemical or (early) biological effects, (3) reliable analytical methods which are kept under control by quality assurance, and (4) reference and limit values which enable the interpretation of results in terms of health relevance (Bergamaschi et al., 2014).

High-throughput (omics) methods, if applied in a rigorous manner, hold great promise for the development of (novel) biomarkers and biomarker signatures. Achievements in this field may make more consistent also the regulatory approach to hazard based on dynamic adverse outcome pathway (AOP) models (Labib et al., 2016; Vietti et al., 2016) with the aim to evaluate the application of global gene expression data in deriving pathway-based points of departure (PODs) for MWCNT-induced lung fibrosis, as a noncancer endpoint of regulatory importance, Labib et al. (2016) showed an early perturbation of similar biological pathways regardless of MWCNT types across the doses and postexposure time points studied. The authors also showed that transcriptional benchmark dose (BMD) values for pathways associated with fibrosis (4.0–30.4 μg per mouse) were comparable to the BMDs derived by NIOSH for MWCNT-induced lung fibrotic lesions (namely, 21.0–27.1 μg per mouse), thus suggesting that transcriptomic data can be used to derive acceptable levels of exposure to NM in product development (Labib et al., 2016). Assessing, for example, changes in mRNA levels profile both in vitro and in vivo test models may help link altered gene expression profiles with the dose level at which these changes occur, thus allowing the identification of the underlying mechanisms of toxicity and to predict disease outcomes.

The knowledge-based body of data generated by systems toxicology approaches will certainly improve the safety assessment, but the complex and multifaceted nature of events occurring at the nano-biointerfaces (at the cell, tissue, organ, and system level) also means that the full replacement of in vivo assessment is not yet possible. For a reliable risk assessment (RA), the data generated by omics approaches should be validated in the real exposure scenarios where more complex and unpredictable interactions can occur and the human behavior can affect the outputs. In the guidance on the protection of the health and safety of workers from the potential risks related to NMs at work recently issued by the European Commission (2014), RA is still described as a formal procedure involving various steps in risk prevention when working with NMs. The RA and the efficacy of the implemented risk management measures must be reviewed periodically and before any changes are made to either the chemical agents being used or working conditions (in compliance with Article 4(5) of the Chemical Agents Directive 98/24/EC). Whatever the approach adopted and the outputs generated, the general principles for prevention of risks associated with hazardous chemical agents still rely on

well-established hierarchy of controls that should be followed. A suitable combination of the risk control measures suggested should be adopted by employers to ensure the safe handling of NMs. The core of the NM risk management program, as with all occupational safety and health (OSH) risk management programs, is implementation of the well-established hierarchy of controls. NM risk management should be part of an overall company health and safety program. Elements such as BM are not actually part of the classical hierarchy of control measures, rather they may help in evaluating controls, and thus can be viewed as a part of hierarchy of prevention that include controls as evaluative strategy (Schulte et al., 2014). Given that epidemiological research and interventional studies are a necessary prerequisite for health programs and prevention, they should involve BM and lead to identification, selection, and validation of candidate biomarkers for generalized exposure and health effects surveillance. In such studies, BM could help to circumvent the issues of the heterogeneity of NM, making difficult to identify and recruit enough workers with the same exposure pattern, considering that exposures to different nanoparticles may lead to the same pathway for disease, or share common mechanisms (Schulte and Hauser, 2012). In conclusion, BM of exposure and effect should represent a valuable component of an integrated strategy and a proactive approach to RA and management, an opportunity for companies committed with the responsible development of nanotechnology, and also an ethical obligation toward all worker population that everything is done for a safe work environment (Bergamaschi et al., 2015; Seaton et al., 2010).

TAKE-HOME MESSAGES

- There is a pressing need to overcome pitfalls in RA for NMs.
- Inherent properties of NMs are subject to changes in the environmental settings.
- Air pollution particle and nanoparticle-induced biological effects can share similar mechanisms.
- Lack of specificity should not hamper the use of biomarkers to assess associations with exposure since, if negative health impacts are detected, further investigations can be focused to determine causality.
- Within the hierarchy of biomarker development, for efficiency exploratory and candidate biomarkers should be drawn from other fields of particle toxicology and air pollution studies, and should be validated in observational epidemiological and interventional studies of NP-exposed populations.

References

Aitken, R.A., Bassan, A., Friedrichs, S., Hankin, S.M., Hansen, S.F., Holmqvist, J., Peters, S.A.K., Poland, C.A., Tran, C.L., 2011. Specific Advice on Exposure Assessment and Hazard/Risk Characterisation for Nanomaterials under REACH (RIP-oN3)-Final Project Report. Available from: http://ec.europa.eu/environment/chemicals/nanotech/reach-clp/ripon_en.htm

Alessandrini, F., Weichenmeier, I., van Miert, E., Takenaka, S., Karg, E., Blume, C., Mempel, M., Schulz, H., Bernard, A., Behrendt, H., 2010. Effects of ultrafine particles-induced oxidative stress on Clara cells in allergic lung inflammation. Part. Fibre Toxicol. 7, 11.

Andreoli, R., Spatari, G., Pigini, D., Poli, D., Banda, I., Goldoni, M., Riccelli, M.G., Petyx, M., Protano, C., Vitali, M., Barbaro, M., Mutti, A., 2015. Urinary biomarkers of exposure and of oxidative damage in children exposed to low airborne concentrations of benzene. Environ. Res. 142, 264–272.

Antonini, J.M., Roberts, J.R., Schwegler-Berry, D., Mercer, R.R., 2013. Comparative microscopic study of human and rat lungs after overexposure to welding fume. Ann. Occup. Hyg. 57 (9), 1167–1179.

Bachler, G., Losert, S., Umehara, Y., von Goetz, N., Rodriguez-Lorenzo, L., Petri-Fink, A., Rothen-Rutishauser, B., Hungerbuehler, K., 2015. Translocation of gold nanoparticles across the lung epithelial tissue barrier: combining in vitro and in silico methods to substitute in vivo experiments. Part. Fibre Toxicol. 12, 18.

Barath, S., Mills, N.L., Adelroth, E., Olin, A.C., Blomberg, A., 2013. Diesel exhaust but not ozone increases fraction of exhaled nitric oxide in a randomized controlled experimental exposure study of healthy human subjects. Environ. Health 12, 36.

Barregard, L., Sallsten, G., Andersson, L., Almstrand, A.C., Gustafson, P., Andersson, M., Olin, A.C., 2008. Experimental exposure to wood smoke: effects on airway inflammation and oxidative stress. Occup. Environ. Med. 65, 319–324.

Bergamaschi, E., 2009. Occupational exposure to nanomaterials: present knowledge and future development. Nanotoxicology 3 (3), 194–201.

Bergamaschi, E., 2012. Human biomonitoring of engineered nanoparticles: an appraisal of critical issues and potential biomarkers. J. Nanomater. 12. doi: 10.1155/2012/564121.

Bergamaschi, E., Gulumian, M., Kanno, J., Savolainen, K., 2014. Engineered nanomaterials: biomarkers of exposure and effect. In: Gupta, R. (Ed.), Biomarkers in Toxicology. Elsevier Inc., New York, NY, pp. 697–716.

Bergamaschi, E., Poland, C., Canu, I.G., Prina-Mello, A., 2015. The role of biological monitoring in nano-safety. Nano Today 10, 274–277.

Bianchi, M.G., Allegri, M., Costa, A.L., Blosi, M., Gardini, D., Delpivo, C., Prina-Mello, A., DiCristo, L., Bussolati, O., Bergamaschi, E., 2015. Titanium dioxide nanoparticles enhance macrophage activation by LPS through a TLR4-dependent intracellular pathway. Toxicol. Res. 4, 385–398.

Boland, S., Hussain, S., Baeza-Squiban, A., 2014. Carbon black and titanium dioxide nanoparticles induce distinct molecular mechanisms of toxicity. Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol. 6 (6), 641–652.

Bollati, V., Marinelli, B., Apostoli, P., Bonzini, M., Nordio, F., Hoxha, M., Pegoraro, V., Motta, V., Tarantini, L., Cantone, L., Schwartz, J., Bertazzi, P.A., Baccarelli, A., 2010. Exposure to metal-rich particulate matter modifies the expression of candidate microRNAs in peripheral blood leukocytes. Environ. Health Perspect. 118, 763–768.

Bourdon, J.A., Williams, A., Kuo, B., Moffat, I., White, P.A., Halappanavar, S., Vogel, U., Wallin, H., Yauk, C.L., 2013. Gene expression profiling to identify potentially relevant disease outcomes and support human health risk assessment for carbon black nanoparticle exposure. Toxicology 303, 83–93.

Bregoli, L., Movia, D., Gavigan-Imedio, J.D., Lysaght, J., Reynolds, J., Prina-Mello, A., 2016. Nanomedicine applied to translational oncology: a future perspective on cancer treatment. Nanomedicine 12 (1), 81–103.

Broday, D.M., Agnon, Y., 2007. Asymmetric human lung morphology induce particle deposition variation. J. Aerosol. Sci. 38, 701–718.

Broeckaert, F., Arsalane, K., Hermans, C., Bergamaschi, E., Brustolin, A., Mutti, A., Bernard, A., 2000. Lung epithelial damage at low concentrations of ambient ozone. Lancet 353, 900–901.

Brown, T.A., Lee, J.W., Holian, A., Porter, V., Fredriksen, H., Kima, M., Cho, Y.H., 2016. Alterations in DNA methylation corresponding with lung inflammation and as a biomarker for disease development after MWCNT exposure. Nanotoxicology 10 (4), 453–461.

Council Directive, 1989. Council Directive of 12 June 1989 on the introduction of measures to encourage improvements in the safety and health of workers at work (89/391/EEC). Off. J. L 183, 0001–0008. Available from: http://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A31989L0391

Council Directive, 1998. Council Directive 98/24/EC of 7 April 1998 on the protection of the health and safety of workers from the risks related to chemical agents at work (fourteenth individual Directive within the meaning of Article 16(1) of Directive 89/391/EEC). Off. J. L 131, 0011–0023. Available from: http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A31998L0024

Chew, W.S., Poh, K.W., Siddiqi, N.J., Alhomida, A.S., Yu, L.E., On, W.Y., 2012. Short- and long-term changes in blood miRNA levels after nanogold injection in rats–potential biomarkers of nanoparticle exposure. Biomarkers 17, 750–757.

Cho, W.S., Duffin, R., Thielbeer, F., Bradley, M., Megson, I.L., Macnee, W., Poland, C.A., Tran, C.L., Donaldson, K., 2012. Zeta potential and solubility to toxic ions as mechanisms of lung inflammation caused by metal/metal oxide nanoparticles. Toxicol. Sci. 126 (2), 469–477.

Cho, W.S., Kang, B.C., Lee, J.K., Jeong, J., Che, J.H., Seok, S.H., 2013. Comparative absorption, distribution, and excretion of titanium dioxide and zinc oxide nanoparticles after repeated oral administration. Part. Fibre Toxicol. 10, 9.

Choi, H.S., Ashitate, Y., Lee, J.H., Kim, S.H., Matsui, A., Insin, N., Bawendi, M.G., Semmler-Behnke, M., Frangioni, J.V., Tsuda, A., 2010. Rapid translocation of nanoparticles from the lung airspaces to the body. Nat. Biotechnol. 28 (12), 1300–1303.

Cooke, M.S., Henderson, P.T., Evans, M.D., 2009. Sources of extracellular, oxidatively modified DNA lesions: implications for their measurement in urine. J. Clin. Biochem. Nutr. 45, 255–270.

Costa, P.M., Fadeel, B., 2016. Emerging systems biology approaches in nanotoxicology: towards a mechanism-based understanding of nanomaterial hazard and risk. Toxicol. Appl. Pharmacol. 299, 101–111.

Delfino, R.J., Staimer, N., Tjoa, T., Arhami, M., Polidori, A., Gillen, D.L., Kleinman, M.T., Schauer, J.J., Sioutas, C., 2010. Association of biomarkers of systemic inflammation with organic components and source tracers in quasi-ultrafine particles. Environ. Health Perspect. 118, 756–762.

Di Cristo, L., Movia, D., Bianchi, M.G., Allegri, M., Mohamed, B.M., Bell, A.P., Moore, C., Pinelli, S., Rasmussen, K., Riego-Sintes, J., Prina-Mello, A., Bussolati, O., Bergamaschi, E., 2015. Proinflammatory effects of pyrogenic and precipitated amorphous silica nanoparticles in innate immunity cells. Toxicol. Sci. 150, 40–53.

Donaldson, K., Poland, C.A., 2012. Inhaled nanoparticles and lung cancer. What we can learn from conventional particle toxicology. Swiss Med. Wkly. 142, w13547.

Donaldson, K., Poland, C.A., 2013. Nanotoxicity: challenging the myth of nano-specific toxicity. Curr. Opin. Biotechnol. 24 (4), 724–734.

Donaldson, K., Schinwald, A., Murphy, F., Cho, W.S., Duffin, R., Tran, L., Poland, C., 2013. The biologically effective dose in inhalation nanotoxicology. Acc. Chem. Res. 46 (3), 723–732.

Donaldson, K., Seaton, A., 2012. A short history of the toxicology of inhaled particles. Part. Fibre Toxicol. 9, 13.

Elsabahy, M., Wooley, K.L., 2013. Cytokines as biomarkers of nanoparticle immunotoxicity. Chem. Soc. Rev. 42, 5552.

Eom, H.J., Chatterjee, N., Lee, J., Choi, J., 2014. Integrated mRNA and micro RNA profiling reveals epigenetic mechanism of differential sensitivity of Jurkat T cells to AgNPs and Ag ions. Toxicol. Lett. 229 (1), 311–318.

Erdely, A., Liston, A., Salmen-Muniz, R., Hulderman, T., Young, S.H., Zeidler-Erdely, P.C., Castranova, V., Simeonova, P.P., 2011. Identification of systemic markers from a pulmonary carbon nanotube exposure. J. Occup. Environ. Med. 53 (6), S80–S86.

European Commission, 2014. Guidance on the protection of the health and safety of workers from the potential risks related to nanomaterials at work. Guidance for employers and health and safety practitioners. November 2014.

Fadeel, B., Feliu, N., Vogt, C., Abdelmonem, A.M., Parak, W.J., 2013. Bridge over troubled waters: understanding the synthetic and biological identities of engineered nanomaterials. WIREs Nanomed. Nanobiotechnol. 5, 111–129.

Fatkhutdinova, L.M., Khaliullin, T.O., Vasil'yeva, O.L., Zalyalov, R.R., Mustafin, I.G., Kisin, E.R., Birch, M.E., Yanamala, N., Shvedova, A.A., 2016. Fibrosis biomarkers in workers exposed to MWCNTs. Toxicol. Appl. Pharmacol. 299, 125–131.

Fubini, B., Ghiazza, M., Fenoglio, I., 2010. Physico-chemical features of engineered nanoparticles relevant to their toxicity. Nanotoxicology 4, 347–363.

Geiser, M., Kreyling, W.G., 2010. Deposition and biokinetics of inhaled nanoparticles. Part. Fibre Toxicol. 7, 2.

Gioria, S., Lobo Vicente, J., Barboro, P., La Spina, R., Tomasi, G., Urbán, P., Kinsner-Ovaskainen, A., Rossi, F., Chassaigne, H., 2015. A combined proteomics and metabolomics approach to assess the effects of gold nanoparticles in vitro. Nanotoxicology 9, 1–59.

Goldoni, M., Caglieri, A., Poli, D., Vettori, M.V., Corradi, M., Apostoli, P., Mutti, A., 2006. Determination of hexavalent chromium in exhaled breath condensate and environmental air among chrome plating workers. Anal. Chim. Acta 562, 229–235.

Goldoni, M., Catalani, S., De Palma, G., Manini, P., Acampa, O., Corradi, M., Bergonzi, R., Apostoli, P., Mutti, A., 2004. Exhaled breath condensate as a suitable matrix to assess lung dose and effects in workers exposed to cobalt and tungsten. Environ. Health Perspect. 112, 1293–1298.

Gonzalez, L., Lison, D., Kirsch-Volders, M., 2008. Genotoxicity of engineered nanomaterials: a critical review. Nanotoxicology 2 (4), 252–273.

Gulumian, M., Borm, P.J., Vallyathan, V., Castranova, V., Donaldson, K., Nelson, G., Murray, J., 2006. Mechanistically identified suitable biomarkers of exposure, effect, and susceptibility for silicosis and coal-worker's pneumoconiosis: a comprehensive review. J. Toxicol. Environ. Health B 9 (5), 357–395.

Guseva Canu, I., Boutou-Kempf, O., Delabre, L., Ducamp, S., Iwatsubo, Y., Marchand, J.L., Imbernon, E., 2013. Medical surveillance and epidemiologic studies of engineered nanomaterials (enm) workers in France. Occup. Environ. Med. 70 (Suppl. 1), A66.

Guseva Canu, I., Jezewski-Serra, D., Delabre, L., Ducamp, S., Iwatsubo, Y., Audignon-Durand, S., Ducros, C., Radauceanu, A., Witschger, O., Durand, C., Flahaut, E., 2016. Qualitative and semi-quantitative assessment of exposure to engineered nanomaterials within the French EpiNano program: inter- and intra-method reliability study (submitted).

Hermans, C., Bernard, A., 1999. Lung epithelium-specific proteins: characteristics and potential applications as markers. Am. J. Respir. Crit. Care Med. 159, 646–678.

Higashisaka, K., Chui, K., Yoshioka, Y., Yamashita, Y., Morishita, Y., Fujimura, M., Nabeshi, H., Nagano, K., Abe, Y., Kamada, Y., Tsunoda, S., Yoshikawa, T., Itoh, I., Tsutsumi, Y., 2011. Acute phase proteins as biomarkers for predicting the exposure and toxicity of nanomaterials. Biomaterials 32, 3–9.

Higashisaka, K., Yoshioka, Y., Yamashita, K., Morishita, Y., Pan, H., Ogura, T., Nagano, T., Kunieda, A., Nagano, K., Abe, Y., Kamada, H., Tsunoda, S., Nabeshi, H., Yoshikawa, T., Tsutsumi, Y., 2012. Hemopexin as biomarkers for analyzing the biological responses associated with exposure to silica nanoparticles. Nanoscale Res. Lett. 7, 555.

Hoffmeyer, F., Raulf-Heimsoth, M., Weiss, T., Lehnert, M., Gawrych, K., Kendzia, B., Harth, V., Henry, J., Pesch, B., Brüning, T., WELDOX Study Group, 2012. Relation between biomarkers in exhaled breath condensate and internal exposure to metals from gas metal arc welding. J. Breath Res. 6 (2), 027105.

Holgate, S., 2010. Exposure, uptake distribution and toxicity of nanomaterials in humans. J. Biomed. Nanotechnol. 6 (1), 1–19.

Horvath, I., Hunt, J., Barnes, P.J., Alving, K., Antczak, A., Baraldi, E., Becher, G., vanBeurden, W.J., Corradi, M., Dekhuijzen, R., Dweik, R.A., Dwyer, T., Effros, R., Erzurum, S., Gaston, B., Gessner, C., Greening, A., Ho, L.P., Hohlfeld, J., Jobsis, Q., Laskowski, D., Loukides, S., Marlin, D., Montuschi, P., Olin, A.C., Redington, A.E., Reinhold, P., van Rensen, E.L., Rubinstein, I., Silkoff, P., Toren, K., Vass, G., Vogelberg, C., Wirtz, H., ATS/ERS Task Force on Exhaled Breath Condensate, 2005. Exhaled breath condensate: methodological recommendations and unresolved questions. Eur. Respir. J. 26 (3), 523–548.

Iavicoli, I., Leso, V., Manno, M., Schulte, P.A., 2014. Biomarkers of nanomaterial exposure and effect: current status. J. Nanopart. Res. 16, 2302.

International Commission on Occupational Health (ICOH), 2014. International code of ethics for occupational health professionals, 2014. Available from: http://www.icohweb.org/site/multimedia/code_of_ethics/code-of-ethics-en.pdf

International Programme on Chemical Safety (IPCS), 1993. Biomarkers and Risk Assessment: Concepts and Principles. Environmental Health Criteria *n*. 155. World Health Organization, Geneva, 1993

Johnston, H.J., Hutchison, G., Christensen, F.M., Peters, S., Hankin, S., Stone, V., 2010. A review of the in vivo and in vitro toxicity of silver and gold particulates: particle attributes and biological mechanisms responsible for the observed toxicity. Crit. Rev. Toxicol. 40 (4), 328–346.

Jun, E.A., Lim, K.M., Kim, K., Bae, O.N., Noh, J.I.Y., Chung, K.H., Chung, J.H., 2011. Silver nanoparticles enhance thrombus formation through increased platelet aggregation and procoagulant activity. Nanotoxicology 5 (2), 157–167.

Karlsson, H.L., 2010. The comet assay in nanotoxicology research. Anal. Bioanal. Chem. 398, 651–666.

Karlsson, H.L., Di Bucchianico, S., Collins, A.R., Dusinska, M., 2015. Can the comet assay be used reliably to detect nanoparticle-induced genotoxicity? Environ. Mol. Mutagen. 56 (2), 82–96.

Karlsson, H.L., Gustafsson, J., Cronholm, P., Möller, L., 2009. Size-dependent toxicity of metal oxide particles—a comparison between nano- and micrometer size. Toxicol. Lett. 188 (2), 112–118.

Khatri, M., Bello, D., Pal, A.K., Cohen, J.M., Woskie, S., Gassert, T., Lan, J., Gu, A.Z., Demokritou, P., Gaines, P., 2013. Evaluation of cytotoxic, genotoxic and inflammatory responses of nanoparticles from photocopiers in three human cell lines. Part. Fibre Toxicol. 10, 42.

Kilinç, E., Schulz, H., Kuiper, J.A.J.M., Spronk, H.M.H., Cate, H., Upadhyay, S., Ganguly, K., Stoeger, T., Semmler-Bhenke, M., Takenaka, S., Kreyling, W.G., Pitz, M., Reitmeir, P., Peters, A., Eickelberg, O., Wichmann, H.E., 2011. The procoagulant effects of fine particulate matter in vivo. Part. Fibre Toxicol. 8, 12.

Klaper, R., Arndt, D., Bozich, J., Dominguez, G., 2014. Molecular interactions of nanomaterials and organisms: defining biomarkers for toxicity and high-throughput screening using traditional and next-generation sequencing approaches. Analyst 139, 882–895.

Kreyling, W.G., Abdelmonem, A.M., Ali, Z., Alves, F., Geiser, M., Haberl, N., Hartmann, R., Hirn, S., de Aberasturi, D.J., Kantner, K., Khadem-Saba, G., Montenegro, J.M., Rejman, J., Rojo, T., de Larramendi, I.R., Ufartes, R., Wenk, A., Parak, W.J., 2015. In vivo integrity of polymer-coated gold nanoparticles. Nat. Nanotechnol. 10 (7), 619–623.

Kreyling, W.G., Semmler, M., Erbe, F., Mayer, P., Takenaka, S., Schulz, H., Oberdörster, G., Ziesenis, A., 2002. Translocation of ultrafine insoluble iridium particles from lung epithelium to extrapulmonary organs is size dependent but very low. J. Toxicol. Environ. Health A 65 (20), 1513–1530.
Kreyling, W.G., Semmler-Behnke, M., Seitz, J., Scymczak, W., Wenk, A., Mayer, P., Takenaka, S., Oberdörster, G., 2009. Size dependence of the translocation of inhaled iridium and carbon nanoparticle aggregates from the lung of rats to the blood and secondary target organs. Inhal. Toxicol. 21 (S1), 55–60.
Krug, H.F., 2014. Nanosafety research—are we on the right track? Angew. Chem. Int. 53, 12304–12319.
Kuhlbusch, T.A., Asbach, C., Fissan, H., Göhler, D., Stintz, M., 2011. Nanoparticle exposure at nanotechnology workplaces: a review. Part. Fibre Toxicol. 8, 22.
Labib, S., Williams, A., Yauk, C.L., Nikota, J.K., Wallin, H., Vogel, U., Halappanavar, S., 2016. Nano-risk science: application of toxicogenomics in an adverse outcome pathway framework for risk assessment of multi-walled carbon nanotubes. Part. Fibre Toxicol. 13, 15.
Lakind, J.S., Holgate, S.T., Ownby, D.R., Mansur, A.H., Helms, P.J., Pyatt, D., Hays, S.M., 2007. A critical review of the use of Clara cell secretory protein (CC16) as a biomarker of acute or chronic pulmonary effects. Biomarkers 12 (5), 445–467.
Lee, J.S., Choi, Y.C., Shin, J.H., Lee, J.H., Lee, Y., Park, S.Y., Baik, J.E., Ahn, K., Yu, I.J., 2015. Health surveillance study of workers who manufacture multi-walled carbon nanotubes. Nanotoxicology 9, 802–811.
Lee, J.H., Mun, J., Park, J.D., Yu, I.J., 2012. A health surveillance case study on workers who manufacture silver nanomaterials. Nanotoxicology 6, 667–669.
Li, Y., Boraschi, D., 2016. Endotoxin contamination: a key element in the interpretation of nanosafety studies. Nanomedicine 11 (3), 269–287.
Li, N., Nel, A., 2011. Feasibility of biomarker studies for engineered nanoparticles. What can be learned from air pollution research. JOEM 53 (6), S74–S79.
Liao, H.Y., Chung, Y.T., Lai, C.H., Lin, M.H., Liou, S.H., 2014a. Sneezing and allergic dermatitis were increased in engineered nanomaterial handling workers. Ind. Health 52, 199–215.
Liao, H.Y., Chung, Y.T., Tsou, T.C., Wang, S.L., Li, L.A., Chiang, H.C., Li, W.F., Lai, C.H., Lee, H.L., Lin, M.H., Hsu, J.H., Ho, J.J., Chen, C.J., Shih, T.S., Lin, C.C., Liou, S.H., 2014b. Six-month follow-up study of health markers of nanomaterials among workers handling engineered nanomaterials. Nanotoxicology 8, 100–110.
Lin, J., Li, J., Huang, B., Liu, J., Chen, X., Chen, X.M., Xu, Y.M., Huang, L.F., Wang, X.Z., 2015. Exosomes: novel biomarkers for clinical diagnosis. Scientific World J. 657086. doi: 10.1155/2015/657086.
Liou, H.S., Tsai, C., Pelclova, D., Schubauer-Berigan, M., Schulte, P., 2015. Assessing the first wave of epidemiological studies of nanomaterial workers. J. Nanopart. Res. 17, 413.
Liou, S.H., Tsou, T.C., Wang, S.L., Li, L.A., Chiang, H.C., Li, W.F., Lin, P.P., Lai, C.H., Lee, H.L., Lin, M.H., Hsu, J.H., Chen, C.R., Shih, T.S., Liao, H.Y., Chung, Y.T., 2012. Epidemiological study of health hazards among workers handling engineered nanomaterials. J. Nanopart. Res. 14, 878–892.
Liu, H.H., Chen, C.Y., Chen, G.I., Lee, L.H., Chen, H.L., 2012. Relationship between indium exposure and oxidative damage in workers in indium tin oxide production plants. Int. Arch. Occup. Environ. Health 85, 447–453.
Loft, S., Danielsen, P.O., Mikkelsen, L., Risom, L., Forchhammer, L., Møller, P., 2008. Biomarkers of oxidative damage to DNA and repair. Biochem. Soc. Trans. 36, 1071–1076.
Luo, J.C., Hsu, K.H., Shen, W.S., 2009. Inflammatory responses and oxidative stress from metal fume exposure in automobile welders. J. Occup. Environ. Med. 51 (1), 95–103.
Lv, M., Huang, W., Chen, Z., Jiang, H., Chen, J., Tian, Y., Zhang, Z., Xu, F., 2015. Metabolomics techniques for nanotoxicity investigations. Bioanalysis 7 (12), 1527–1544.
Lynch, I., Weiss, C., Valsami-Jones, E., 2014. A strategy for grouping of nanomaterials based on key physico-chemical descriptors as a basis for safer-by-design NMs. Nano Today 9, 266–270.
Madl, A.K., Pinkerton, K.E., 2009. Health effects of inhaled engineered and incidental nanoparticles. Crit. Rev. Toxicol. 39 (8), 629–658.
Magdolenova, Z., Collins, A., Kumar, A., Dhawan, A., Stone, V., Dusinska, M., 2013. Mechanisms of genotoxicity. A review of in vitro and in vivo studies with engineered nanoparticles. Nanotoxicology 8 (3), 233–278.
Manno, M., Viau, C., Cocker, J., Colosio, C., Lowry, L., Mutti, A., Nordberg, M., Wangh, S., 2010. Biomonitoring for occupational health risk assessment (BOHRA). Toxicol. Lett. 192, 3–16.
Merget, R., Bauer, T., Küpper, H.U., Philippou, S., Bauer, H.D., Breitstadt, R., Bruening, T., 2002. Health hazards due to the inhalation of amorphous silica. Arch. Toxicol. 75 (11–12), 625–634.

Migliore, L., Saracino, D., Bonelli, A., Colognato, R., D'Errico, M.G., Magrini, A., Bergamaschi, A., Bergamaschi, E., 2010. Carbon nanotubes induce oxidative DNA damage in RAW 264.7 cells. Environ. Mol. Mutagen. 51 (4), 294–302.
Mills, N.L., Donaldson, K., Hadoke, P.W., Boon, N.A., MacNee, W., Cassee, F.R., Sandström, T., Blomberg, A., Newby, D.E., 2009. Adverse cardiovascular effects of air pollution. Nat. Clin. Pract. Cardiovasc. Med. 6, 36–44.
Mohamed, B.M., Movia, D., Knyazev, A., Langevin, D., Davies, A.M., Prina-Mello, A., Volkov, Y., 2013. Citrullination as early-stage indicator of cell response to single-walled carbon nanotubes. Sci. Rep. 3, 1124.
Mohamed, M.B., Verma, N.K., Davies, A.M., McGowan, A., Crosbie-Staunton, K., Prina-Mello, A., Kelleher, D., Botting, C.H., Causey, C.P., Thompson, P.R., Pruijn, G.J.M., Kisin, E.R., Tkach, A.V., Shvedova, A.A., Volkov, Y., 2012. Citrullination of proteins: a common post-translational modification pathway induced by different nanoparticles in vitro and in vivo. Nanomedicine 7 (8), 1181–1195.
Møller, P., Jacobsen, N.R., Folkmann, J.K., Danielsen, P.H., Mikkelsen, L., Hemmingsen, J.G., Vesterdal, L.K., Forchhammer, L., Wallin, H., Loft, S., 2010. Role of oxidative damage in toxicity of particulates. Free Radic. Res. 44 (1), 1–46.
Møller, P., Loft, S., 2010. Oxidative damage to DNA and lipids as biomarkers of exposure to air pollution. Environ. Health Perspect. 118, 1126–1136.
Monopoli, M.P., Aberg, C., Salvati, A., Dawson, K.A., 2012. Biomolecular coronas provide the biological identity of nanosized materials. Nat. Nanotechnol. 7 (12), 79–786.
Montecalvo, A., Larregina, A.T., Shufesky, W.J., Stolz, D.B., Sullivan, M.L., Karlsson, J.M., Baty, C.J., Gibson, G.A., Erdos, G., Wang, Z., et al., 2012. Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. Blood 119, 756–766.
Montuschi, P., 2007. Analysis of exhaled breath condensate in respiratory medicine: methodological aspects and potential clinical applications. Ther. Adv. Resp. Dis. 1 (1), 5–23.
Muller, J., Decordier, I., Hoet, P.H., Lombaert, N., Thomassen, L., Huaux, F., Lison, D., Kirsch-Volders, M., 2008. Clastogenic and aneugenic effects of multi-wall carbon nanotubes in epithelial cells. Carcinogenesis 29, 427–433.
Mutti, A., 2001. Biomarkers of exposure and effect for non carcinogenic end-points. International programme on chemical safety. Biomarkers in risk assessment: validity and validation. Environmental Health Criteria 222, World Health Organization, Geneva, 130–136.
Nagano, T., Higashisaka, K., Kunieda, A., Iwahara, Y., Tanaka, K., Nagano, K., Abe, Y., Kamada, H., Tsunoda, S., Nabeshi, H., Yoshikawa, T., Yoshioka, Y., Tsutsumi, Y., 2013. Liver-specific microRNAs as biomarkers of nanomaterial-induced liver damage. Nanotechnology 24 (40), 405102.
Nasterlack, M., 2011. Role of medical surveillance in risk management. J. Occup. Environ. Med. 53, S18–S21.
Nasterlack, M., Zober, A., Oberlinner, C., 2008. Considerations on occupational medical surveillance in employees handling nanoparticles. Int. Arch. Occup. Environ. Health 81 (6), 721–726.
Nikota, J., Williams, A., Yauk, C.L., Wallin, H., Vogel, U., Halappanavar, S., 2016. Meta-analysis of transcriptomic responses as a means to identify pulmonary disease outcomes for engineered nanomaterials. Part. Fibre Toxicol. 13, 25.
National Institute for Occupational Safety and Health (NIOSH), 2013. Current Intelligence Bulletin 65: Occupational Exposure to Carbon Nanotubes and Nanofibers. DHHS Publication No. 145, 2013.
Ng, C.T., Dheen, S.T., Yip, W.C., Ong, C.N., Bay, B.H., Lanry Yung, L.Y., 2011. The induction of epigenetic regulation of PROS1 gene in lung fibroblasts by gold nanoparticles and implications for potential lung injury. Biomaterials 32, 7609–7615.
Nolte-'t Hoen, E.N., van der Vlist, E.J., Aalberts, M., Mertens, H.C., Bosch, B.J., Bartelink, W., Mastrobattista, E., van Gaal, E.V., Stoorvogel, W., Arkesteijn, G.J., Wauben, M.H., 2012. Quantitative and qualitative flow cytometric analysis of nanosized cell-derived membrane vesicles. Nanomedicine 8, 712–720.
Oberdörster, G., 2010. Safety assessment for nanotechnology and nanomedicine: concepts of nanotoxicology. J. Intern. Med. 267, 89–105.
Oberdörster, G., Sharp, Z., Atudorei, V., Elder, A., Gelein, R., Kreyling, W., Cox, C., 2004. Translocation of inhaled ultrafine particles to the brain. Inhal. Toxicol. 16, 437–445.
Pacher, P., Beckman, J.S., Liaudet, L., 2007. Nitric oxide and peroxynitrite in health and disease. Physiol. Rev. 87, 315–424.
Palomäki, J., Sund, J., Vippola, M., Kinaret, P., Greco, D., Savolainen, K., Puustinen, A., Alenius, H., 2015. A secretomics analysis reveals major differences in the macrophage responses towards different types of carbon nanotubes. Nanotoxicology 9 (6), 719–728.

Palomäki, J., Välimäki, E., Sund, J., Vippola, M., Clausen, P.A., Jensen, K.A., Savolainen, K., Matikainen, S., Alenius, H., 2011. Long, needle-like carbon nanotubes and asbestos activate the NLRP3 inflammasome through a similar mechanism. ACS Nano 5 (9), 6861–6870.

Patel, M.M., Chillrud, S.N., Deepti, K.C., Ross, J.M., Kinney, P.L., 2013. Traffic-related air pollutants and exhaled markers of airway inflammation and oxidative stress in New York City adolescents. Environ. Res. 121, 71–78.

Patti, G.J., Yanes, O., Siuzdak, G., 2012. Innovation: Metabolomics: the apogee of the omics trilogy. Nat. Rev. Mol. Cell Biol. 13, 263–269.

Pelclova, D., Barosova, H., Kukutschova, J., Zdimal, V., Navratil, T., Fenclova, Z., Vlckova, S., Schwarz, J., Zikova, N., Kacer, P., Komarc, M., Belacek, J., Zakharov, S., 2015a. Raman microspectroscopy of exhaled breath condensate and urine in workers exposed to fine and nano TiO_2 particles: a cross-sectional study. J. Breath Res. 9 (3), 036008.

Pelclova, D., Zdimal, V., Fenclova, Z., Vlckova, S., Turci, F., Corazzari, I., Kacer, P., Schwarz, J., Zikova, N., Makes, O., Syslova, K., Komarc, M., Belacek, J., Navratil, T., Machajova, M., Zakharov, S., 2015b. Markers of oxidative damage of nucleic acids and proteins among workers exposed to TiO_2 (nano)particles. Occup. Environ. Med. 73 (2), 110–118.

Pelclova, D., Zdimal, V., Kacer, P., Fenclova, Z., Vlckova, S., Syslova, K., Navratil, T., Schwarz, J., Zikova, N., Barosova, H., Turci, F., Komarc, M., Pelcl, T., Belacek, J., Kukutschova, J., Zakharov, S., 2016. Oxidative stress markers are elevated in exhaled breath condensate of workers exposed to nanoparticles during iron oxide pigment production. J. Breath Res. 10 (1), 016004.

Pisani, C., Gaillard, J.-C., Nouvel, V., Odorico, M., Armengaud, J., Prat, O., 2015. High-throughput, quantitative assessment of the effects of low-dose silica nanoparticles on lung cells: grasping complex toxicity with a great depth of field. BMC Genomics 16 (1), 315.

Poli, D., Goldoni, M., Corradi, M., Acampa, O., Carbognani, P., Internullo, E., Casalini, A., Mutti, A., 2010. Determination of aldehydes in exhaled breath of patients with lung cancer by means of on fiber derivatisation SPME–GC/MS. J. Chromatogr. B 878 (27), 2643–2651.

Radomski, A., Jurasz, P., Alonso-Escolano, D., Drews, M., Morandi, M., Malinski, T., Radomski, M.W., 2005. Nanoparticle-induced platelet aggregation and vascular thrombosis. Br. J. Pharmacol. 146, 882–893.

Raposo, G., Stoorvogel, W., 2013. Extracellular vesicles: exosomes, microvesicles, and friends. J. Cell Biol. 200 (4), 373–383.

Riediker, M., Schubauer-Berigan, M.K., Brouwer, D.H., Nelissen, I., Koppen, G., Frijns, E., Clark, K.A., Hoeck, J., Liou, S.H., Ho, S.F., Bergamaschi, E., Gibson, R., 2012. A road map toward a globally harmonized approach for occupational health surveillance and epidemiology in nanomaterial workers. J. Occup. Environ. Med. 54, 1214–1223.

Rotoli, B.M., Gatti, R., Movia, D., Bianchi, M.G., Di Cristo, L., Fenoglio, I., Sonvico, F., Bergamaschi, E., Prina-Mello, A., Bussolati, O., 2015. Identifying contact-mediated, localized toxic effects of MWCNT aggregates on epithelial monolayers: a single-cell monitoring toxicity assay. Nanotoxicology 9 (2), 230–241.

Roux, A., Lison, D., Junot, C., Heilier, J.F., 2011. Applications of liquid chromatography coupled to mass spectrometry-based metabolomics in clinical chemistry and toxicology: A review. Clin. Biochem. 44, 119–135.

Roy, A., Gong, J., Thomas, D.C., Zhang, J., Kipen, H.M., Rich, D.Q., et al., 2014. The cardiopulmonary effects of ambient air pollution and mechanistic pathways: a comparative hierarchical pathway analysis. PLoS One 9 (12), e114913.

Saber, A.T., Lamson, J.S., Jacobsen, N.R., Ravn-Haren, G., Hougaard, K.S., Nyendi, A.N., Wahlberg, P., Madsen, A.M., Jackson, P., Wallin, H., Vogel, U., 2013. Particle-induced pulmonary acute phase response correlates with neutrophil influx linking inhaled particles and cardiovascular risk. PLoS One 8 (7), e69020.

Sarnat, S.E., Raysoni, A.U., Li, W.W., Holguin, F., Johnson, B.A., Flores Luevano, S., Garcia, J.H., Sarnat, J.A., 2011. Air pollution and acute respiratory response in a panel of asthmatic children along the U.S.-Mexico border. Environ. Health Perspect. 120, 437–444.

Schulte, P.A., Geraci, C.L., Murashov, V., Kuempel, E.D., Zumwalde, R.D., Castranova, V., Hoover, M.D., Hodson, L., Martinez, K.F., 2014. Occupational safety and health criteria for responsible development of nanotechnology. J. Nanopart. Res. 16, 2153.

Schulte, P.A., Hauser, J.E., 2012. The use of biomarkers in occupational health research, practice, and policy. Toxicol. Lett. 213 (1), 91–99.

Seaton, A., Tran, L., Aitken, R., Donaldson, K., 2010. Nanoparticles, human health hazard and regulation. J. R. Soc. Interface 7, S119–S129.

Shvedova, A., Kagan, V.E., Fadeel, B., 2010. Close encounters of the small kind: adverse effects of man-made materials interfacing with the nano-cosmos of biological systems. Annu. Rev. Pharmacol. Toxicol. 50, 63–88.
Shvedova, A.A., Yanamala, N., Kisin, E.R., Khailullin, T.O., Birch, M.E., Fatkhutdinova, L.M., 2016. Integrated analysis of dysregulated ncRNA and mRNA expression profiles in humans exposed to carbon nanotubes. PLoS One 11, e0150628.
Shyamasundar, S., Ng, C.T., Yung, L.Y., Dheen, S.T., Bay, B.H., 2015. Epigenetic mechanisms in nanomaterial-induced toxicity. Epigenomics 7 (3), 395–411.
Simpson, C.A., Huffman, B.J., Cliffel, D.E., 2013. In vivo testing for gold nanoparticle toxicity. Methods Mol. Biol. 1026, 175–186.
Singh, N., Manshian, B., Jenkins, G.J.S., Griffiths, S.M., Williams, P.M., Maffeis, T.G.G., Wright, C.J., Doak, S.H., 2009. NanoGenotoxicology: the DNA damaging potential of engineered nanomaterials. Biomaterials 30, 3891–3914.
Smolders, R., Bartonova, A., Boogaard, P.J., Dusinska, M., Koppen, G., Merlo, F., Sram, R.J., Vineis, P., Schoeters, G., 2010. The use of biomarkers for risk assessment: reporting from the INTARESE/ENVIRISK Workshop in Prague. Int. J. Hyg. Environ. Health 213 (5), 395–400.
Søs Poulsen, S., Jacobsen, N.R., Labib, S., Wu, D., Husain, M., Williams, A., Bøgelund, J.P., Andersen, O., Købler, C., Mølhave, K., Kyjovska, Z.O., Saber, A.T., Wallin, H., Yauk, C.L., Vogel, U., Halappanavar, S., 2013. Transcriptomic analysis reveals novel mechanistic insight into murine biological responses to multi-walled carbon nanotubes in lungs and cultured lung epithelial cells. PLoS One 8 (11), e80452.
Steenhof, M., Mudway, I.S., Gosens, I., Hoek, G., Godri, K.J., Kelly, F.J., et al., 2013. Acute nasal pro-inflammatory response to air pollution depends on characteristics other than particle mass concentration or oxidative potential: the RAPTES project. Occup. Environ. Med. 70, 341–348.
Stoccoro, A., Karlsson, H.L., Coppedè, F., Migliore, L., 2013. Epigenetic effects of nano-sized materials. Toxicology 313 (1), 3–14.
Sturla, S.J., Boobis, A.R., FitzGerald, R.E., Hoeng, J., Kavlock, R.J., Schirmer, K., Whelan, M., Wilks, M.F., Peitsch, M.C., 2014. Systems toxicology: from basic research to risk assessment. Chem. Res. Toxicol. 27 (3), 314–329.
Sung, J.H., Ji, J.H., Park, J.D., Yoon, J.U., Kim, D.S., Jeon, K.S., Song, M.Y., Jeong, J., Han, B.S., Han, J.H., Chung, Y.H., Chang, H.K., Lee, J.H., Cho, M.H., Kelman, B.J., Yu, I.J., 2009. Subchronic inhalation toxicity of silver nanoparticles. Toxicol. Sci. 108, 452–461.
Tarantini, L., Bonzini, M., Apostoli, P., Pegoraro, V., Bollati, V., Marinelli, B., Cantone, L., Rizzo, G., Hou, L., Schwartz, J., Bertazzi, P.A., Baccarelli, A., 2009. Effects of particulate matter on genomic DNA methylation content and iNOS promoter methylation. Environ. Health Perspect. 117, 217–222.
Teeguarden, J.G., Webb-Robertson, B.J., Waters, K.M., Murray, A.R., Kisin, E.R., Varnum, S.M., Jacobs, J.M., Pounds, J.G., Zanger, R.C., Shvedova, A.A., 2011. Comparative proteomics and pulmonary toxicity of instilled single-walled carbon nanotubes, crocidolite asbestos, and ultrafine carbon black in mice. Toxicol. Sci. 120 (1), 123–135.
Théry, C., Zitvogel, L., Amigorena, S., 2002. Exosomes: composition, biogenesis and function. Nat. Rev. Immunol. 2 (8), 569–579.
Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M., Lee, J.J., Lötvall, J.O., 2007. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat. Cell Biol. 9, 654–659.
van Niel, G., Porto-Carreiro, I., Simoes, S., Raposo, G., 2006. Exosomes: a common pathway for a specialized function. J. Biochem. 140 (1), 13–21.
Vesterdal, L.K., Folkmann, J.K., Jacobsen, N.R., Sheykhzade, M., Wallin, H., Loft, S., Møller, P., 2010. Pulmonary exposure to carbon black nanoparticles and vascular effects. Part. Fibre Toxicol. 7, 33.
Vietti, G., Lison, D., van den Brule, S., 2016. Mechanisms of lung fibrosis induced by carbon nanotubes: towards an Adverse Outcome Pathway (AOP). Part. Fibre Toxicol. 13, 11.
Vrijens, K., Bollati, V., Nawrot, T.S., 2015. MicroRNAs as potential signatures of environmental exposure or effect: a systematic review. Environ. Health Perspect. 123 (5), 399–411.
Wang, S.X., Liu, P., Wei, M.T., Chen, L., Guo, Y., Wang, R.Y., Tu, Z.G., Liang, X.G., 2007. Roles of serum Clara cell protein 16 and surfactant protein-D in the early diagnosis and progression of silicosis. J. Occup. Environ. Med. 49, 834–839.
Waters, K.M., Masiello, L.M., Zangar, R.C., Tarasevich, B.J., Karin, N.J., Quesenberry, R.D., Bandyopadhyay, S., Teeguarden, J.G., Pounds, J.G., Thrall, B.D., 2008. Macrophage responses to silica nanoparticles are highly conserved across particle sizes. Toxicol. Sci. 104 (1), 155–162.

Wu, W.T., Liao, H.Y., Chung, Y.T., Li, W.F., Tsou, T.C., Lin, M.H., Ho, J.J., Wu, T.N., Liou, S.H., 2014. Effect of nanoparticles exposure on fractional exhaled nitric oxide (FENO) in workers exposed to nanomaterials. Int. J. Mol. Sci. 15, 878–894.

Yokel, R.A., Hussain, S., Garantziotis, S., Demokritou, P., Castranova, V., Cassee, F.R., The, Yin., 2014. An adverse health perspective of nanoceria: uptake, distribution, accumulation, and mechanisms of its toxicity. Environ. Sci. Nano 1 (5), 406–428.

Zhang, R., Dai, Y., Zhang, X., Niu, Y., Meng, T., Li, Y., Duan, H., Bin, P., Ye, M., Jia, X., Shen, M., Yu, S., Yang, X., Gao, W., Zheng, Y., 2014. Reduced pulmonary function and increased pro-inflammatory cytokines in nanoscale carbon black-exposed workers. Part. Fibre Toxicol. 11, 73.

Zhu, M., Li, Y., Shi, J., Feng, W., Nie, G., Zhao, Y., 2012. Cellular responses to nanomaterials: exosomes as extrapulmonary signaling conveyors for nanoparticle-induced systemic immune activation. Small 8 (3), 404–412.

CHAPTER

7

Regulation and Legislation

Kirsten Rasmussen, Birgit Sokull-Klüttgen*, Il Je Yu**, Jun Kanno†, Akihiro Hirose†, Maureen R. Gwinn‡*

***Institute for Health and Consumer Protection, European Commission, Joint Research Centre, Ispra, Italy; **Institute of Nanoproduct Safety Research, Hoseo University, Asan, Korea; †The National Institute of Health Sciences, Kamiyoga, Setagaya-ku, Tokyo, Japan; ‡US Environmental Protection Agency, Washington, DC, United States**

OUTLINE

Background 160

Risk Assessment of Nanomaterials 160

Regulation Areas for Nanomaterials 162

United States 163
- *Environmental/Human Health* 166
- *Food Safety and Medical Applications* 167
- *Consumer Products* 167
- *Occupational* 168

European Union 168
- *Chemicals* 170
- *Plant Protection Products and Biocidal Products* 171
- *Worker Protection* 172
- *Consumer Products* 174
- *Cosmetic Products Regulation* 174
- *Food Legislation* 174
- *Medicinal Products and Medical Devices* 176
- *Environmental Protection* 176

Korea 177
- *Master Plans on Nanosafety Management* 177
- *Nanotechnology Relevant Standard Development* 177
- *Research Activities to Support Safety of Nanomaterials* 179

Japan 180
- *Regulatory Framework* 180
- *Nanotechnology Relevant Standard Development* 181
- *Research Activities* 181

Take-Home Messages 182

Acknowledgments 183

Disclaimer 183

References 183

Adverse Effects of Engineered Nanomaterials. http://dx.doi.org/10.1016/B978-0-12-809199-9.00007-0
Copyright © 2017 Elsevier Inc. All rights reserved.

BACKGROUND

Regulation of nanomaterials is still debated; the European Union has started to introduce specific requirements for nanomaterials in some of its legislation. It is recognized that questions related to the risks of exposure to nanomaterials are still unanswered. The focus of this chapter is on the existing legislations currently used for regulation of nanomaterials. This includes the Toxic Substances Control Act (TSCA) in the USA and the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) in the European Union, as well as Toxic Chemical Control Act and the Chemical Registration and Assessment Act in Korea and the Act on the Evaluation of Chemical Substances and Regulation of their Manufacture, and so on, in Japan. The OECD is the global forum for information exchange between regulators and other stakeholders on safety of nanomaterials (OECD, 2016). As described in the earlier chapters, nanomaterials are produced for a variety of uses, and the main parameters that make them so appealing in some applications may also make them more potentially more hazardous to human health and the environment (the so-called nanomaterials paradox). Information is beginning to be available regarding human health effects associated with exposure to nanomaterials during manufacturing or use in consumer products. The information on the effects of the nanomaterials as compared to many of the macroscale materials is increasing and the OECD published the raw data from its testing program. At the time of this review, in the United States there is an ongoing National Toxicology Program (NTP) bioassay on a variety of nanomaterials that will inform some of these issues (NTP, webpage). There have been some occupational exposure studies performed, but these are small-scale studies limited to a particular production method or use of a specific nanomaterial. These data limitations impact the ability to adequately assess the risks associated with nanomaterials, making it difficult to evaluate nanomaterials beyond a case-by-case basis. Recent exposure and toxicity studies have moved beyond analysis of pristine nanomaterials to better understand the impact of environmental contaminants on the nanomaterials we are exposed to and the potential effects on human health.

RISK ASSESSMENT OF NANOMATERIALS

The Organization for Economic Cooperation and Development (OECD) established a Working Party on Manufactured Nanomaterials (WPMN) (OECD WPMN, webpage) in 2006 in order to collaborate internationally on the safety research of nanomaterials. The WPMN is a key example of the interdisciplinary nature of the work needed in nanomaterial research, including scientists (e.g., chemists, physicists, biologists, and toxicologists) and regulators working on the evaluation of environmental health and safety, exposure, and testing of nanomaterials; in addition, the WPMN covers a vast geographical area that enables it to harvest many different approaches. The WPMN are looking at different issues such as Testing and Assessment, Risk Assessment & Regulatory Programmes, Exposure Measurement & Exposure Mitigation, and Environmentally Sustainable Use of Manufactured Nanomaterials. Of particular importance is the analysis of the outcomes of the task "Testing a Representative Set of Manufactured Nanomaterials." The testing was finalized in 2013 and the raw data was made available through the OECD web site during 2015 (OECD,

webpage) for the following 11 nanomaterials: fullerenes, single-walled carbon nanotubes (SWCNT), multiwalled carbon nanotubes (MWCNT), silver, gold, titanium dioxide, silicon dioxide, nanoclays, dendrimers, zinc oxide, and cerium dioxide. The objective was to test a representative set of manufactured nanomaterials in accordance with the Guidance Manual for Sponsors (OECD, 2010) leading to an understanding of relevant additional physical chemical characterization of nanomaterials, and furthermore practical experience and evidence on how far current OECD Test Guidelines and other methods are applicable. An overview of the outcomes of the WPMN testing program is presented by Rasmussen et al. (2016). The analysis to date indicates that many existing OECD test guidelines are suitable for nanomaterials. Consequently, hazard data collected using such guidelines will fall under OECD's system of Mutual Acceptance of Data (MAD) which is a legally binding instrument to facilitate the international acceptance of information for the regulatory safety assessment of chemicals. To cover testing of all end-points for nanomaterials, the WPMN cooperates with the OECD Test Guidelines Programme on adapting selected OECD test guidelines to testing of nanomaterials and also to develop relevant nanospecific test guidelines. An overview of test guidelines to be modified or recently developed for nanomaterials is found (Rasmussen et al., 2016). There is also an ongoing NTP bioassay on a variety of nanomaterials that will inform some of the issues related to the effects of nanomaterials (NTP, webpage). These efforts will help to design standardized tests, which require well-controlled and defined conditions and the availability of well-characterized manufactured nanomaterials.

Various attempts to analyze the safety of nanomaterials have focused on combining the risk assessment paradigm with a life cycle framework (LCA). The use of a comprehensive environmental assessment (CEA) entails all life cycle stages in the context of hazard identification and risk assessment. CEA involves both qualitative and quantitative approaches to risk characterization of human or ecological risk (Davis, 2007). Case studies and workshops have addressed the issues of nanomaterial risk assessment using this methodology (US EPA, 2011, 2012). Also the WPMN has looked into links between life cycle assessment and risk assessment, providing a Guidance Manual to further improve and facilitate the international scientific dialogue in the field of life-cycle aspects of manufactured nanomaterials (OECD, 2015). It aims to support decision-making in various situations of manufactured nanomaterials throughout their whole life cycle from research to final product phase. It serves as supplementary guidance to conduct a LCA for manufactured nanomaterials, being neither a new guideline nor a "super tool."

Risk assessment is generally performed based on the chemical composition of the material in question. The OECD WPMN and its member countries came to the conclusion "*… that the approaches for the testing and assessment of traditional chemicals are in general appropriate for assessing the safety of nanomaterials, but may have to be adapted to the specificities of nanomaterials. As with other chemicals, it is clear that each nanomaterial may pose specific challenges, but in most instances, they can be addressed with existing test methods and assessment approaches. In some cases, it might be necessary to adapt methods of sample preparation and dosimetry for safety testing. Similarly, adaptations may be needed for certain Test Guidelines. But it will not be necessary to develop completely new approaches for nanomaterials.…*" (OECD, 2012). However, as chemicals generally considered nontoxic in macroscale ranges may become toxic in the nanoscale range a case-by-case approach is prudent.

A key area of nanomaterial safety research is characterization of physicochemical properties. While most researchers agree that characterization of nanomaterials is a necessary step in toxicological analysis, needs regarding the level, type, and timing of characterization are not as clear; this is further discussed in Chapter 2. The increasing use of some materials in their nanoform(s) takes advantage of the differences between the nanoform(s) of the materials and their larger scale counterparts. Research has focused on many of these characteristics and their potential role in toxicity, generally compared to their larger scale counterparts. Studies have examined the impact of their smaller size, increased surface area, and increased surface reactivity.

REGULATION AREAS FOR NANOMATERIALS

As nanomaterials are used in an increasing range of high-tech applications and consumer products, concern remains about their safe use and possible impact on human health and the environment. Current and future legislation will have to take this into account and address nanomaterials more specifically. The need for a standard regulatory definition for nanomaterials has been debated (Maynard, 2011). Currently the EU is the only geographical area that has proposed and recommended a general regulatory definition of the term "nanomaterial" (European Commission, 2011).

In the recent update of the US National Nanotechnology Initiative Environmental Health and Safety Research Strategy and subsequent progress review (NNI, 2011; NNI, 2014), nanomaterials are defined to more specifically include all materials *designed* to have at least one dimension less than 100 nm and exhibit unique qualities determined by this size. This definition may not apply to regulatory statutes or policies relevant to nanotechnology.

In East Asia, Korea is addressing the safety of nanotechnology and nanomaterials through application of existing legislation regarding safety of chemicals and its management. At present (2016), Korea has not initiated new legislation for safe management of nanomaterials. Also Japan addresses nanomaterials through application of existing legislation: the Act on the Evaluation of Chemical Substances and Regulation of their Manufacture, and Acts relating to food, drugs, medical devices, cosmetics and pesticides are the most relevant regulation.

In the European Union, the European Parliament considered it important to address nanomaterials explicitly within the scope of certain legislation and called for the introduction of a comprehensive science-based definition of nanomaterials in Community legislation (European Parliament, 2009). Following this, nanomaterials have been explicitly addressed in updates of some legislation, for example in the Biocidal Products Regulation (European Union, 2012a), Cosmetic Products Regulation (European Union, 2009a), Food Information to Consumers (European Union, 2011a), Novel Food legislation (European Union, 2015), Active and Intelligent Materials and Articles (European Union, 2009b), and Plastic Food Contact Materials (European Union, 2011b).

Based on a Reference Report on "Considerations on a Definition of Nanomaterial for Regulatory Purposes" (Lövestam et al., 2010) of the European Commission's Joint Research Centre (JRC) and the opinion of the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), entitled Scientific Basis for the Definition of the Term "Nanomaterial"

(European Commission, 2010), the European Commission published a recommendation on the definition of nanomaterial (European Commission, 2011) in the following called "EC Definition": "*'Nanomaterial' means a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as en agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm–100 nm.*" In specific cases and where warranted by concerns for the environment, health, safety, or competitiveness the number size distribution threshold of 50% may be replaced by a lower threshold. Fullerenes, grapheme flakes and single wall carbon nanotubes with one or more external dimensions below 1 nm should also be considered as nanomaterials. The terms "particle," "agglomerate," and "aggregate" are defined. For a further discussion on the definition of nanomaterials, see Chapter 2. This definition is broadly applicable across different regulatory sectors and its use is recommended to promote consistency in the interpretation of the term "nanomaterial" for legislative and policy purposes in the EU. The EC Definition contained a clause that mandates its review in 2014, and the JRC provided the scientific basis with three reports (Rauscher et al., 2014, 2015; Roebben et al., 2014). The review itself has been deferred to 2016.

The Biocidal Products Regulation (EU) 528/2012/EC (European Union, 2012a) is the first legal act that contains a definition of nanomaterial which is based on the EC Definition. Some EU legislation also addressing nanomaterials was adopted before the EC Definition and therefore use different definitions, and the Novel Food Regulation (European Union, 2015) amends the food legislation definition [including the Food Information to Consumers (European Union, 2011a)] to be closer to the recommendation; an overview of these different definitions is found in (Rauscher et al., 2015).

Some of the key questions surrounding the regulation of nanomaterials relates to whether these should be considered a new material, or if they can be regulated based on their bulk counterparts (if these exist). The following section will describe the current legislation that specifically mentions nanomaterials or may play a role in the regulation of nanomaterials in the United States and the European Union.

UNITED STATES

Nanotechnology policy in the United States is primarily coordinated through the National Science and Technology Council (http://whitehouse.gov/administration/eop/ostp/nstc) through the Nanoscale Science, Engineering, and Technology (NSET) Subcommittee (www.nano.gov). The NSET Subcommittee and its working groups, with support from the National Nanotechnology Coordination Office (NNCO), prepare the multiagency planning, budget, and assessment documents related to the National Nanotechnology Initiative (NNI). NNI is a United States government-wide effort to coordinate federally funded nanomaterial research focusing on multiple topics, including metrology, environmental health and safety, and exposure to nanomaterials. Coordination of efforts is an important step in making sure the most useful research is done in an efficient manner. An overview of the United States' legislation is given in Table 7.1.

The NNI releases an Environmental, Health and Safety Strategy document regularly providing guidance to federal agencies involved the development of nanotechnology.

TABLE 7.1 Legislative Authorities that may Encompass Nanomaterial Usage in the United States

Regulation	Description	Potential use	Governing body	Websites
TSCA	TSCA provides EPA with the authority to require reporting, record-keeping and testing requirements, and restrictions relating to chemical substances and/or mixtures.	Proposed case-by-case determination of nanomaterials as new materials.	United States Environmental Protection Agency (US EPA)	http://www.epa.gov/lawsregs/laws/tsca.html
Federal Insecticide, Fungicide and Rodenticide Act (FIFRA)	FIFRA provides the basis for regulation, sale, distribution, and use of pesticides in the United States.	Determination of unreasonable adverse human health risks with the addition of nanomaterials to existing pesticides.	United States Environmental Protection Agency (US EPA)	http://www.epa.gov/opp00001/regulating/laws.htm
Safe Drinking Water Act (SDWA)	The main federal law that ensures the quality of America's drinking water.	Currently no maximum contaminant level goals set for nanoparticles.	United States Environmental Protection Agency (US EPA)	http://www.epa.gov/OGWDW/sdwa/
Comprehensive Environmental Response, Compensation, and Liability Act and Resource Conservation and Recovery Act (CERCLA/RCRA)	Commonly known as Superfund, CERCLA was enacted in 1980 and created a tax on the chemical and petroleum industries and provide a broad Federal Authority to response directly to releases or threatened releases of hazardous substances that may endanger public health or the environment.	This would cover the evaluation and control of nanomaterials in waste sites.	United States Environmental Protection Agency (US EPA)	http://www.epa.gov/superfund/policy/cercla.htm

Clean Air Act (CAA)/ Clean Water Act (CWA)	The Clean Air Act and Clean Water Act both allow the EPA to set limits on certain air and water pollutants in the United States. Under these Acts, the EPA also review and approve permit applications for industries or chemical processes.	Release of nanomaterials into the air or water would fall under these Acts.	United States Environmental Protection Agency (US EPA)	http://www.epa.gov/air/caa/peg/understand.html http://www.epa.gov/oecaagct/lcwa.html
Food, Drug and Cosmetic Act (FDCA)	The FDCA is a set of laws giving FDA the authority to oversee the safety or food, drugs and cosmetics in the United States.	Already in use for premarket testing of food additives, this may cover regulation of the use of nanomaterials in food additives and cosmetics.	United States Food and Drug Administration (US FDA)	http://www.fda.gov/nanotechnology/regulation.html
Occupational Safety and Health Act of 1970 (29 US 654)	Section 5(a)(1) of the OSH Act requires employers to "furnish each of his employees employment and a place of employment, which are free from recognized hazards that are causing or likely to cause death or serious physical harm to his employees."	OSHA has approved plans for 24 states to adopt federal safety standards for workers exposed to nanomaterials in private industry.	United States Occupational Safety and Health Administration (OSHA)	http://www.osha.gov/dsg/nanotechnology/nanotech_standards.html

Written by members of the various federal agencies involved in NNI, these strategies are based on the state of the science, ongoing research activities, and input from the public through workshops and public review (NNI, 2011). The NNI is not a regulatory body; however, one goal of the NNI is to give recommendations on the research needed for risk assessment and risk management of nanomaterials to ensure responsible development of nanotechnology.

Environmental/Human Health

Toxic Substances Control Act (TSCA)

The TSCA (EPA, 1976) is the main legislative authority under which the US EPA requires manufacturers of new chemicals to submit specific information on the effects of these materials (http://www.epa.gov/oppt/nano/). Under TSCA, the US EPA has taken steps to limit the use and exposure to nanomaterials, including carbon nanotubes (CNTs). EPA has required the use of personal protective equipment and limitations on the use and environmental exposures of nanomaterials. The US EPA's Office of Pollution Prevention and Toxics recently proposed one-time reporting and record keeping requirements for new and existing chemical substances that are nanoscale materials under TSCA (EPA, webpage).

Under TSCA, the US EPA has reviewed new nanoscale chemical premanufacture notices for carbon nanotubes, nanosilver and other materials required prior to the manufacture or introduction of new chemicals. This process allows for the US EPA to control and/or limit exposures to these chemicals through a variety of mechanisms (e.g., limiting use and release of materials, requiring use of personal protective equipment). US EPA has permitted the manufacture of new nanoscale chemical materials through the use of the Significant New Use Rules (SNUR) under TSCA. Through this process, the US EPA is provided with a basic set of information on nanoscale materials, including the chemical properties, toxicity data, and production volume, which would allow the US EPA to determine an unreasonable risk of these new materials to human health or the environment.

Although not specific to nanomaterial regulation, it is important to note that the United States has recently passed the Frank R. Lautenberg Chemical Safety for the 21st Century Act (EPA, 2016), which will amend TSCA. These changes are the first such amendment to TSCA in 40 years. These amendments address the review and regulation of new and existing chemicals, and in that way may impact the regulation of nanomaterials.

Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA)

Nanomaterials added to pesticides would fall under the mandates of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) to determine whether their addition leads to adverse health risks (EPA, 2008a). Under FIFRA, the US EPA held its first Science Advisory Panel (SAP) review of nanomaterials in 2009, specifically examining nanosilver and other nanometal pesticide products. The panel was designed to address issues related to whether nanosilver and other nanometals in the 1–100 nm have unique behavior under conditions relevant to human health and ecological risk. The SAP review focused mainly on use of nanosilver in pesticide products, with recommendations on how to conduct risk assessments of pesticide products containing nanosilver, particularly related to what types

of data would be needed to evaluate the risks. The panel determinations related to the role of the silver ion release in the toxicity of silver (macroscale and nanoscale), and the lack of available data demonstrating any variability in the release of silver ions from nanomaterials as opposed to macroscale silver, and the specific biological impacts of particle size (both individual or agglomerated particles). In general, for risk assessment the SAP review recommended the need for a case-by-case evaluation of pesticide products containing nanosilver www.epa.gov.

Other Regulations

There are other environmental regulations that may play a key role in regulating the use and disposal of nanomaterials. The Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) and Resource Conservation and Recovery Act (RCRA) would be used to evaluate the risks of nanomaterials at waste sites, although this has not yet been performed (EPA, 2008b). The 1990 Clean Air Act Amendments (CAAA) (EPA, 1990) and the 1996 Clean Water Act Amendments (CWAA) (EPA, 1996) would be used for regulation of nanomaterials in ambient air or ambient water and wastewater. For CAAA, the levels transported in air would be regulated based on their danger to public health. The CWAA would regulate the effluent limits of nanomaterials in the wastewater.

Food Safety and Medical Applications

The Food, Drug and Cosmetic Act (FDCA) has been used by the FDA to address the impact and use of nanomaterials in food, including additives and contaminants, medicines and cosmetics (FAD, 1938). The FDCA requires premarket testing for food and color additives, as well as drugs, but not for cosmetics. The FDA can require information on the identity and properties of materials, regardless of the particle size of a material. FDA also regulates cosmetics, including sunscreens, for example, the use of nanosized titanium dioxide and other nanomaterials in sunscreens. The FDA generally does not require premarket safety data on cosmetics.

There are currently no regulations specific for nanomaterials related to food or drug safety. Currently, all medical products that contain nanomaterials go through traditional regulatory process and include requirements for clinical, toxicological, and other studies for determining the safety and efficacy of these materials. FDA has recently released final guidance documents on the use of nanotechnology in FDA-regulated products (FDA, 2014). These guidance documents inform the consideration of nanotechnology in FDA-regulated products, including both cosmetics and food.

Consumer Products

The use of nanomaterials in consumer products in the United States is under the oversight of the Consumer Product Safety Commission (CPSC) (http://www.cpsc.gov/). The CPSC mission is protecting the public from unreasonable risks of serious injury or death from the products under their jurisdiction. Although there is no specific ruling for nanomaterials, if any of these products contain nanomaterials, they would fall under the regulation of CPSC.

Occupational

The purpose of the Occupational Safety and Health Act of 1970 (OSHA, 1970) is to assure safe and healthy working conditions in all occupational settings, generally based on recommendations from the National Institute for Occupational Safety and Health (NIOSH) and enforced by the Occupational Safety and Health Administration (OSHA). Generally, occupational exposure to nanomaterials falls under OSHA's General Industry standards. OSHA has approved plans for 24 states to adopt federal safety standards for workers exposed to nanomaterials in private industry (http://www.osha.gov/dsg/nanotechnology/nanotech_standards.html).

In 2011, NIOSH released a Current Intelligence Bulletin on the scientific hazards of occupational exposure to titanium dioxide (TiO_2), including engineered nanosized particles (http://www.cdc.gov/niosh/docs/2011-160/pdfs/2011-160.pdf). This bulletin performed a risk assessment of the currently available scientific information on TiO_2, including experimental animal toxicity and human health data, dose-response information and exposure monitoring techniques and control strategies. This NIOSH bulletin recommends exposure limits for both fine TiO_2 (2.4 mg/m^3) and ultrafine TiO_2 (including engineered nanoscale TiO_2; 0.3 mg/m^3) as time-weighted average (TWA) concentrations for up to 10 h per day during a 40-hour workweek. Further, NIOSH has categorized ultrafine TiO_2 as a potential occupational carcinogen, but there was insufficient data to conclude on carcinogenicity of fine TiO_2. NIOSH has released a similar draft guidance document examining the health effects of occupational exposure to silver nanomaterials, which is currently under review (USA Federal Register, 2016).

Examples of occupational exposures and human health effects reported in the literature include two pulmonary disease occurrences in conjunction with exposure to nanomaterials: workplace exposures to multiple chemicals with limited ventilation and personal protective equipment (Song et al., 2009) and exposures of volunteers to dust following the World Trade Center collapse in 2001 (Wu et al., 2010). Although limited, these are examples of adverse human health effects following exposure to nanomaterials in the workplace (see Chapter 5 for a further discussion on exposure to nanomaterials). However, these two examples demonstrate the need for clear investigation of the causal factors for these events which can provide insight into whether production and use of nanoscale engineered materials are creating undue risk that needs to be controlled. As such, NIOSH has taken steps to create guidance documents for workplace safety specifically related to nanotechnology. The first focused on the safe handling of nanomaterials in the workplace in general (https://www.cdc.gov/niosh/docs/2009-125/pdfs/2009-125.pdf), with the second focused specifically on safety for small and medium-sized enterprises (http://www.cdc.gov/niosh/docs/2016-102/pdfs/2016-102.pdf). Both guidance documents stress the importance of responsible development of nanotechnology in the workplace, by being prepared for potential unintended consequences of possible scenarios to human health and the environment.

EUROPEAN UNION

The main legal instruments in the European Union (EU) are Regulations and Directives. A regulation is directly applicable in all the Member States, while a Directive is addressed to the Member States and leaves them the choice how to adopt the Community objectives

TABLE 7.2 European Union Legislation Relevant for Nanomaterials

Horizontal legislation	Product legislation
Chemicals legislation (REACH: Regulation (EC) No 1907/2006, CLP: Regulation (EC) No 1272/2008)	*General Product Safety* (Directive 2001/95/EC of the European Parliament and of the Council)
Worker protection (for example: Council Directive 89/391/EEC, Council Directive 89/655/EEC, Council Directive 89/656/EEC, Council Directive 98/24/EC, Commission Directive 2000/39/EC, Commission Directive 2006/15/EC)	*Cosmetic Products* (Regulation (EC) No 1223/2009 of the European Parliament and of the Council)
Environmental Legislation	*Biocidal products* (Regulation (EU) No 528/2012 of the European Parliament and of the Council)
Integrated Pollution Prevention and Control	*Plant Protection Products* (Regulation 1107/2009 of the European Parliament and the Council)
Major Accidents Hazards Directive (Directive 2012/18/EU of the European Parliament and of the Council)	*Food Legislation* (e.g., Regulation (EU) No 1169/2011 of the European Parliament and of the Council, Regulation (EU) 2015/2283 of the European Parliament and of the Council, Commission Regulation (EU) No 10/2011)
Waste (Council Directive 91/689/EEC, Directive 2006/12/EC of the European Parliament and of the Council)	*Medicinal Devices* (fe.g., Council Directive 90/385/EEC on Active Implantable Medical Devices (AIMDD), Council Directive 93/42/EEC on Medical Devices (MDD), Council Directive 98/79/EC on In Vitro Diagnostic Medical Devices (IVDMD)
Water (Directive 2000/60/EC)	*Electronic Industry* See http://ec.europa.eu/growth/sectors/electrical-engineering/index_en.htm

within their national legal order. EU legislation is accessible and searchable online at http://EUR-LEX.europa.eu/. An overview of the main pieces of EU legislation is given in Table 7.2.

Regulatory aspects of nanomaterials have been intensively discussed in the European Parliament (EP), for example, the EP resolution of 2009 (European Parliament, 2009). The EP considered it important to address nanomaterials explicitly within the scope of certain legislation for areas, such as chemicals, biocides, and food. Besides the introduction of a definition of nanomaterials in Community legislation, the EP called for introduction of, for example, an inventory of the different types and uses of nanomaterials on the European market, and for better information to consumers on the use of nanomaterials in consumer products: all ingredients present in the form of nanomaterials should be clearly indicated in the label of the product.

Based on the resolutions from the EP the Commission of the European Communities (the European Commission) undertook a review of regulatory aspects of nanomaterials within the European Union. A first and second regulatory review of EU legislation was published in 2009 and 2012. The outcome of the first review is available in a Communication from 2008 (European Commission, 2008a), which came to the result that: *"Overall, it can be concluded*

that current legislation [in the EU] covers to a large extent risks in relation to nanomaterials and that risks can be dealt with under the current legislative framework. However, current legislation may have to modified in the light of new information becoming available, for example as regards thresholds used in some legislation." It was recognized that knowledge on essential questions, such as characterization of nanomaterials, their hazards, exposure, risk assessment and risk management need to be improved. The outcomes of the second regulatory review and an accompanying staff working document confirmed the conclusions of the first review (European Commission, 2012a,b).

By 2016, provisions for nanomaterials have been introduced for biocidal products and cosmetic products, as well as in food legislation. For chemicals, the introduction of specific references nanomaterials in the legislation has been debated at length. The EC Definition will be reviewed in 2016. The Annexes to REACH (European Union, 2006a) are planned to be adapted to technical progress in general, and provided that an agreed definition is available, references to nanomaterials will be introduced as relevant.

The following section summarizes the main outcomes of the aforementioned reviews (European Commission, 2008a, 2012a) by the European Commission concerning legislation relevant for health, safety and environment of nanomaterials, as well as information from accompanying Commission staff working documents (European Commission, 2008b, 2012b), which provides additional details, and information from other relevant documents; the information is brought up to date as relevant. Further reviews and other activities are expected by 2016, and thus the amount of information is increasing in a very dynamic way.

Overview publications on the-state-of-the-art of various aspects of nanomaterials and regulation have been published by the JRC, for example (Aschberger et al., 2014; Rauscher et al., 2014, 2015; Roebben et al., 2014).

Chemicals

Industrial Chemicals

The REACH Regulation applies to chemical substances on their own, in preparations or in articles (European Union, 2006a). It is based on the principle that manufacturers, importers and downstream users have to ensure that they manufacture, place on the market or use substances that do not adversely affect human health or the environment. Its provisions are underpinned by the precautionary principle. The European Chemicals Agency (ECHA) (European Chemicals Agency (ECHA), webpage) is responsible for the implementation of REACH. Guidance documents (European Chemicals Agency (ECHA), webpage) for industry and authorities support the implementation.

There are no provisions in REACH referring explicitly to nanomaterials. However, the "substance" definition in REACH states: "*Substance means a chemical element and its compounds in the natural state or obtained by any manufacturing process, including any additive necessary to preserve its stability and any impurity deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition*." Thus, REACH addresses chemical substances, in whatever size, shape, or physical state (European Commission, 2008c). Substances at the nanoscale are therefore covered by REACH and its provisions apply.

Under REACH, manufacturers and importers have to submit a registration dossier to ECHA for substances that they manufacture or import at or above 1 ton/year. REACH has staggered information requirements, increasing at the thresholds at or above 10, 100, and 1000 ton/year. At or above 10 ton/year the registrant is also required to submit a chemical safety report.

If a nanomaterial is considered to be a substance on its own, a registration dossier addressing the nanomaterial needs to be submitted. If a nanomaterial is considered to be a nanoform of a nonnano (bulk) substance, it can be registered within the registration dossier of this substance; the dossier must then contain all relevant information for all forms of the substance. The *total* volume (i.e., the sum of the nano and the nonnano volume per manufacturer or importer) determines the need and the timing for the registration and the information requirements. The registration dossier has to include all relevant information on the bulk and the nanomaterial covering the properties, uses, effects, and exposure related information. REACH does currently not include specific information requirements for nanomaterials; however, as the listed information is stated to be minimum information requirements (REACH Art. 12(1)) additional testing or information to determine the specific properties and hazards of the nanomaterial may therefore be required. Furthermore, standard test guidelines may not be fully suitable for the testing of nanomaterials. The information on the nanoform needs to be adequate for its Chemical Safety Assessment, if required (>10 t/a), as well as for the classification and labelling [according to The Classification, Labelling and Packaging (CLP) Regulation] (European Union, 2008a). Any different classification and labelling of the nanoform and additional risk management measures, if needed, have to be included in the registration dossier and have to be communicated to the supply chain via the safety data sheet (European Commission, 2009).

While the content of the registration dossier is the responsibility of the registrant (industry), a minimum of 5% of the dossiers will be evaluated by ECHA to ensure compliance with REACH (dossier evaluation). Furthermore, EU Member State Authorities may examine any information on a substance (substance evaluation). If deemed necessary, ECHA can require any information on the substance, that is, nanomaterial, in addition to the standard information requirements of REACH. Authorizations for substances of very high concern or restrictions for substances which pose an unacceptable risk are the regulatory instruments for risk management under REACH; they are independent from tonnage triggers or registration duty and could therefore, as for any other substance, also apply to nanomaterials of low tonnage level.

In conclusion, though there are no provisions in REACH referring explicitly to nanomaterials, they are substances, and thus covered by the REACH Regulation. ECHA provided guidance for nanomaterials under REACH for example on information requirements and chemical safety assessment (European Chemicals Agency, 2012a,b,c).

As for all substances, REACH is fundamental to obtain information on properties, including hazardous properties, of nanomaterials, and the CLP Regulation is essential to transfer this knowledge along the supply chain. The resulting regulatory hazard classification has consequences for many other legal instruments, such as occupational and environmental legislation.

Plant Protection Products and Biocidal Products

The placing on the European Union market of Plant Protection Products (PPP) and Biocidal Products is regulated by the PPP Regulation (European Union, 2009c) and the Biocidal

Products Regulation (BPR) (European Union, 2012a). The PPP Regulation covers "pesticides" for agriculture, such as insecticides and fungicides, applied mainly by professional users. The BPR covers 23 different product types, varying from disinfectants to antifouling agents and rodenticides, and the biocidal products may be used by both professional users and by consumers. PPPs and Biocidal Products contain active substances, which due to their nature have the potential to be (eco)toxic, and they are evaluated and approved at European Union level. Only PPPs and Biocidal Products containing these approved active substances can be authorized.

The BPR uses the same and the PPP Regulation a similar substance definition as REACH. Thus, both legal instruments address chemical substances, in whatever size, shape, or physical state, and substances at the nanoscale are therefore covered; the BPR specifically mentions nanomaterials, which are defined based on the EC Definition.

Nanomaterials could also be used as coformulants in PPPs and Biocidal Products. Full composition of the products needs to be presented when applying for product authorization, and additional data can be requested for substances of concern, and certain coformulants can be prohibited in case of concern for human and animal health and/or the environment.

The implementation of the PPP Regulation at EU level is guided and monitored under the responsibility of the European Food Safety Authority (EFSA) (European Food Safety Authority (EFSA), webpage). The European Chemicals Agency (ECHA) manages the same aspects for the Biocidal Products Regulation. EFSA has provided guidance on the risk assessment of the application of nanoscience and nanotechnologies in the Food and Feed Chain (European Food Safety Authority, 2011). There may be a need to be review and amend current guidance documents for PPPs, for example, on data requirements, risk assessment, and decision making, in order to highlight that the legislation covers nanomaterials and to ensure that all aspects of risks of nanomaterials are adequately addressed. An overview of regulatory aspects of nanotechnology in the agri/feed/food sector in European Union and non-European Union countries is provided (Amenta et al., 2015). For the BPR, ECHA provides the OECD WPMN guidance documents (OECD, 2010, 2012a) addressing nanomaterials, and has not developed nanospecific BPR guidance. According to the BPR, approval of an active substance does not cover the nanoform of the active substance except where explicitly mentioned, and for active substances that are nanomaterials a separate dossier, addressing all data requirements is required. Furthermore, a dedicated risk assessment is needed when a biocidal product contains active and/or nonactive substances that are nanomaterials. The label of the biocidal product must show the name of each nanomaterial followed by the word "nano" in brackets. Products containing nanomaterials are excluded from the simplified authorization procedure.

Worker Protection

Framework Directive 89/391/EEC on the introduction of measures to encourage improvements in the safety and health of workers (European Union, 1989a) places a number of obligations on employers to take measures necessary for the safety and health protection of workers. It applies to all substances and work activities including manufacturing and use of chemicals

at all levels of the production process. Although this Directive does not specifically address nanomaterials, it fully applies to nanomaterials. Employers, therefore, must carry out a risk assessment and, where a risk is identified, take measures to eliminate this risk. The Directive foresees the possibility to adopt individual "daughter Directives" laying down more specific provisions with respect to particular aspects of safety and health. These "daughter" Directives relate, for example, to risks related to chemical agents at work (European Union, 1998a), the use of work equipment by workers at work (European Union, 1989b), and the use of personal protective equipment at the workplace (European Union, 1989c). As these Directives introduce minimum requirements, national authorities have the possibility to introduce, more stringent rules.

Directive 98/24/EC on the protection of the health and safety of workers from the risks related to chemical agents (European Union, 1998b) is aimed at controlling and reducing occupational exposure risks. This is achieved, among others, by establishing indicative and binding Occupational Exposure Limit (OEL) values, as well as biological limit values for specific substances. The term "OEL value" is defined in the Directive, article 2(d), as "… *the limit of the time-weighted average of the concentration of a chemical agent in the air within the breathing zone of a worker in relation to a specified reference period.*" In this directive, the term "Biological limit value" is defined as "… *the limit of the concentration in the appropriate biological medium of the relevant agent, its metabolite, or an indicator effect.*" While OEL values address the concentration of the chemical in the air inhaled by workers, the biological limit is an internal dose of a chemical or its metabolite for which the route of entry is not defined (so it may be oral, dermal and/or inhalation). OEL values are given as 8-h time weighted average for airborne concentrations and/or as a short-term period of 15 min. Directive 98/24/EC has been amended several times, for example, to include lists with specific substances for which limit values have been agreed. Specific OEL values for nanoparticles do not yet exist. Currently the limit values for amorphous silica (2–6 mg/m^3) (European Union, 2000a) and silver (European Union, 2006b) are also applicable to their nanoforms, although it would be relevant to confirm whether these mass-based OELs established for "bulk" substances would be appropriate for nanomaterials.

The EU Commission published guidance for employers and workers on the safe use of nanomaterials (European Commission, 2014a,b). The Guidance document offered an overview of the issues surrounding the safe use of nanomaterials at the workplace, set out the broad outlines of preventive action and provided a practical tool for complying with specific aspects of ensuring workers' safety, such as risk assessment and risk management.

The European Agency for Safety and Health at Work (EU-OSHA) (EU-OSHA, webpage) deals with worker protection on Community level, including protection from nanomaterials, and has published a series of expert forecasts on emerging risks for the working environment including a report on chemical risks related to occupational safety and health (EU-OSHA, 2009a). The experts agreed that nanoparticles and ultrafine particles pose the most significant emerging risk.

Furthermore, EU-OSHA has published a literature review on workplace exposure to nanoparticles (EU-OSHA, 2009b). This report focuses on exposure measurements and assessments, health effects of nanomaterials, and possible subsequent activities to manage the risk.

Consumer Products

Consumer products may be covered by product legislation that addresses a specific area of application and lays down requirements regarding products, such as biocidal products, cosmetic products, and medical products. Consumer products, not covered by product legislation are covered by the Directive on General Safety of Consumer Products (European Union, 2002a). In many cases, more than one piece of legislation applies to the same product. Where regulation contains requirements of a general nature, they will cover also risks related to nanotechnology, even if they have been adopted without specifically intending to address risks associated with nanomaterials and nanotechnologies.

Cosmetic Products Regulation

The Cosmetic Products Regulation from 2009 was the first legal act in the EU that included specific provisions for nanomaterials (European Union, 2009a). It introduced the following definition: *"nanomaterial" means an insoluble or biopersistent and intentionally manufactured material with one or more external dimensions, or an internal structure, on the scale from 1 to 100 nm'.* Cosmetic products containing nanomaterials shall be notified to the European Commission electronically 6 months prior to being placed on the market. This notification shall contain additional information on the nanomaterial, such as the specification of the nanomaterial including size of particles, physical and chemical properties; an estimate of the quantity of nanomaterial contained in cosmetic products; the toxicological profile of the nanomaterial; safety data and the reasonably foreseeable exposure conditions. A catalogue of nanomaterials used in cosmetic products was required for 2014, but is delayed and not yet published. Furthermore, the Cosmetic Products Regulation included a labeling obligation, to inform consumers, for ingredients present in the form of nanomaterials, that is, in the list of ingredients the names of such substances shall be followed by the word *nano* in brackets.

The Scientific Committee on Consumer Safety (SCCS) (European Commission, webpage) provides guidance for the testing of cosmetic ingredients and their safety evaluation. The most recent 9th revision of its Notes of Guidance addresses, among others, more detailed requirements for nanomaterials (European Commission, 2015). The SCCS has provided its opinion on several nanomaterials, such as nanoforms of carbon black, titanium dioxide, and zinc oxide (European Commission, webpage).

Food Legislation

Regulation 178/2002 lays down the general principles and requirements of food law, establishes the European Food Safety Authority (EFSA) (European Food Safety Authority, 2011) and applies horizontally to all foods and feed (European Union, 2002b). More particularly relevant in relation to risks associated with nanomaterials are the following Regulations and Directives:

Regulation (EU 2015/2283 concerning novel foods and novel food ingredients) (European Union, 2015). The first Novel Foods legislation entered into force in 1997 and was amended in 2015. "Novel food" can be newly developed, innovative food; food produced using new technologies and production processes or food, which has been traditionally consumed outside

of the European Union. The year 2015 revision of the Novel Food Regulation amended the definition of "nanomaterial" for food and included specific requirements for food containing, or consisting of, nanomaterials, such as demonstrating that the test guidelines used are applicable to nanomaterials and contained a requirement for labeling to inform consumers.

Food additives. Directive 89/107/EEC (European Union, 1989d) on food additives covers substances added to food for a "technological purpose in the manufacture, processing, preparation, treatment, packaging, transport, or storage of such food." Some of the currently used food additives in particle form, such as anticacking powders (SiO_2, $CaCO_3$) may contain a fraction in the nanosize.

Food supplements. Framework Directive 2002/46/EC (and its daughter Directives) on food supplements provides a definition of food supplements, specific rules on labelling, presentation, and advertising of these products and regulates their compositions in vitamins and minerals (European Union, 2002c). Positive lists of vitamins and minerals and of their sources that can be used in food supplements are given in the Annexes of the Directive. Prior to the authorization and inclusion in the positive lists every vitamin and mineral and/or every source need to undergo a safety assessment carried out by EFSA. It can be envisaged that substances produced in "nano" form or packed into nanoglobules (such as vitamins) to improve their solubility or bioavailability could be used as food supplements.

Food contact materials. Regulation (EC) 1935/2004 relates to materials and articles intended to come into contact with food (European Union, 2004). It covers also articles for contact with water intended for human consumption. Prior to authorization for their use in food contact materials substances need to undergo a safety assessment carried out by EFSA.

Products deriving from nanotechnology seem to be promising for food contact materials, such as materials with barriers or coatings against humidity, UV light or oxygen, or antibacterial materials. Regulation (EC) 10/2011 on plastic materials and articles intended to come into contact with food (European Union, 2011b) clarifies that authorizations which are based on the risk assessment of the conventional particle size of a substance do not cover engineered nanoparticles. Nanoparticles' risk should be assessed on a case-by-case basis. Nanoparticles are not covered by the functional barrier concept (i.e., the use of nonauthorized substances behind a functional barrier, which separate the food from plastic by a multimaterial multilayer materials or article). An example for an authorized nanomaterial in a food contact material on the EU market is nano titanium nitrite. It has a defined primary particle size (20 nm) and is authorized for use in PET bottles up to 20 ng/kg.

EFSA has published a *Guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain* (Amenta et al., 2015). The guidance covers risk assessments for food and feed applications including food additives, enzymes, flavorings, food contact materials, novel foods, feed additives, and pesticides. It concludes that the risk assessment paradigm (hazard identification and hazard characterization followed by exposure assessment and risk characterization) is appropriate also for nanomaterials. The report provides guidance on nanospecific considerations that need to be assessed in addition to conventional aspects. It stipulates the additional data needed for the physical and chemical characterization of nanomaterials in comparison with conventional applications and outlines different toxicity testing approaches.

Furthermore, EFSA's nanonetwork precised in its report for the year 2015 that also soft nanomaterials (the term soft nanomaterials is generally used for liposomes, nanoemulsions,

and (bio)polymer based encapsulates) would require a risk assessment if used, for example, as novel food (European Food Safety Authority, 2016).

In 2011, the European Parliament adopted rules to provide more and better information to consumers so they can make informed choices when buying food (European Union, 2011a). Besides information, such as nutrition values, allergens, or country of origin, all ingredients present in the form of engineered nanomaterials shall be clearly indicated in the list of ingredients. The names of such ingredients shall be followed by the word "nano" in brackets.

An overview of regulatory aspects of nanotechnology in the agri/feed/food sector in EU and non-EU countries is found in (Amenta et al., 2015).

Medicinal Products and Medical Devices

Nanotechnology and nanomaterials are promising for new medicinal products, medical devices, and diagnostic agents. There are currently no specific provisions for "nano" products used in the aforementioned areas. However, the usual comprehensive legal frame applies and details are published on the homepage of the (www.ema.europa.eu) in London (UK). EMA has created an Innovation Task Force to ensure coordination of scientific and regulatory competence in the field of emerging therapies and technologies, including nanotechnologies, and to provide a forum for an early dialogue with applicants on regulatory, scientific, or other issues that may arise from the development. In the absence of specific guidance, applicants are encouraged to contact the EMA from the early stages of the development of their products.

Environmental Protection

Relevant environmental regulation relates in particular to integrated pollution prevention and control (IPPC), the control of major accident hazards involving dangerous substances (Seveso III), the water framework directive and a number of waste directives.

The IPPC Directive (European Union, 2008b) covers approximately 52,000 industrial installations across the European Union and requires installations falling under its scope to operate in accordance with permits including emission limit values based on the application of best available techniques (BAT). In principle, the IPPC Directive could be used to control environmental impacts of nanomaterials.

The Seveso III Directive (European Union, 2012b) applies to companies where named dangerous substances or substances falling within certain CLP classification categories are present above specific quantities; its definition of "substance" is identical to the REACH definition, thus by analogy covering nanomaterials. It imposes a general obligation on operators to take all measures necessary to prevent major accidents and to limit their consequences for man and the environment. If certain nanomaterials are found to demonstrate a major accident hazard, they may be categorized, together with appropriate thresholds, in the context of the Directive.

The Water Framework Directive (European Union, 2000b) sets common principles and an overall framework for action to improve the aquatic environment and to progressively reduce the pollution from priority substances and phasing out emissions, discharges, and losses of

priority hazardous substances to water. Nanomaterials could be included among the Priority Substances depending on their hazardous properties. Environment Quality Standards would in these cases be proposed by the Commission.

The Waste Framework Directive (European Union, 2006c) on waste sets the general framework and imposes an obligation on EU Member States to ensure that waste treatment does not adversely affect health and the environment. The Hazardous Waste Directive defines which wastes are hazardous and lays down stricter provisions regarding such waste (European Union, 1991). Wastes containing nanomaterials could be classified as hazardous, if the nanomaterial displays relevant properties, which render the waste hazardous.

The legislation addressing environmental areas thus has no specific provisions addressing nanomaterials, which are simply covered in general by these directives and regulations.

KOREA

The legislation addressing safety of chemicals, and thus relevant for nanomaterials, is the following acts: Industrial Safety & Health Act (MOEL), Quality Management & Safety Control of Industrial Products Act (MOTIE), Pharmaceutical Act, Cosmetic Act, and Food Hygiene Act (MFDS), Toxic Chemical Control Act and Chemical Registration and Assessment Act (MOE), and Nanotechnology Promotion Act (MSIP). An amendment to the Nanotechnology promotion act to include and to promote nanosafety was notified in 2013 and is still under review in the Korean National Assembly. An overview of all relevant Korean legislation is given in Table 7.3.

Master Plans on Nanosafety Management

The Korean government has promoted the safety of nanomaterials through master plans. The first 5-year master plan from 2012 to 2016 aimed to establish a foundation of nanosafety and nanosafety management and had five major goals: (1) analytics and measurement technologies of nanomaterial, and database, (2) nanosafety assessment, (3) preparation of nanosafety legislation, and (4) training and partnership establishment. It involved 5 ministries; MSIP (Ministry of Science, ICT and Future Planning), MOTIE (Ministry of Trade, Industry, and Energy), MFDS (Ministry of Food and Drug Safety), MOEL (Ministry of Employment and Labor), and MOE (Ministry of Environment). The second 5-year master plan from 2012 to 2017 aims to institutionalize nanosafety, and based on the policy and research infrastructures developed in the 1st Master Plan at interministerial levels, the Korean government will establish the goal, vision, and implementation plan of the 2nd Master Plan by end 2015. The 3rd 5-year master plan (2022–2026) plans to build market competitiveness through safe nanotechnology commercialization and a first version of the detailed implementation projects have been developed by end 2015.

Nanotechnology Relevant Standard Development

To support the implementation of legislation and to guide industry, Korea has supported standardization of nanotechnology through KATS (Korea Agency for Technology

TABLE 7.3 Regulatory acts relevant to nanotechnology in Korea

Act	Ministry	Purpose	Subjects	Relevance to NT
Industrial Safety & Health Act	MOEL	Protection of worker safety & health	Hazardous factor	High
Quality Management & Safety Control of Industrial Products Act, Framework Act on Product Safety	MOTIE	Protection of consumers and children	Consumer products, Products for children, packaging products	High
Pharmaceutical Act, Cosmetic Act, Food Hygiene Act	MFDS	Safety of Drugs, cosmetics, and food	Drugs, cosmetics, and food	High
Medical devices Act	MFDS	Safety of medical devices	Safety of medical devices	High
Toxic Chemical Control Act	MOE	Protection of citizens from hazardous chemicals	Hazardous Chemicals	High
Chemical Registration and Assessment Act	MOE	Protection of citizens from hazardous chemicals	Hazardous chemicals	High
Environmental Health Act	MOE.	Ensure health of citizens and ecosystem	Environmental pollutants	Medium
Pesticide control act	MOAF	Safety of pesticides	Pesticides	Medium
Laboratory safety Act	MOEd,	Laboratory safety	Hazardous and Dangerous materials	Medium
Nanotechnology Promotion Act	MSIP	Promotion of Nanotechnology	All	High
Maritime safety act	MMAF	Management of maritime safety	Dangerous, toxic chemicals	Low

MOEL, ministry of employment and labor; MOTIE, ministry of trade, industry and energy; MFDS, ministry of food and drug safety; MOE, ministry of environment; MOAF, ministry of agriculture and forestry; MOEd, ministry of education; MSIP, ministry of science, ICT and future planning.

and Standards), which has led the development of two international standards and two other documents in ISO/TC 229 (Nanotechnologies), already published (ISO, webpage). KATS is developing 4 additional documents under ISO/TC 229 (Nanotechnologies) relevant to nanomaterial safety testing: "Aerosol generation for NOAA (Nanoobjects, and their aggregates and agglomerates) air exposure studies" and "Electron spin resonance (ESR) as a method for measuring reactive oxygen species (ROS) generated by metal oxide nanomaterials", "Aquatic Toxicity Assessment of Nanomaterials using *Artemia* sp." and "Materials specification—Antibacterial silver nanoparticles."

Research Activities to Support Safety of Nanomaterials

The Korean legislation will rely on guidelines to ensure the safety of nanomaterials is adequately addressed within the existing legislation, and a number of initiatives have been undertaken regarding the development of such guidelines. The program "Strategy on Nano Convergence Industry Development" was initiated to strengthen research on the safety and social impact of nanomaterials. After completion of "Risk Management Platform Technology for Nanoproducts (2009–2013)", which provided an infrastructure for the certification of nanoproducts based on a risk management system including characterization, efficacy quality, and safety assessment along with standard development, MOTIE launched (2013–2015) "Development of safety evaluation based technology for nanoproduct to promote commercialization", which had 3 parts. Part 1 "Establishment of database for product containing nanomaterials and inventory" included nanomaterial/product safety inventory and safety data sheets for nanomaterials, and developing a process for certification of nanoproduct safety. Part 2 "Nanoproduct safety assessment by case studies" included in vivo and ecotoxicological safety data generation for nanoproducts, data for (1) biopersistence of antimicrobial nanomaterials, and (2) and on effects of ionization on the toxicity of nanoparticles, as well as preparation of a recommendation for a reference dose for products containing nanomaterials. Part 3 "Development of testing methods and standardization of nanomaterials and product containing nanomaterials" included development of (1) analytical chemistry methods for nanoproducts, (2) assessment of exposure to nanoproducts, (3) test methods for nanorelease from nanoproducts, as well as research into effects of ionization on the toxicity of nanoparticles. A follow-up project from 2015–2017 also called "Development of safety evaluation based technology for nanoproduct to promote commercialization" has started.

Since 2005, MFDS and NIFDS (National Institute of Food and Drug Safety Evaluation) have conducted safety studies on manufactured nanomaterials in order to evaluate the safety of manufactured nanomaterials and nanoproducts. Since 2007 NIFDS has run the Nanotoxicology Project, which mainly focuses on providing toxicity data for preparing guidelines to evaluate safety and nano risk management associated with food, drugs, medical devices, and cosmetics using nanomaterials. The Project research encompasses a wide range of nanosafety issues including toxicological evaluation, risk assessment, ADME (absorption, distribution, and metabolism, excretion), kinetics, and physicochemical characterization. The safety of nanomaterials, such as SiO_2, silver, gold, ZnO, and nanocalcium, and so on has been evaluated, based also on own studies. Effects of size, shape, and surface properties of nanomaterials on general toxicity, genotoxicity, immune response, developmental, and reproductive toxicity, Central Nervous System (CNS) uptake mechanism, interaction with biomaterials are being investigated. Since 2015, MFDS and NIFDS have conducted the planning research for nanomedicines, mainly focusing on establishing an evaluation system for nanomedicines for preparing approval and reviews. A validation study on internal absorption assessment methods of organic nanofoods has been conducted to ensure nanofood safety. In addition, the possibility of migration of nanomaterials from food packaging material to foods will be investigated to ensure safety of food products.

JAPAN

Regulatory Framework

The Act on the Evaluation of Chemical Substances and Regulation of their Manufacture is highly relevant to nanomaterials and nanotechnology, and additional legal acts relevant to nanomaterials are addressing food, drugs, medical devices, cosmetics, and pesticides, see Table 7.4. Three ministries, Ministry of Economy, Trade and Industry (METI), Ministry of Health, Labour and Welfare (MHLW), and Ministry of Environment (MOE), are responsible

TABLE 7.4 Regulatory Acts Relevant to Nanotechnology in Japan

Act	Ministry	Purpose	Subject materials	Relevance to NT
Act on the Evaluation of Chemical Substances and Regulation of their Manufacture, etc	METI, MHLW and MOE	To prevent environmental pollution by chemical substances that pose a risk of impairing human health or the ecological system.	Chemical compound created by causing chemical reactions to occur to elements or compounds, excluding radioactive substances and the substances regulated by other Acts[a].	High
Industrial Safety and Health Act	MHLW	Worker protection	Hazardous chemicals in workplace	High
Food Sanitation Act	MHLW	To prevent the sanitation hazards resulting from eating and drinking	Food products, food additives, apparatus, containers and packaging	High
Pharmaceutical and Medical Device Act[b]	MHLW	Safety of Drug and Medical Devices	Drugs, quasi-drugs and medical devices	High
Act on Control of Household Products Containing Harmful Substances	MHLW	To protect the health of citizens by imposing necessary controls on household products containing harmful substances	Hazardous materials	High
Household Goods Quality Labeling Act	METI	To ensure proper labeling of the quality of household goods and to protect the interests of general consumers.	Chemicals contained in the consumer products	High
Agricultural Chemicals Control Act	MAFF	Safety of farmer, consumer and environment	Agricultural Chemicals	Medium

METI, ministry of economy, trade and industry; MHLW, ministry of health, labor and welfare; Ministry of Agriculture, Forestry, and Fisheries; NT: nanotechnology

[a]*Other acts include "Poisonous and Deleterious Substances Control Act", "Stimulants Control Act", "Narcotics and Psychotropics Control Act"*

[b]*Act on Securing Quality, Efficacy and Safety of Pharmaceuticals, Medical Devices, Regenerative and Cellular Therapy Products, Gene Therapy Products, and Cosmetics*

for the Act on the Evaluation of Chemical Substances and Regulation of their Manufacture, which regulates importation, marketing, and usage, and has clauses on premarket registration/notification, although currently not specifically to regulate nanomaterials. The Industrial Safety and Health Act operated by MHLW ensure the safety and health of workers in workplaces handling nanomaterials.

Several guidelines concerning nanomaterials have been developed or are under development. MHLW published a Notification on Precautionary Measures for Prevention of Exposure, and so on to nanomaterials in 2009 (MHLW Japan, 2009) to protect workers at the workplace. In 2012, a committee established by MHLW began consideration of risk assessment for the prevention of impairment of workers' health caused by exposure to TiO_2 at the nanoscale. MOE issued the "Guidelines for preventing the environmental impact of manufactured nanomaterials" to provide manufacturers with currently available information for the environmentally sound management of manufactured nanomaterials, in 2009 (MOE Japan, 2009). MOE has been focusing their efforts on environmental risk of manufactured nanomaterials via understanding of their environmental fate and ecotoxicity focusing at developing methodologies for measurement of manufactured nanomaterials in the environment (i.e., ambient air and surface water). MOE also started a project on measuring nanoscale TiO_2 in a closed system and then in the open air outside of the waste shredders. MOE has also been reviewing and analyzing existing data on ecotoxicity of manufactured nanomaterials, such as TiO_2, silver, and CNTs to identify any harmful effects attributed to their particle size (OECD, 2015).

Nanotechnology Relevant Standard Development

Japan has supports standardization of nanotechnology as one means of enhancing nanosafety and the Japanese Industrial Standards Committee (JISC) is member of ISO/TC229 (Nanotechnologies), and was nominated the Convenor and Secretary of TC229/JWG2 (Measurement and characterization), where it leads revision of ISO/TS 10868 "Nanotechnologies—Characterization of single-wall carbon nanotubes using ultraviolet-visible-near infrared (UV-Vis-NIR) absorption spectroscopy" and a Preliminary Work Item "Application of field flow fractionation for characterization of nanomaterial contents." Furthermore, jointly with ANSI (the American National Standards Institute), Japan leads the Study Group on Particle Size Distribution by Transmission Electron Microscopy, and a Preliminary Work Item "Determination of size and size distribution of nanoobjects by scanning electron microscopy". Japan is also deeply involved in ISO/TC 229 WG3 (Health, Safety, and Environmental Aspects of Nanotechnologies), leading several projects, "ISO 29701: Endotoxin test on nanomaterials", and ISO/TS 19337 "Characteristics of working suspensions of nanoobjects for in vitro assays to evaluate inherent nanoobject toxicity."

Research Activities

In 2011, METI launched a 5-year program for the "Development of Innovative Methodology for Safety Assessment of Industrial Nanomaterials", coordinated by the National Institute of Advanced Industrial Science and Technology (AIST), which aims to develop fundamental hazard assessment methodology leading to a tiered risk assessment approach for industrial

nanomaterials. The program has two R&D themes: (1) Determination of whether the subject nanomaterials are deemed substantially equivalent to nanomaterials previously subject to hazard assessment (Tier 0); if not deemed substantially equivalent to the existing nanomaterials, implementation of intratracheal administration testing as toxicity screening (Tier 1), both of which are for regulatory purposes.

METI also launched a 5-year program "Development of Innovation Carbon Nanotube Composite Materials for a Low Carbon Emission Society" in 2010. One R&D theme of the program was "techniques suitable for voluntary safety management of CNTs by industries", which focused on the development of toxicity testing and exposure assessment protocols for ensuring safety of CNTs and their applications. In mid-2014, this program was converted into a 3-year program "Commercialising Carbon Nanomaterials for a Low Carbon Emission Society." Under one of the R&D themes of this successive program, two documents (only in Japanese) regarding the safety data and management of two types of SWCNTs were released by the AIST (OECD, 2015).

MHLW has promoted research on the human health aspect of several nanomaterials since 2003 through the Health and Labour Sciences Research Grants, and so on. In 2015, a basic research project on development of methods for evaluating chronic health effects and disposition of nanomaterials lead by the National Institute of Health Sciences (NIHS), one of a total of seven research projects, proved carcinogenicity of MWCNT for the first time in the world (Takagi et al., 2008). The Japan Bioassay Research Centre launched a "Research project on the potential hazards, etc. of nanomaterials", commissioned by MHLW, which focuses on carcinogenicity of nanomaterials used/manufactured in the workplace (6-year program, 2009–2015). Under the project, a 2-year inhalation study of MWCNT was completed in 2015, and the final results indicating lung carcinogenicity was reported at the Investigative Committee of Chemical Risk Assessment for the Workplaces, MHLW (in Japanese).

The National Institute of Occupational Safety and Health, Japan (JNIOSH) has conducted two project studies "Toxicological Study on Ultrafine Particles of Metal Oxides (2012–2014)" and "Study on collection and analysis procedures of airborne particulate matters in nanomaterial-handling workplaces (2013–2015)." The National Institute for Environmental Studies (NIES) conducted two consecutive nanotoxicology programs (years 2006–2010 and 2011–2015) which included toxicological studies of MWCNTs and silver nanoparticles, toxicokinetic studies of polystyrene, gold nanoparticles and dendrimers, and ecotoxicological study of TiO_2 nanoparticles (OECD, 2015).

TAKE-HOME MESSAGES

Risk assessment and regulation of nanomaterials has benefited from the continued efforts to address the many research needs to inform the differential responses to nanoscale materials, which allowed the OECD WPMN to conclude "*…that the approaches for the testing and assessment of traditional chemicals are in general appropriate for assessing the safety of nanomaterials…*". Of course, there are still many issues to address in further detail in future research, for example, the actual exposure to nanomaterials, the impact of the environment on the physicochemical properties of nanomaterials, the environmental occurrence of nanomaterials from waste, and the environment (soil and sediment) acting as a sink for (hazardous)

nanomaterials. Regarding human health, the OECD WPMN focus was on especially on inhalatory exposure, given the particulate nature of many nanomaterials, leading to discussion and updating of the OECD test guidelines of inhalation toxicity (OECD, 2012b).

With the increased use of nanomaterials, it is important that the ongoing research is designed to address the risk assessment needs involving manufacture, use, and disposal of nanomaterials. Though the OECD confirmed that the standard chemicals methodology is applicable to nanomaterials, risk assessment of nanomaterials is complicated by limited exposure and hazard information and no clear dose metric; the importance of characterizing the studied materials has been stressed. There are clear data gaps in exposure data and assessment of nanomaterials, particularly moving beyond occupational exposures. The Danish initiative "Better control of nanomaterials" (The Danish nano-initiative, 2015) focused on consumer use of nanomaterials in Denmark, and the areas of likely consumer risks identified were inhalatory exposure. The lack of a dose metric for the analysis of toxic effects of nanomaterials is another large data gap. Once a risk assessment framework for nanomaterials can be designed (see Chapter 8), it will be possible to conclude on the adequacy of the legislative frame for nanomaterials.

Acknowledgments

The authors are grateful to their colleagues in the USA, Korea, Japan, and Europe for their input and useful suggestions, with special thanks to Karin Aschberger.

Disclaimer

The views expressed in this chapter are that of the authors and do not represent the views and/or policies of the US Environmental Protection Agency, the European Commission or any European Agency, or the Korean government, or the Japanese government. The authors are not responsible for any use, which might be made of this text.

References

Amenta, V., Aschberger, K., Arena, M., Bouwmeester, H., Botelho Moniz, F., Brandhoff, P., Gottardo, S., Marvin, H.J.P., Mech, A., Quiros Pesudo, L., Rauscher, H., Schoonjans, R., Vettori, M.V., Weigel, S., Peters, R.J., 2015. Regulatory aspects of nanotechnology in the agri/feed/food sector in EU and non-EU countries. Regul. Toxicol. Pharmacol. 73 (2015), 463–476.

Aschberger, K., Rauscher, H., Crutzen, H., Rasmussen, K., Christensen, F.M., Sokull-Klüttgen, B., Stamm, H., 2014. Information needs for nanomaterials in consumer products. An appraisal and considerations for a labelling and reporting scheme regarding nanomaterials in consumer products in the EU. EUR report 26560.

Davis, J.M., 2007. How to assess risks of nanotechnology: learning from past experience. J. Nanosci. Nanotechnol. 37, 186–191.

EPA, 1976. The Toxic Substances Control Act (TSCA).

EPA, 1990. Clean Air Act Amendments (CAAA).

EPA, 1996. Clean Water Act Amendments (CWAA).

EPA, 2008a. Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA).

EPA, 2008b. Comprehensive Environmental Response, Compensation, and Liability Act and Resource Conservation and Recovery Act (CERCLA/RCRA).

EPA, 2016. Available from: https://www.epa.gov/assessing-and-managing-chemicals-under-tsca/frank-r-lautenberg-chemical-safety-21st-century-act

European Agency for Safety and Health at Work (EU-OSHA), 2009a. Expert forecast on emerging chemical risks related to occupational safety and health. Available from: http://osha.europa.eu/en/publications/reports/TE-3008390ENC_chemical_risks/view

European Agency for Safety and Health at Work (EU-OSHA), 2009b. Workplace exposure to nanoparticles. Available from: https://osha.europa.eu/en/tools-and-publications/publications/literature_reviews/workplace_exposure_to_nanoparticles

European Agency for Safety and Health at Work (EU-OSHA), webpage. Available from: http://osha.europa.eu/en

European Chemicals Agency, 2012a. Guidance on information requirements and chemical safety assessment. Appendix R7-2 Recommendations for nanomaterials applicable to Chapter R7c Endpoint specific guidance. Available from: http://echa.europa.eu/documents/10162/13632/appendix_r7c_nanomaterials_en.pdf

European Chemicals Agency, 2012b. Guidance on information requirements and chemical safety assessment. Recommendations for nanomaterials applicable to Chapter R7b Endpoint specific guidance. Available from: http://echa.europa.eu/documents/10162/13632/appendix_r7b_nanomaterials_en.pdf

European Chemicals Agency, 2012c. Guidance on information requirements and chemical safety assessment. Appendix R7-1 Recommendations for nanomaterials applicable to Chapter R7a Endpoint specific guidance. Available from: http://echa.europa.eu/documents/10162/13632/appendix_r7a_nanomaterials_en.pdf

European Chemicals Agency (ECHA), webpage. Available from: http://ec.europa.eu/echa/, http://echa.europa.eu/support/guidance

European Commission, 2008a. Communication from the Commission to the European Parliament, the Council and the European Economic and Social Committee, Regulatory Aspects of Nanomaterials—Summary of legislation in relation to health, safety and environment aspects of nanomaterials, regulatory research needs and related measures, COM (2008) 366 final of 17.06.2008.

European Commission, 2008b. Commission staff working document, Accompanying document to Communication from the Commission to the European Parliament, the Council and the European Economic and Social Committee, Regulatory Aspects of Nanomaterials, COM (2008) 366 final, SEC(2008) 2036 of 17 6 2008. Available from: http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:52008SC2036&from=EN

European Commission, 2008c. Nanomaterials in REACH (CA/59/2008 rev.1). Available from: http://ec.europa.eu/environment/chemicals/reach/reach_intro.htm

European Commission, 2009. C&L of nanomaterials (CA/90/2009 Rev.2). Available from: http://ec.europa.eu/environment/chemicals/reach/reach_intro.htm

European Commission, 2010. Scientific Committee on Emerging and Newly Identified Health Risks. Scientific Basis for the Definition of the Term "Nanomaterial". Available from: http://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_o_030.pdf

European Commission, 2011. Commission Recommendation of 18 October 2011 on the definition of nanomaterial, 2011/696/EU. Official Journal of the European Union. L275/38-40.

European Commission, 2012a. COM(2012) 572 final. Communication from the Commission to the European Parliament, the Council and the European Economic and Social Committee. Second Regulatory Review on Nanomaterials (Text with EEA relevance) {SWD(2012) 288 final. Available from: http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:52012DC0572&from=EN

European Commission, 2012b. Commission Staff Working paper, SWD(2012) 288 final. Types and uses of nanomaterials, including safety aspects. Accompanying the Communication from the Commission to the European Parliament, the Council and the European Economic and Social Committee on the Second Regulatory Review on Nanomaterials {COM(2012) 572 final}. Available from: http://ec.europa.eu/health/nanotechnology/docs/swd_2012_288_en.pdf

European Commission, 2014a. DG Employment, Social Affairs & Inclusion. Guidance on the protection of the health and safety of workers from the potential risks related to nanomaterials at work. Available from: https://osha.europa.eu/en/oshnews/eu-safe-use-of-nanomaterials-commission-publishes-guidance-for-employers-and-workers

European Commission, 2014b. DG Employment, Social Affairs & Inclusion. Working Safely with Manufactured Nanomaterials. Available from: https://osha.europa.eu/en/oshnews/eu-safe-use-of-nanomaterials-commission-publishes-guidance-for-employers-and-workers

European Commission, 2015. Scientific Committee on Consumer Safety (2015). The SCCS's Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation, 9th Revision. SCCS/1564/15. Available from: http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_190.pdf

European Commission, webpage. Public Health. Scientific committees. Scientific Committee on Consumer Safety (SCCS). DG Health and Food Safety,1; Scientific Committees, opinions of SCCP. Available from: http://ec.europa.eu/health/scientific_committees/consumer_safety/opinions/index_en.htm#page2, http://ec.europa.eu/health/scientific_committees/consumer_safety/index_en.htm

European Food Safety Authority, Scientific Committee, 2011. Guidance on the Risk Assessment of the Application of Nanoscience and Nanotechnologies in the Food and Feed Chain from. Available from: http://www.efsa.europa.eu/en/efsajournal/doc/2140.pdf

European Food Safety Authority, 2016. Annual report of the EFSA Scientific Network of Risk Assessment of Nanotechnologies in Food and Feed for 2015. Available from: http://www.efsa.europa.eu/sites/default/files/scientific_output/files/main_documents/939e.pdf

European Food Safety Authority (EFSA), In Press. Available from: http://www.efsa.europa.eu/

European Parliament, 2009. European Parliament resolution of 24 April 2009 on regulatory aspects of nanomaterials, P6_TA(2009)0328. Available from: http://www.europarl.europa.eu/sides/getDoc.do?pubRef=-//EP//TEXT+TA+P6-TA-2009-0328+0+DOC+XML+V0//EN

European Union, 1989a. Council Directive 89/391/EEC of 12 June 1989 on the introduction of measures to encourage improvements in the safety and health of workers at work. Official Journal of the European Union. L 183.

European Union, 1989b. Council Directive 89/655/EEC of 30 November 1989 concerning the minimum safety and health requirements for the use of work equipment by workers at work (second individual Directive). Official Journal of the European Union. L 393.

European Union, 1989c. Council Directive 89/656/EEC of 30 November 1989 on the minimum health and safety requirements for the use of personal protective equipment at the workplace (third individual Directive). Official Journal of the European Union. L 393.

European Union, 1989d. Council Directive 89/107/EEC of 21 December 1988 on the approximation of the laws of the Member States concerning food additives authorized for use in foodstuffs intended for human consumption. Official Journal of the European Union. L 40.

European Union, 1991. Council Directive 91/689/EEC of 12 December 1991 on hazardous waste. Official Journal of the European Union. L 377.

European Union, 1998a. Council Directive 98/24/EC of 7 April 1998 on the protection of the health and safety of workers from the risks related to chemical agents at work (fourteenth individual Directive within the meaning of Article 16(1) of Directive 89/391/EEC). Official Journal of the European Union. L 131.

European Union, 1998b. Council Directive 98/24/EC of 7 April 1998 on the protection of the health and safety of workers from the risks related to chemical agents at work (fourteenth individual Directive within the meaning of Article 16(1) of Directive 89/391/EEC). Official Journal of the European Union. L 131.

European Union, 2000a. Commission Directive 2000/39/EC of 8 June 2000 establishing a first list of indicative occupational exposure limit in implementation of Council Directive 98/24/EC on the protection of the health and safety of workers from the risks related to chemical agents at work. Official Journal of the European Union. L 142/47–50.

European Union, 2000b). Water Framework Directive 2000/60/EC. EU Official Journal of the European Union. L 327.

European Union, 2002a. Directive 2001/95/EC of the European Parliament and of the Council of 3 December 2001 on general product safety. Official Journal of the European Union. L 11.

European Union, 2002b. Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. Official Journal of the European Union. L 31.

European Union, 2002c. Directive 2002/46/EC of the European Parliament and of the Council of 10 June 2002 on the approximation of the laws of the Member States relating to food supplements. Official Journal of the European Communities L 183/5.

European Union, 2004. Regulation (EC) No 1935/2004 of the European Parliament and of the Council of 27 October 2004 on materials and articles intended to come into contact with food and repealing Directives 80/590/EEC and 89/109/EEC. Official Journal of the European Union. L 338.

European Union, 2006a. Regulation (EC) No 1907/2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals. Official of the European Union, Publications Office of the European Union, L 136/3.

European Union, 2006b. Commission Directive 2006/15/EC of 7 February 2006 establishing a second list of indicative occupational exposure limit in implementation of Council Directive 98/24/EC and amending Directives 91/322/EEC and Directive 2000/39/EC. Official Journal of the European Union. L 38/36–39.

European Union, 2006c. Directive 2006/12/EC of the European Parliament and of the Council of 5 April 2006 on waste. Official Journal of the European Union. L 114.
European Union, 2008a. Regulation (EC) No 1272/2008 on Classification, Labelling and Packaging of substances and mixtures. Official Journal of the European Union. L 353.
European Union, 2008b. Council Directive 2008/1/EC on integrated pollution prevention and control. Official Journal of the European Union. L 24 of 29.1.2008.
European Union, 2009a. Regulation (EC) No. 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products. Official Journal of the European Union. L342/59–209.
European Union, 2009b. Commission Regulation (EC) No. 450/2009 of 29 May 2009 on active and intelligent materials and articles intended to come into contact with food. Official Journal of the European Union. L 135/3–11.
European Union (2009). Regulation 1107/2009 of the European Parliament and the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council directives79/117/EEC and 91/414/EEC. Official Journal of the European Union. L 309/1.
European Union, 2011a. Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004. Official Journal of the European Union. L304/18–63.
European Union, 2011b. Commission Regulation (EU) No 10/2011 of 14 January 2011 on plastic materials and articles intended to come into contact with food. Official Journal of the European Union. L 12/1–89.
European Union, 2012a. Regulation (EU) No 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products. Official Journal of the European Union. L 167, 1–123.
European Union, 2012b. Directive 2012/18/EU of the European Parliament and of the Council of 4 July 2012 on the control of major-accident hazards involving dangerous substances, amending and subsequently repealing Council Directive 96/82/EC. Official Journal of the European Union. L 197/1–37.
European Union, 2015. Regulation (EU) 2015/2283 of the European Parliament and of the Council of 25 November 2015 on novel foods, amending Regulation (EU) No 1169/2011 of the European Parliament and of the Council and repealing Regulation (EC) No 258/97 of the European Parliament and of the Council and Commission Regulation (EC) No 1852/2001. Official Journal of the European Union. L 327/1–22.
FAD, 1938. The Food, Drug and Cosmetic Act (FDCA).
FDA, 2014. FDA Issues Three Final Guidances Related to Nanotechnology Applications in Regulated Products, Including Cosmetics and Food Substances. Available from: http://www.fda.gov/ScienceResearch/SpecialTopics/Nanotechnology/ucm301093.htm
ISO webpage. ISO 10801, Generation of Metal Nanoparticles with the Evaporation/Condensation Method for Inhalation Toxicity Testing, ISO 10808, Characterization of Nanoparticles in Inhalation Exposure Chambers for Inhalation Toxicity Testing, ISO/TR 13329, Nanomaterials—Preparation of MSDS for nanomaterials, ISO/TS 14101, Surface characterization of gold nanoparticles for nanomaterial specific toxicity screening: FT-IR method. Available from: http://www.iso.org/iso/home.html
Lövestam, G., Rauscher, H., Roebben, G., Sokull-Klüttgen, B., Gibson, N., Putaud, J.-P., Stamm, H., 2010. Considerations on a Definition of Nanomaterial for Regulatory Purposes. Publications Office of the European Union, Luxembourg, EUR 24403 EN.
Maynard, A.D., 2011. Don't define nanomaterials. Nature 475, 31.
MHLW Japan (Ministry of Health, Labor and Welfare, Japan), 2009. Notification on Precautionary Measures for Prevention of Exposure etc. to Nanomaterials. Available from: http://www.jniosh.go.jp/en/publication/nanomaterial.html
MOE Japan (Ministry of Environment, Japan), 2009. Guidelines for preventing the environmental impact of manufactured nanomaterials. Available from: https://www.env.go.jp/chemi/nanomaterial/eibs-conf/guideline_0903_enab.pdf
National Nanotechnology Initiative (NNI), 2011. Available from: http://www.nano.gov/sites/default/files/pub_resource/nni_2011_ehs_research_strategy.pdf
National Nanotechnology Initiative (NNI), 2014. Available from: http://www.nano.gov/sites/default/files/pub_resource/2014_nni_ehs_progress_review.pdf

NTP, webpage. Nanotechnology Safety Initiatives. National Toxicology Program, National Institute of Environmental Health Services, Research Triangle Park, NC. Available from: http://ntp.niehs.nih.gov/results/nano/index.html, http://ntp.niehs.nih.gov/results/nano/projects/index.html

OECD, 2010. Guidance Manual for the Testing of Manufactured Nanomaterials: OECD's Sponsorship Programme; first revision. ENV/JM/MONO(2009)20/REV. Available from: http://www.oecd.org/officialdocuments/displaydocumentpdf/?cote=env/jm/mono(2009)20/rev&doclanguage=en

OECD, 2012. Six years of OECD Work on the Safety of Manufactured Nanomaterials: Achievements and Future Opportunities. Available from: http://www.oecd.org/env/ehs/nanosafety/Nano%20Brochure%20Sept%202012%20for%20Website%20%20%282%29.pdf

OECD, 2015. Guidance Manual towards the Integration of Risk Assessment into Life Cycle Assessment of Nano-enabled Applications. ENV/JM/MONO(2015)30. Available at http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono%282015%2930&doclanguage=en.

OECD, 2016. Working Party on Manufactured Nanomaterials. Development in delegations on the safety of manufactured nanomaterials—Tour de Table. ENV/JM/MONO(2016)11. Available from: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono%282016%2911&doclanguage=en

OECD WPMN, webpage. Available from: http://www.oecd.org/chemicalsafety/nanosafety/testing-programme-manufactured-nanomaterials.htm

Organization for Economic Cooperation and Development, 2012a. Guidance on sample preparation and dosimetry for the safety testing of manufactured nanomaterials. ENV/JM/MONO(2012)40. Available from: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO%282012%2940&docLanguage=En

Organization for Economic Cooperation and Development, 2012b. Inhalation Toxicity Testing: Expert Meeting on Potential Revisions to OECD Test Guidelines and Guidance Document. ENV/JM/MONO(2012)14. Available from: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2012)14&doclanguage=en

Organization for Economic Cooperation and Development, 2015. Working Party on Manufactured Nanomaterial (2015). Development in delegations on the safety of manufactured nanomaterials—Tour De Table. ENV/CHEM/NANO(2015)24, OECD, Paris.

Organization for Economic Cooperation and Development. Working Party on Manufactured Nanomaterials. Homepage on internet for the outcomes of the "Testing Programme of Manufactured Nanomaterials". Available from: http://www.oecd.org/chemicalsafety/nanosafety/testing-programme-manufactured-nanomaterials.htm

OSHA, 1970. Occupational Safety and Health Act.

Rasmussen, K., González, M., Kearns, P., Riego Sintes, J., Rossi, F., Sayre, P., 2016. Review of Achievements of the OECD Working Party on Manufactured Nanomaterials' Testing and Assessment Programme. From Exploratory Testing to Test Guidelines. Regul. Toxicol. Pharmacol. 74, 147–160.

Rauscher, H., Roebben, G., Amenta, V., Boix Sanfeliu, A., Calzolai, L., Emons, H., Gaillard, C., Gibson, N., Linsinger, T., Mech, A., Quiros Pesudo, L., Rasmussen, K., Riego Sintes, J., Sokull-Klüttgen, B., Stamm, H., 2014. Towards a review of the EC Recommendation for a definition of the term "nanomaterial" Part 1 Compilation of information concerning the experience with the definition EUR Report 26567 EN.

Rauscher, H., Roebben, G., Boix Sanfeliu, A., Emons, H., Gibson, N., Koeber, R., Linsinger, T., Rasmussen, K., Riego Sintes, J., Sokull-Klüttgen, B., Stamm, H., 2015. Towards a review of the EC Recommendation for a definition of the term "nanomaterial" Part 3 Scientific-technical evaluation of options to clarify the definition and to facilitate its implementation. EUR Report 27240 EN.

Roebben, G., Rauscher, H., Amenta, V., Aschberger, K., Boix Sanfeliu, A., Calzolai, L., Emons, H., Gaillard, C., Gibson, N., Holzwarth, U., Koeber, R., Linsinger, T., Rasmussen, K., Sokull-Klüttgen, B. Stamm, H., 2014. Towards a review of the EC Recommendation for a definition of the term "nanomaterial" Part 2 Assessment of collected information concerning the experience with the definition. EUR Report 26744 EN.

Song, Y., Li, X., Du, X., 2009. Exposure to nanoparticles is related to pleural effusion, pulmonary fibrosis and granuloma. Eur. Respir. J. 34 (3), 559–567.

Takagi, et al., 2008. Induction of mesothelioma in p53+/− mouse by intraperitoneal application of multi-wall carbon nanotube. J. Toxicol. Sci. 33, 105–116.

The Danish nano-initiative, report "Better control of nanomaterials and their safety", 2015. Available from: http://www2.mst.dk/Udgiv/publications/2015/12/978-87-93352-89-6.pdf

US EPA, 2011. Nanomaterial case study: Nanoscale titanium dioxide in water treatment and topical sunscreen (final). 2011. Available from: http://cfpub.epa.gov/ncea/cfm/recordisplay.cfm?deid=230972

US EPA, 2012. Nanomaterial Case Study: Nanoscale Silver in Disinfectant Spray (Final Report). 2012. Available from: http://cfpub.epa.gov/ncea/risk/recordisplay.cfm?deid=241665

USA Federal Register (2016). Draft Current Intelligence Bulletin: Health Effects of Occupational Exposure to Silver Nanomaterials. Available from: https://www.federalregister.gov/articles/2016/02/10/2016-02647/draft-current-intelligence-bulletin-health-effects-of-occupational-exposure-to-silver-nanomaterials

Wu, M., Gordon, R.E., Herbert, R., Padilla, M., Moline, J., Mendelson, D., Litle, V., Travis, W.D., Gil, J., 2010. Environ. Health Perspect 118 (4), 499–504.

CHAPTER

8

Risk Assessment and Risk Management

*Robert Landsiedel**, Ursula G. Sauer**, Wim H. de Jong*†

***BASF SE, Experimental Toxicology and Ecology, Ludwigshafen am Rhein, Germany; **Scientific Consultancy - Animal Welfare, Neubiberg, Germany; †National Institute for Public Health and the Environment, Bilthoven, The Netherlands**

OUTLINE

Introduction 190

Consideration of Exposure for Nanomaterial Risk Assessment 191

Estimation of Nanomaterial Organ Burden at the Primary Site of Contact 192

Estimation of Nanomaterial Systemic Exposure 193

Nanomaterial Hazard Assessment by Toxicological Properties 194

Nanomaterial Hazard Assessment by Local Toxicity 194

Nanomaterial Hazard Assessment by Short-Term Toxicity 195

Nanomaterial Hazard Assessment by Carcinogenesis 198

Nanomaterial Hazard Assessment by Reproductive and Developmental Toxicity 201

Grouping of Nanomaterials to Streamline Hazard and Risk Assessment 201

Consideration of Cellular and Apical Effects for Nanomaterial Grouping 206

Nanomaterial Risk Assessment and Risk Management 208

Conclusions 212

Take-Home Messages 212

References 212

Adverse Effects of Engineered Nanomaterials. http://dx.doi.org/10.1016/B978-0-12-809199-9.00008-2
Copyright © 2017 Elsevier Inc. All rights reserved.

INTRODUCTION

Risk assessment implies the evaluation of the likelihood and severity of the occurrence of harmful effects on humans, animals, or the environment. The traditional risk-assessment paradigm consists of four specific steps that serve to elucidate the following questions: (1) Exposure assessment: Does exposure to the substance under evaluation occur and, if so, to which extent? Discretionarily, the internal organ exposure after substance uptake into the body is assessed. (2) Hazard identification: Does the substance elicit a toxic effect, and, if so, which effect? (3) Hazard characterization: What is the dose-response, that is, the relationship between the administered dose and the toxic effect? (4) Risk characterization: How does the potential hazardous dose relate to the actual exposure? This traditional risk assessment considers a spectrum of possible toxicological endpoints, and the most sensitive one is selected for the hazard characterization, which, in turn, defines the point of departure for the risk assessment (Meek et al., 2011). It is expected that risk assessment will increasingly shift from a hazard-driven to an exposure-driven process (SCHER, SCENIHR, SCCS, 2013), since the absence of exposure translates into an absence of risk. However, exposure determination is generally one of the more difficult and complex processes involved in risk assessment, and this is even more so true for nanomaterials. By comparing the given exposure to a substance to its dose that initiates a toxic insult (and further applying several uncertainty factors for intra- and interspecies variation and extrapolation), an overall estimation of the risk can be made. During the final stage of the risk assessment, that is, during risk characterization, the overall risk for harm that may evolve by using the given substance in occupational settings or consumer products is assessed. Generally, this risk is a combination of the extent of the exposure and the severity of the harm. Accordingly, a low risk can be present if a very low (negligible) exposure coincides with a very serious adverse effect (e.g., death), as well as if a very high exposure coincides with a relatively innocent adverse effect (e.g., temporary skin rashes or itching). Based on the final risk assessment, risk managers decide whether the risk is acceptable for the intended uses of the substance. If the risk is concluded to be unacceptable, risk management is performed to reduce it to an acceptable level.

While ongoing initiatives aim at modernizing the traditional risk-assessment paradigm (Dix et al., 2007; Krewski et al., 2009; Rovida et al., 2015; Rudén and Hansson, 2010; Tice et al., 2013), it has been and is still being widely used for the risk assessment of substances. In the European Union, the legal provisions for the risk assessment of chemicals have been implemented in *Regulation (EC) No. 1907/2006 on the Registration, Evaluation, Authorisation, and Restriction of Chemicals (REACH)*. Just as nanomaterials are covered by the REACH regulation (see Chapter 7), also the traditional risk-assessment paradigm is generally applicable to nanomaterials although some modifications that take into account the specific characteristics of nanomaterials may be necessary (Anzai et al., 2012; ECHA, 2012; EFSA, 2009; Hankin et al., 2011; Landsiedel, 2015; OECD, 2012a; SCENIHR, 2006, 2007, 2009; Wohlleben et al., 2013a). For several product categories (e.g., food and feed, cosmetics, and medical devices), guidance documents on nanomaterial risk assessment have been published (EFSA, 2011; SCCS, 2012; SCENIHR, 2015). The physicochemical characteristics and the particulate nature of nanomaterials pose a challenge for their application in various toxicological assays that may need to be modified to accommodate nanomaterial testing. In view of their possible variations, nanomaterials have to be characterized and identified in the test system during hazard identification (SCENIHR, 2009) to demonstrate that the nanomaterial used in a given product is indeed the

same as the one that is evaluated for safety. An abundance of published studies address the potential human health hazards of nanomaterials, and these studies cover the spectrum of toxicological properties laid down in the International Uniform Chemical Information Database (IUCLID; www.iuclid.eu) that is dedicated to the REACH information requirements.

However, application of the full REACH information requirements for every single variant of a given nanomaterial regarding particle size, shape, or surface properties (Stark et al., 2015) would lead to an insurmountable amount of testing. This would further stand in contradiction to the legal requirement to replace, reduce, and refine animal testing (3Rs principle; Russell and Burch, 1959) that has been implemented in *EU Directive 2010/63/EU on the protection of animals used for scientific purposes*. At the same time, the development of risk-assessment procedures for nanomaterials offers the opportunity to apply modern concepts, which are evolving for the general risk assessment of substances. Modern concepts provide flexible frameworks for identifying and collecting the data that truly serve to ensure substance safety. In this respect, the alignment of information needs to realistic exposure scenarios has been suggested as an important means to improve the risk assessment for nanomaterials (Bos et al., 2015; Landsiedel, 2015; Oomen et al., 2014a, 2015). Part of the information for the risk assessment can be obtained in in vitro high or medium throughput systems that allow for the evaluation of large series of nanomaterials (Nel et al., 2013b) and by applying integrated testing strategies (Nel et al., 2013a; Oomen et al., 2014a). Furthermore, the *grouping* of substances is widely recognized as a potentially powerful tool to streamline the collection of data for regulatory hazard and risk assessment. General grouping approaches for substances, regardless of their physical form, have been implemented in the REACH Regulation. The European Chemicals Agency (ECHA, 2013) describes grouping as the process of uniting substances into a common group *if they are structurally similar with physico-chemical, toxicological, ecotoxicological and/or environmental fate properties that are likely to be similar or to follow a regular pattern*. Within a group, each individual substance may not need to be tested. Endpoint-specific effects of an unknown substance may be derived from the endpoint-specific effects of further substances within the group. This grouping concept is generally applicable to nanomaterials; merely the prerequisite of "structural similarity" may need to be adapted to appropriate descriptors of nanomaterials.

Against this background, the following section of this chapter discusses exposure assessment as the first step of nanomaterial risk assessment. Then, we present an overview of available toxicological studies sorted by different toxicological properties as they are in the REACH information requirements. Based on this overview, the next section elucidates how the grouping of nanomaterials may be applied to streamline testing for nanomaterial risk assessment, and we then provide further details on how in vitro and in vivo data may be used for nanomaterial grouping. The final section discusses how relevant information on nanomaterial exposure and hazard may be evaluated for risk assessment and risk management.

CONSIDERATION OF EXPOSURE FOR NANOMATERIAL RISK ASSESSMENT

The likelihood of the occurrence of adverse effects on humans, animals, or the environment is primarily dependent on the extent of exposure to the given substance. Obviously, when there is no exposure, there is no risk for harmful effects. However, the likelihood of exposure may change during different life cycle stages of a nanomaterial or nanomaterial-containing

product, that is, during production, use, or waste disposal at the end of the life cycle. Two different aspects of exposure may be considered for risk assessment, that is, the external and the internal exposure. The external exposure addresses, for example, the concentration of a nanomaterial in the environment (or microenvironment, such as the breathing air) or its intake dose (such as the concentration present in food). The internal exposure relates to the concentration of the nanomaterial in different organs, that is, the organ burden at the primary site of contact, as well as in secondary organs provided that the nanomaterial is taken up into the body. Accordingly, the internal exposure is governed by the nanomaterial's biopersistence, systemic uptake, and biodistribution, that is, biokinetics or toxicokinetics (Braakhuis et al., 2015a; Burden et al., 2016; Kuempel et al., 2015; Oomen et al., 2014a,b).

For risk assessment, the internal exposure to a nanomaterial is most relevant in view of the dose present in a given organ over time and its relationship to the induction of toxic effects. Toxicokinetics may also indicate potential target organs for toxicity. Specifically, the biopersistence of a nanomaterial may affect its retention and clearance (and hence organ burden) at the primary site of contact, as well as systemic uptake from the primary site of contact and biodistribution (Oberdörster et al., 1994). In vivo, nanomaterials may be nondegradable, nonsoluble, or poorly soluble (i.e., biopersistent) or of moderate or high solubility. Specific particle- or fiber-like toxicity is largely expected for biopersistent particles, whereas the toxicity of soluble nanomaterials is dominated by the dissolved ions, and the potential of soluble nanomaterials to reach target organs is the result of the biodistribution of the dissolved ions and the particulate nanomaterials before dissolution occurs (Mercer, 1967).

Estimation of Nanomaterial Organ Burden at the Primary Site of Contact

Determination of the lung burden of inhaled nanoparticles over time is especially relevant to determine whether pulmonary particle overload is likely, which may lead to lung cell damage and systemic uptake (Cassee et al., 2015; Kuempel et al., 2014). Depending upon their size, nanomaterials may reach the alveolar regions of the lung and are deposited there (Elder and Oberdörster, 2006). Generally, the alveolar macrophages sequester inhaled nanoparticles, and lung cell damage is only prone to evolve if the macrophages' clearance capacity is overwhelmed (Morrow, 1988). Under overload conditions, nanomaterial contact with the epithelium is likely, resulting in inflammatory reactions and translocation of particles to the interstitium and lung-associated lymph nodes (Elder and Oberdörster, 2006). Particle size (and, accordingly, surface area) also appears to affect pulmonary retention. In a rat short-term inhalation study (STIS) assessing silver nanomaterials of different primary particle sizes (PPSs: 18, 34, 60, and 160 nm, respectively), the various pulmonary effects correlated well with the particle surface area-expressed dose (Braakhuis et al., 2015b). Upon 12-week inhalation exposure of rats to 20- or 250-nm anatase TiO_2, 20-nm TiO_2 was cleared from the lungs much more slowly than its larger counterpart, and a significantly larger fraction of the nano-sized material was transferred to the lung-associated lymph nodes (Oberdörster et al., 1994).

Also nanomaterial agglomeration, either in the aerosol or upon contact with protein- and lipid-containing lung lining fluid, will affect lung burden, and lipophilic nanomaterials appear to exhibit a higher lung burden than hydrophilic counterparts (Landsiedel et al., 2014a,b; Sauer et al., 2014; Wohlleben et al., 2016). Cumulative lung burden appears dependent upon the size of agglomerated particles and the likelihood of their disintegration. Agglomerates

that do not disintegrate in the lung are unlikely to become systemically available (Konduru et al., 2014; Landsiedel et al., 2014a). Similarly, a nanomaterial's dissolution rate in the lung will affect lung burden (Mercer, 1967). Overall, lung burden in rats upon short-term inhalation exposure is low (Morfeld et al., 2012), ranging from approximately 1% for surface-functionalized ZrO_2 to approximately 15% for ZnO and CeO_2 nanomaterials immediately after 5 days of exposure (Landsiedel et al., 2014a). Upon intratracheal instillation in mice, ^{14}C-labeled few-layer graphene (0.1 mg/mL; 50 µL) was mainly retained in the lung with 47% of the administered dose remaining 4-weeks postexposure (Mao et al., 2016).

Nanomaterial deposition and retention in the lung may affect the evolvement of apical effects, that is, the observable outcome of (nano-)material exposure to test animals (OECD, 2012b). Upon inhalation exposure to CeO_2 NM-211, the dose rate of CeO_2 deposition drove an initial neutrophil-dominated inflammatory reaction (Keller et al., 2014). During 4 weeks of exposure, cell counts shifted to a macrophage-dominated inflammation that progressed toward a granulomatous reaction depending on the duration and amount of particles retained in the lung (Keller et al., 2014; Pauluhn, 2014).

Estimation of Nanomaterial Systemic Exposure

Typically, systemic uptake of nanomaterials upon inhalation lies in ranges below 1% of the (already low) dose retained in the lung, and it is most likely to occur under high-dose conditions. Nevertheless, translocation of small fractions of iridium, carbon, and ceria nanoparticles has also been observed at low concentrations (Geraets et al., 2012; Kreyling et al., 2009). Nanomaterial translocation appears to depend on chemistry, surface area or shape, and the duration of exposure (Elder and Oberdörster, 2006; Oberdörster et al., 1994; Yokel et al., 2014). Generally, a relevant different translocation rate between nanomaterials and their bulk counterparts has not been observed. If taken up systemically, nanoparticles are prone to lymphatic transport via the mononuclear phagocytic system (MPS), but they may also be translocated in the blood (Albanese et al., 2012; Landsiedel et al., 2012b; Mao et al., 2016). By contrast, absorption via the olfactory system, if it occurs, does not seem to be specific for the nanosized variants of a material (Arts et al., 2015, 2016). Just as lung burden, also secondary organ burden is dependent upon nanomaterial transport to and clearance from the respective organs. Primarily, nanomaterial organ uptake does not appear to be based on concentration-driven diffusion, but it is most likely based on an active uptake by phagocytic cells of the MPS. This is indicated by high nanomaterial uptake into organs of the MPS, such as liver and spleen that act as a depot for nanomaterials, as was observed upon intravenous application to rats resulting in immediate systemic availability of the entire dose (De Jong et al., 2008; Fabian et al., 2008; Geraets et al., 2014). Similarly, decrease in organ concentrations may not be diffusion-driven resulting in potentially long organ biopersistence of nonsoluble nanomaterials, such as TiO_2 (Geraets et al., 2014).

Also the risk assessment of nanomaterials with likely dermal or gastrointestinal exposure should address their potential to become systemically available. Industrially used nanomaterials, just as nanomaterials that are applied as sunscreens in cosmetic products, may penetrate the corneal layers of the skin (Butz et al., 2007). However, as a rule, they neither penetrate the intact porcine skin (Butz et al., 2007; Gamer et al., 2006), nor sun-burnt porcine skin. As a result, they are not taken up into living tissues (Monteiro-Riviere et al., 2011). Only very small

nanoparticles, such as carboxyl quantum dots, were found to penetrate the barrier-compromised (UV-radiated) skin of SKH-1 mice in vivo, thereby reaching the lower epidermal layers and the dermis (Mortensen et al., 2008). Similarly, in an in vitro flow-through diffusion cell culture system, quantum dots coated with carboxylic acid penetrated into the viable dermal layers of abraded rat skin after both 8- and 24-h incubation (Zhang and Monteiro-Riviere, 2008). It has been discussed that specifically designed nanoparticles may constitute efficient carriers for drug delivery by enhancing the penetration and storage of pharmacological compounds in hair follicles (Lademann et al., 2007). Accordingly, specific nanoparticles and formulations have been developed for medicinal purposes that promote the dermal penetration and absorption of nanoparticles (Küchler et al., 2009; Prabhakar et al., 2013; Schäfer-Korting and Romero, 2016).

Seven days after a single oral application of 5 mg/kg body weight (bw) $^{131}BaSO_4$ nanomaterials, only 0.15% of the dose was detected in all organs (mainly blood, bone, and bone marrow). Two hours after intravenous injection (1 mg/kg bw), this $^{131}BaSO_4$ was predominantly localized in MPS organs with access to the circulation, that is, the liver, spleen, and bone and bone marrow. Within the 7-day postexposure period, injected $^{131}BaSO_4$ was found to redistribute from the liver to lungs, bone and bone marrow. For either route of exposure, fecal excretion was the dominant elimination pathway (Konduru et al., 2014). Similarly, 7 days after oral administration of 5 mg/kg bw $^{141}CeO_2$ nanomaterials to rats, nearly 100% of the material was excreted in the feces, and intravenously applied $^{141}CeO_2$ nanomaterials were predominantly retained in the liver, bone, and spleen (Molina et al., 2014). Upon oral administration of ^{14}C-labeled few-layer graphene (100 µL; 0.1 g/mL) to mice, gastrointestinal absorption was undetectable (Mao et al., 2016).

Very low levels of silver were taken up systemically upon 28-day oral administration of 90 mg/kg bw/day silver nanomaterials (PPS: 20 nm, uncoated; and 15 nm, PVP-coated) to rats. The absorbed silver appeared mainly to account from dissolved ions, and it was eliminated rapidly (Van Der Zande et al., 2012). Upon 90-day oral administration of pyrogenic SiO_2 nanomaterials (PPS: 7 nm) to rats, total silica content was increased in the spleen (Van Der Zande et al., 2014). Even though organ burden was not analyzed, the lack of effects upon 28-day oral exposure to 1000 mg/kg bw/day of four amorphous SiO_2 with or without surface functionalization, two surface-functionalized ZrO_2, or $BaSO_4$ (Buesen et al., 2014) was assessed as a further indication of the low systemic availability of orally administered nanomaterials.

NANOMATERIAL HAZARD ASSESSMENT BY TOXICOLOGICAL PROPERTIES

Nanomaterial Hazard Assessment by Local Toxicity

Generally, the (few) available in vitro and in vivo studies assessing nanomaterial local toxicity point to a low eye or skin-irritating potential of a broad spectrum of nanomaterials. Kolle et al. (2016) evaluated 20 nanosized and 3 nonnanosized materials in a two-tier testing strategy combining the EpiOcular eye-irritation test [performed in accordance with the Organization for Economic Co-operation and Development (OECD) test guideline (TG) 492] and the

Bovine Corneal Opacity and Permeability (BCOP; OECD TG 437). None of the dry-powder test items [covering different TiO_2, ZnO, CeO_2, SiO_2 nanomaterials, multiwalled carbon nanotubes (MWCNTs), and organic pigments] revealed eye-irritating potential, which was consistent with available in vivo data (Kolle et al., 2016). Similarly, two different MWCNTs did not elicit eye or skin irritation in vivo, nor in vitro in the hen's egg—chorio-allantoic membrane test or in in vitro skin-irritation studies (Kishore et al., 2009). However, a silver nanomaterial supplied as dispersion (PPS: <20 nm) was tested positive in the EpiOcular eye-irritation test, and it produced highly variable results in the BCOP assay with dark-brown patches remaining on the corneal surface (Kolle et al., 2016). As reviewed by Monteiro-Riviere (2016), carbon-coated silver nanomaterials (PPSs: 25 and 35 nm) or uncoated silver nanomaterials (PPSs: 20, 50, and 80 nm) did not elicit skin irritation upon 14-day topical application onto the backs of weanling pigs, and the test materials were only observed in the superficial layers of the stratum corneum (Samberg et al., 2010). Similarly, 4-day application of pristine hydrophobic C_{60} fullerenes did not elicit local skin toxicity in pigs (Xia et al., 2010). Chronic human exposure to silver has been reported to elicit permanent bluish-gray discoloration of the eyes (Wijnhoven et al., 2009). Local toxicity for particles including nanomaterials is most prominent in the lung (see below).

Nanomaterial Hazard Assessment by Short-Term Toxicity

Inhalation is the predominant route of exposure for most nanomaterials as was recognized already quite early when assessing the role of ultrafine (nano-)particles in ambient air pollution (Donaldson et al., 2005; Klein et al., 2012; Landsiedel et al., 2012a, 2014a; Oberdörster et al., 2005). Accordingly, the majority of available studies investigating nanomaterial hazard potential addresses the inhalation route of exposure. For regulatory purposes, substance-induced effects upon subacute or subchronic inhalation exposure are assessed according to OECD TG 412 and 413. By contrast, the acute inhalation toxicity of nanomaterials in accordance with OECD TG 403 is scarcely investigated and may indeed provide very little useful information for nanomaterial risk assessment (Landsiedel et al., 2014b). With the rat STIS, a reduction and refinement alternative to repeated-dose subacute inhalation studies for nanomaterial hazard assessment has been developed. The STIS is essentially an adaptation of the OECD TG 412 *Subacute inhalation toxicity: 28-day study*. The STIS (short-term inhalation study) protocol foresees 5 days of exposure (6 h/day) and a mandatory postexposure observation (or recovery) period of up to 13 weeks. It includes appropriate aerosol generation and characterization, bronchoalveolar lavage parameters and lung burden assessments (Arts et al., 2007; Landsiedel et al., 2014a; Ma-Hock et al., 2007, 2009a). The STIS allows determining the nanomaterials' potential to elicit local effects in the respiratory tract, that is, the primary site of contact, and it provides information on the test materials' toxic potency, and the location and reversibility of effects. Moreover, assessment of lung burden and material translocation to extrapulmonary tissues provide preliminary biokinetic information (Landsiedel et al., 2014a). Table 8.1 summarizes rat STIS data for a broad spectrum of nanomaterials (and two nonnanosized materials) that were published by Arts et al. (2007), Ma-Hock et al. (2009a,b, 2013), Bellmann (2011), Treumann et al. (2013), Landsiedel et al. (2014a), Keller et al. (2014), Gosens et al. (2015), and Hofmann et al. (2016). The test materials encompass carbonaceous nanomaterials, metal oxide and metal sulfate nanomaterials, amorphous silica nanomaterials (without and

TABLE 8.1 Nanomaterial Assignment to Main Groups Applying the DF4nanoGrouping Nonanimal Tiers 1 and 2 and Tier 3 STIS Biopersistence or Toxic Potency (NOAEC), Respectively

Test material	Tier 1 and 2 MG assignment	Tier 1 and 2: decisive grouping criterion	Tier 3 STIS NOAEC (mg/m^3)	Tier 3 STIS biopersistence	Tier 3 MG assignment	Reversibility or progression of effects	Subgrouping by NOAEC range	References
ZnO NM-110	MG1 "soluble NMs"	Dissolution in biological media	<8[a]	Rapid clearance	Solubility confirmed	Full reversibility	[Solubility does not per se relate to toxic potency]	1
ZnO NM-111			0.5	Rapid clearance		Partial reversibility		2
10 nm-CuO			0.6	N/A	N/A	Full reversibility		3
SiO_2 NM-200		Dissolution in biological media; water solubility: borderline	1[b]	At 1 and 5 mg/m^3: Below detection limit at all times; at 25 mg/m^3: Full clearance	Solubility supported	Partial reversibility		4
SiO_2 NM-203			1[b]			Partial reversibility		4
MWCNT NM-400	MG2 "biopersistent HAR NMs"	Particle morphology (HAR)	<0.5	d.n.p.	Biopersistence	Progression	[Range I or II]	5
MWCNT NM-402			<0.5					6
LS carbon black	MG 3 "passive NMs"	*All* Tier 1 and Tier 2 grouping criteria indicate "passivity"	≥10	d.n.p.	MG3 "passive NMs"	No adverse effects	[Range IV = MG3]	6
$BaSO_4$ NM-220			≥50	t_{50} <40 days				2[c]
15 nm-Fe_2O_3			≥30	t_{50} <40 days				7
SiO_2.amino			≥50	t_{50} <40 days				2
SiO_2.PEG			≥50	t_{50} <40 days				2
DPP orange (bulk)			≥10	Not decisive for grouping				7
DPP orange (nano)			≥30					7
Pigment red 254-2			≥30					7

SiO_2.phosphate	MG 4 "active NMs"	Dispersibility	≥50	t_{50} <40 days		No adverse effects		2
Pigment blue 15:1		Cellular effects	≥30	N/A				7
Graph. nanoplatelets		Surface reactivity: d.n.p.	≥10	d.n.p.				6
$aSiO_2$-susp		Cellular effects	2.5	N/A	MG4 "active NMs"	Progression	Range III	2
Graphene		Surface reactivity: d.n.p.	<2.5	d.n.p.		Persistence of effects		6
TiO_2 NM-105		Cellular effects	<2	t_{50} >40 days		Partial reversibility		8
SiO_2.acrylate		Dispersibility	0.5 (splenic effects) pulmonary effects: ≥10	t_{50} >40 days		Spleen: full reversibility; no pulmonary effects	Range II	2
CeO_2 NM-211		Cellular effects	<0.5	t_{50} >40 days		Progression		9
CeO_2 NM-212		Cellular effects	<0.5	t_{50} >40 days		Progression		9
Quartz dust DQ12		Surface reactivity and cellular effects	0.1	t_{50} >40 days		Progression	Range I or II	10[d]

d.n.p., Determination not possible for technical reasons; HAR, high aspect ratio; LS, low surface; MG, main group; N/A, not available; NOAEC, no-observed adverse effect concentration; STIS, short-term inhalation study.

STIS data were retrieved from the following sources: (1) Bellmann (2011); (2) Landsiedel et al. (2014a); (3) Gosens et al. (2015); (4) Arts et al. (2007); (5) Ma-Hock et al. (2009b); (6) Ma-Hock et al. (2013) and Treumann et al. (2013); (7) Hofmann et al. (2016); (8) Ma-Hock et al. (2009a); (9) Keller et al. (2014); (10) Henderson et al. (1995).

The STIS NOAEC ranges correspond to: Range I: <0.1 mg/m^3; Range II: <1 mg/m^3; Range III: <10 mg/m^3; Range IV: ≥10 mg/m^3.

In Tier 2, SiO_2.phosphate was assigned to MG 4 on account of its high dispersibility, Pigment blue 15:1 was assigned to MG4 on account of its activity in the in vitro alveolar macrophage assay, and graphite nanoplatelets were assigned to MG4 since determination of surface reactivity was not possible for technical reasons. In Tier 3, high STIS NOAEC (Range IV) are recorded for all three substances indicating MG3 "passivity."

[a]*Fourteen-day exposure, only one test substance concentration (i.e., 8 mg/m^3).*

[b]*For equivalent substance.*

[c]*Furthermore, there are strong indications that $BaSO_4$ is at least partially soluble in vivo after inhalation (Konduru et al., 2014).*

Adapted from Arts, J.H., Hadi, M., Irfan, M.A., Keene, A.M., Kreiling, R., Lyon, D., Maier, M., Michel, K., Petry, T., Sauer, U.G., Warheit, D., Wiench, K., Wohlleben, W., Landsiedel, R., 2015a. A decision-making framework for the grouping and testing of nanomaterials (DF4nanoGrouping). Regul. Toxicol. Pharmacol. 71 (Suppl. 2), S1–S27.

with surface functionalization), nonnanosized and nanosized organic pigments, and nonnanosized crystalline quartz dust. Additionally, Ma-Hock et al. (2012) investigated a further type of organic nanomaterial in the rat STIS, that is, acrylic ester copolymers containing different fractions of nanoparticles, and did not observe adverse effects up to aerosol concentrations of 10 mg/m^3. Low no-observed effect concentrations (NOAECs) of 0.5 mg/m^3 or lower, based upon local, that is, respiratory tract effects, were recorded for ZnO NM-111, MWCNT NM-400 and NM-402, CeO_2 NM-211 and NM-212, and nonnanosized quartz dust DQ12. Apart from ZnO NM-111, for which effects were fully reversible within the 3-week postexposure period, the effects observed for these materials were progressive. Of note, throughout this chapter, all NM-xxx numberings relate to the well-known and widely used codes of representative nanomaterials of the OECD sponsorship program for the testing of nanomaterials (OECD, 2010).

Nanomaterials with high biopersistence in the lung (i.e., pulmonary half-life >40 days) were TiO_2 NM-105, SiO_2.acrylate, CeO_2 NM-211 and NM-212, and nonnanosized quartz dust DQ12. Of the spectrum of nanomaterials assessed in the STIS, the only nanomaterial eliciting alterations outside the respiratory tract was an amorphous SiO_2 with acrylate surface functionalization: For this material, splenic enlargement was observed that was fully reversible within 2-weeks postexposure. None of the nanomaterials caused extrapulmonary effects outside the MPS. Effects on the MPS should be considered seriously since mononuclear phagocytes are the central cells of the immune system and play a key role in the defense against foreign organisms and lung immune homeostasis (Dobrovolskaia et al., 2008; Lucarelli et al., 2004; Mohamud et al., 2014). In a recent risk assessment of systemic, intravenous exposure to silver nanoparticles, immunotoxicological effects were considered the most relevant for the derivation of a tolerable intake level (Vinas et al., 2015). Intravenous systemic exposure to silver nanomaterials induced an increase in spleen weight due to test material accumulation and impairment of immune responses (De Jong et al., 2013; Vandebriel et al., 2014).

By far fewer studies have addressed nanomaterial hazard potential upon oral exposure than upon inhalation exposure. Nanomaterials reaching the gastrointestinal tract are mostly excreted with the feces and generally do not elicit local effects in the gastrointestinal tract, nor effects in secondary organs (Geraets et al., 2014; Yokel et al., 2014). Systemic uptake from the gastrointestinal tract is very low (<1%). In 28-day rat oral toxicity studies performed according to OECD TG 407, 1000 mg/kg bw/day of four amorphous SiO_2 with or without surface functionalization, two surface-functionalized ZrO_2, or $BaSO_4$ NM-220 did not elicit local or systemic effects (Buesen et al., 2014). Upon 90-day oral administration, pyrogenic SiO_2 NM-202 (PPS: 15–25 nm) significantly increased the occurrence of liver fibrosis that, however, was assessed as mild and was not accompanied by altered blood chemistry parameters (Van Der Zande et al., 2014). In a 28-day rat oral toxicity study, 300 and 1000 mg/kg bw silver nanomaterials (average PPS: 60 nm) induced alterations in the blood parameters alkaline phosphatase and cholesterol indicating some liver damage, and silver could be detected in most organs evaluated (Kim et al., 2008). The latter might be due to either nanoparticle or silver ion uptake.

Nanomaterial Hazard Assessment by Carcinogenesis

As is also known for bulk substances, repeated exposure to nanomaterials may lead to tissue accumulation if exposure levels and systemic availability are sufficiently high

(Gebel et al., 2014; Moreno-Horn and Gebel, 2014; Oomen et al., 2014a; Yokel et al., 2014). However, data are sparse whether inhaled nanomaterials may accumulate to an extent that chronic adverse effects may evolve, just as there are limited data on the chronic effects of inhaled nanomaterials (Becker et al., 2011; Gebel et al., 2014; Moreno-Horn and Gebel, 2014). A 2-year combined chronic toxicity–carcinogenicity study performed according to OECD TG 453 assessing $BaSO_4$ NM-220 and CeO_2 NM-212 is expected to be completed in 2016 (Gebel and Landsiedel, 2013; Ma-Hock et al., 2014). Earlier, lung carcinogenicity was shown in the rat upon long-term exposure to high dosages of carbon black (Elder et al., 2005; Nikula et al., 1995) and TiO_2 (Bermudez et al., 2004; Heinrich et al., 1995). By contrast, none of the epidemiological studies examining workers exposed to nanosized carbon black or TiO_2 provide evidence of carcinogenicity in humans (IARC, 2010; MAK, 2013). Carbon black and TiO_2, are examples for "granular biodurable particles without specific toxicity" (GBPs; Gebel, 2012), also referred to as "poorly-soluble low toxicity particles" (PSPs; Kuempel et al., 2012, 2014). Lung tumor formation in rats upon long-term exposure to high levels of GBPs is thought to be caused by reduced particle clearance involving chronic inflammation associated with oxidative stress, secondary genotoxicity, and cell proliferation (Greim and Ziegler-Skylakakis, 2007). It remains unclear why rats develop lung tumors upon long-term exposure to high levels of GBPs, whereas some other animal species (including humans) do not as is indicated by an absence of increased tumor risk of dust-exposed workers (IARC, 2010; MAK, 2013; Morfeld et al., 2015). Based upon an adverse-outcome pathway (AOP; Ankley et al., 2010; OECD, 2012b) approach published by ECETOC (2013), Morfeld et al. (2015) elaborate on the cascade of GBP-initiated cellular and molecular events that lead to pretumor conditions in the lung. In step 1, inflammation-promoting mediators are produced; step 2 involves an increased production of anti-inflammatory mediators; step 3, injury repair; and step 4, intermediate endpoints including DNA mutations and cell proliferation. Following inhalation exposure to high concentrations of GBPs, parameters reflecting these four steps of the AOP were much more strongly affected in rats than in mice, hamsters, or humans. A metaanalysis of chronic rat inhalation studies found the difference in carcinogenic potency between nanosized and micron-sized GBPs to be low (i.e., a factor of 2.0–2.5 referring to mass concentration). Concerning the induction of lung tumors by the so-called GBPs or PSPs, it is still under debate whether particle overload exposure in the rat leads to results regarding tumor induction that are useful for human risk assessment (Borm et al., 2015; Morfeld et al., 2015, 2016; Pauluhn 2014).

With respect to carbon nanotubes, concerns have been raised that these materials may induce lung cancer and mesothelioma by an asbestos-like fiber pathogenesis mechanism (Poland et al., 2009). MWCNT carcinogenic potential appears to depend upon their biopersistence, rigidity, diameter, and length (Toyokuni, 2013). Twenty-four months after a single intraperitoneal administration of up to 20 mg of two different MWCNTs (with or without structural defects; both: 11 nm $\times$ 0.7 µm), neither material induced mesothelioma in rats (Muller et al., 2009). By contrast, 24-months after a single intraperitoneal administration of four different MWCNTs (85 nm $\times$ 8.57 µm; 62 nm $\times$ 9.3 µm; 40 nm $\times$ 10.24 µm; and 37 nm $\times$ 7.91 µm, respectively; target dosages 1×10^9 and 5×10^9 fibers, each), all four test materials induced peritoneal mesothelioma (Rittinghausen et al., 2014). While rigidity was recognized as an important parameter affecting pathogenesis, Rittinghausen et al. (2014) caution that the findings cannot be used for risk assessment since data upon inhalation exposure are unavailable and the MWCNT aerosol concentration, leading to a sufficiently high dose at

the pleura to induce mesothelioma, is unknown. However, these findings indicate that carbon nanotubes or other fibers with certain physicochemical characteristics (i.e., rigidity, length and biopersistence) are able to induce mesothelioma.

During regulatory hazard assessment, in vitro and in vivo genotoxicity is determined to evaluate a substance's potential to induce early mechanisms of toxicity that may ultimately result in tumor formation (or teratogenicity, if the DNA of germ cells is damaged). Predominantly, the in vitro genotoxicity of nanomaterials is being assessed in the alkaline Comet assay or micronucleus test, and many different nanomaterials have been reported to elicit in vitro genotoxic effects in these tests (Arts et al., 2016; Landsiedel et al., 2009, 2010b; Maser et al., 2015; Schulz et al., 2012). For the respiratory route of exposure, in vivo genotoxicity is frequently assessed in lung or bone marrow cells from exposed rats using the alkaline Comet assay or micronucleus test, respectively. By contrast to the numerous positive outcomes of in vitro genotoxicity studies, there is no compelling evidence for in vivo genotoxicity of a broad spectrum of nanomaterials (Arts et al., 2016; Landsiedel et al., 2009, 2010b; Maser et al., 2015; Pfuhler et al., 2013). Also upon 3- or 6-month inhalation exposure to rats, neither 0.1–3 mg/m^3 CeO_2 NM-212 nor 50 mg/m^3 $BaSO_4$ elicited genotoxicity in either the alkaline Comet assay using leukocytes or the *Pig-a* gene mutation assay using erythrocytes (Cordelli et al., 2016). The lack of in vivo findings points to the overall poor correlation between in vitro and in vivo genotoxicity assessments of nanomaterials. Among other issues, this may be explained by the fact that, generally, considerably lower doses of the nanomaterials reach the target cells in vivo than in vitro and that the different time points of in vitro versus in vivo investigation may provide more time for DNA damage repair during in vivo studies (Landsiedel et al., 2009, 2010b; Maser et al., 2015; Pfuhler et al., 2013). Most likely, the available studies further point to the overall low in vivo genotoxic potential of nanomaterials. However, for some carbonaceous nanomaterials, genotoxicity was demonstrated after lung instillation in lung cells and in cells from the bronchoalveolar fluid, being mainly macrophages and neutrophils that generally do not develop tumors (Husain et al., 2015; Kyjovska et al., 2015).

Assessment of a material's genotoxic potential is the only toxicological information required under REACH that does not relate to an apical effect, but to a set of potentially complex cellular mechanisms. As also stands true for nonnanosized substances, the employment of nanomaterial genotoxic effects for hazard and risk assessment may be enhanced when the underlying specific mechanisms are known. However, the elucidation of the mechanisms of genotoxic effects may be challenging. A pragmatic solution may be the application of a battery of standard testing methods covering a wide range of mechanisms. For this purpose, the available standard genotoxicity test methods may require adaptations to ensure applicability for nanomaterials, and the interpretation of test results may require additional considerations, taking into account, for example, effective in vitro dosages or specific system-dependent material properties of the nanomaterials (Doak et al., 2012; Landsiedel et al., 2009, 2014b; Pfuhler et al., 2013). Wills et al. (2015) suggest employing dose–response analyses to improve the utility of in vitro genotoxicity data. Benchmark dose metrics and their confidence intervals can be used to rank test materials by endpoint-specific toxic potency and also to compare potencies recorded in different test systems or after different exposure durations (Slob, 2002; Wills et al., 2015). Negative results of in vivo genotoxicity studies may either indicate that the nanomaterial does not have genotoxic potential or that it did not reach the target organ. Hence, based on the in vivo outcome alone, it cannot be stated that the nanomaterial is

generally nongenotoxic. Conclusions derived from in vivo genotoxicity studies are limited to the fact that the nanomaterial did not show a genotoxic effect in the organ investigated upon exposure by the selected route. Toxicokinetic studies may strengthen the study result by identifying an internal dose in the target organ in which the genotoxicity was studied (cf. paragraph 48 of OECD TG 474 *mammalian erythrocyte micronucleus test* adopted 26 Sep. 2014).

Nanomaterial Hazard Assessment by Reproductive and Developmental Toxicity

Only single reproductive or developmental toxicity studies are available that cover all requirements of current OECD TGs, and none of them revealed any adverse effects. As part of a European Chemical Industry Council Long-Range Research Initiative project, precipitated SiO_2 NM-200 was tested in a two-generation reproduction toxicity study performed according to OECD TG 416. Oral administration of up to 1000 mg/kg bw/day SiO_2 NM-200 had no adverse effects on the reproductive performance of the male or female rats or on the growth and development of the offspring into adulthood for two consecutive generations (Wolterbeek et al., 2015). Hofmann et al. (2015) tested SiO_2 NM-200 for prenatal toxicity in rats according to OECD TG 414. Following oral administration of up to 1000 mg/kg bw/day SiO_2 NM-200 from gestation day 6–19, all pregnant animals were examined by caesarean section at gestation day 20. No compound-related alterations were observed in either the maternal animals or the fetuses. The available data on a limited number of nanomaterials do not indicate primary developmental toxicity of nanomaterials. Further investigations are required to substantiate this for other nanomaterials and to clarify whether adverse effects on the offspring may occur secondarily to maternal inflammatory responses (Hougaard et al., 2015).

GROUPING OF NANOMATERIALS TO STREAMLINE HAZARD AND RISK ASSESSMENT

In view of the economic relevance of nanotechnology-enabled products and the abundance of nanomaterials upcoming for risk assessment, numerous efforts are aiming at developing a generally applicable paradigm for nanomaterial grouping for hazard and risk assessment (Arts et al., 2014; ECHA, 2014). To provide a basis for such regulatory provisions, different jurisdictions have laid down definitions of the term "nanomaterial" (Boverhof et al., 2015). While the precise components of these definitions vary, all of them are based on material characteristics. However, the underlying intrinsic properties of nanomaterials are neither monocausally, nor linearly related to the hazard of nanomaterials (Lynch et al., 2014). Therefore, basing nanomaterial grouping on intrinsic material properties alone is likely to result in the over- or underestimation of hazards or failure to recognize relevant hazards at all (Arts et al., 2014).

To account for nanomaterial complexity, the grouping of nanomaterials should address all relevant aspects of a nanomaterial's life cycle and biological pathway from its release up to its (potential) apical effects (Braakhuis et al., 2015a). Relevant (and quantifiable) aspects include system-dependent properties in addition to intrinsic material properties, specific types of use and exposure, uptake and kinetics, and early cellular and apical effects (Arts et al., 2014, 2015, 2016; Braakhuis et al., 2015a; Landsiedel et al., 2010a; Oomen et al., 2014a, 2015;

Stone et al., 2014). System-dependent (or functionality-driven) properties, such as dissolution, dispersibility, and surface reactivity, form an important pillar of nanomaterial grouping. These properties address a nanomaterial's potential to change with a given environment, be it, for example, a specific product matrix, a cell culture medium, the lung-lining fluid or blood (Potthoff et al., 2015). Accordingly, hazards, exposure scenarios, and risks may change, for example, once a given nanomaterial is embedded in a composite, or when it is dispersed in air or in biological fluids.

Different regulatory authorities and international research consortia have published approaches for the specific grouping of nanomaterials, and preliminary guidance is provided in the context of substance-related legislation or in the occupational setting. These approaches already go beyond the determination of mere structure–activity relationships and are founded on different aspects of the nanomaterial's life cycle or biological pathway (Arts et al., 2014). For instance, material properties and biophysical interactions are addressed in the categorization scheme of the German Federal Institute for Occupational Safety and Health (BAuA, 2013, 2015; Packroff and Gebel, 2014). Nanomaterial exposure is a fundamental component of the grouping concept of the US National Institute for Occupational Safety and Health (DHHS (NIOSH), 2012; Kuempel et al., 2012). Nanomaterial systemic uptake is addressed in the testing approach published by Monteiro-Riviere et al. (2011). Defined mechanisms of nanomaterial toxicity form the basis of the grouping and testing approaches published by Lai (2012), Wang et al. (2014), or Nel et al. (2013a, 2015). However, none of the available approaches cover all relevant aspects of a nanomaterial's life cycle and biological pathway, and most of them have not advanced beyond theoretical, conceptual stages (Arts et al., 2014).

Against this background, the European Centre for the Ecotoxicology and Toxicology of Chemicals (ECETOC) "Nano Task Force" developed a comprehensive functionality-driven concept for the grouping of nanomaterials (Arts et al., 2015, 2016). This *Decision-making framework for the grouping and testing of nanomaterials* (DF4nanoGrouping) consists of three tiers to assign nanomaterials to four main groups (MGs), to perform subgrouping within the MGs, and to determine and refine specific information needs. Addressing all relevant aspects of a nanomaterial's life cycle and biological pathway (Fig. 8.1), the essential grouping criteria encompass intrinsic material properties in Tier 1 (water solubility, particle morphology, chemical composition) and system-dependent properties in Tier 2 (dissolution in biological media, surface reactivity, particle dispersibility, in vitro effects) (Table 8.2). In Tier 3, the Tier 1 and Tier 2 MG assignment that is based upon nonanimal testing alone is confirmed or corrected using data from in vivo short-term studies. For inhalation exposure, the rat STIS is recommended. However, also rat intratracheal instillation studies may be useful to rank nanomaterial hazard potential (Morimoto et al., 2015).

The four MGs of the DF4nanoGrouping encompass (MG1) soluble nanomaterials, (MG2) biopersistent high aspect ratio (HAR) nanomaterials, (MG3) passive nanomaterials that correspond to GBPs, and (MG4) active nanomaterials. The relevance of nanomaterial biopersistence for hazard and risk assessment is underlined by the fact that this parameter is essential for nanomaterial assignment to MG1 and MG2 of the DF4nanoGrouping. While MG1-soluble nanomaterials have low in vivo biopersistence, the MG2 for biopersistent HAR nanomaterials (NMs) takes into account the fibers' high biopersistence. In Tier 3 of the DF4nanoGrouping, STIS data provide valuable information on nanomaterial biopersistence (Table 8.1).

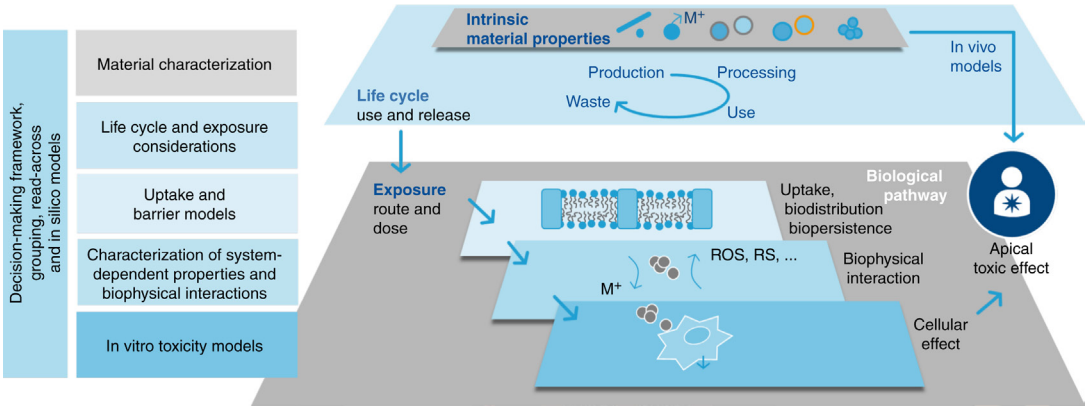


FIGURE 8.1 Comprehensive framework for nanomaterial grouping addressing all relevant aspects of a nanomaterial's life cycle and biological pathway, that is, intrinsic material properties, use, release and exposure, uptake and biokinetics, system-dependent properties (biophysical interactions), cellular and apical toxic effects. *Source: Adapted from Landsiedel, R., Presentation at SENN 2015, Helsinki, Finland.*

Furthermore, the DF4nanoGrouping allows subgrouping active (MG4) nanomaterials by pattern of biodistribution. This serves to identify specific additional information needs. For inhaled nanomaterials, this subgrouping distinguishes between nanomaterials that only become available in the primary organ (i.e., the respiratory tract for the inhalation route of exposure), nanomaterials that are additionally found in the MPS, and, finally, nanomaterials that become systemically available outside the MPS. Given the limited number of potential modes-of-action of nanomaterials in the lung, most of the specific testing needs beyond Tier 3 of the DF4nanoGrouping, that is, the rat STIS, are expected to arise from indications of extrapulmonary effects or the need to consider for distinct biokinetics due to increased lung deposition or prolonged clearance. Of note, however, for none of the nanomaterials for which rat STIS data are available, systemic availability outside the MPS has been recorded (Arts et al., 2015, 2016).

Overall, the tiered framework and four MGs of the DF4nanoGrouping stand in line with the requirements and stipulations from different organizations and authorities, such as the International Standardization Organization (ISO, 2014), the United States Environmental Protection Agency (EPA, 2015), the German Environmental Protection Agency (UBA, 2014), researchers from the German Federal Institute for Occupational Safety and Health (Gebel et al., 2014), or the Dutch National Institute for Public Health and the Environment (RIVM; Sellers et al., 2015). Prior to the application of the DF4nanoGrouping tiers, further intrinsic material properties, such as surface area and surface charge (specified as "supplementary criteria" in Table 8.2), may be used to define if a substance is in fact a nanomaterial, for example, in accordance with the EU recommendation (EU Commission, 2011). Nevertheless, the use of such additional intrinsic properties to identify a nanomaterial and different nanoforms of a substance does not compromise their subsequent grouping by functionality-driven properties for the purpose of hazard assessment.

TABLE 8.2 DF4nanoGrouping: Grouping Criteria, Threshold Values, Relation to Main Group Assignment

Grouping tier	Grouping criterion	Threshold value for grouping	Main group (MG) assignment or indication	Benchmark material; if applicable
Tier 1: Intrinsic material properties	Water solubility	>100 mg/L [1]	Assignment to MG1	ZnO NM-110 and NM-111, 10 nm-CuO: Limited water solubility TiO_2 NM-105: Low water solubility
	Particle size and shape	Aspect ratio >3:1, length >5 µm, diameter <3 µm [2]	Indication for MG2	
	Composition including impurities	≥0.1% of component with GHS classification for systemic effects	Indication for MG4	
Tier 2: System-dependent properties In vitro effects	Dissolution in biological fluids	>100 mg/L [1]	Globular NMs: >100 mg/L: Indication for MG1 Fibres: <100 mg/L: Indication for MG2	10 nm-CuO: High dissolution
	Surface reactivity	≥10% of Mn_2O_3 reactivity, which is equal to: ≥0.19 µUFRAS/m^2*h	Assignment to MG4	Quartz dust DQ12: High surface reactivity $BaSO_4$ NM-220: Not oxidative
	Dispersibility	AAN <3 or diameter <100 nm	Assignment to MG2 or MG4, as applicable	a-SiO_2-susp with acrylate surface functionalization: Dispersible a-SiO_2-susp without surface functionalization: Agglomeration
	Cellular effects	Effect at ≤10 µg/cm^2 [3]	Assignment to MG4	ZnO NM-110 and NM-111: Activity (shedding of ions) CeO_2 NM-211 and NM-212: Activity $BaSO_4$ NM-220: Passivity

Tier 3: In vivo screening	Toxic potency	STIS NOAEC; four ranges: I: <0.5 mg/m^3 and no regression or progression of effects II: <1 mg/m^3; III: <10 mg/m^3 IV: ≥10 mg/m^3	Ranges I–III: Confirmation of MG2 or MG4; subgrouping of MG4; Range IV: Confirmation of MG3	MWCNT NM-400: Range I CeO_2 NM-211 and NM-212: Range II TiO_2 NM-105; a-SiO_2-susp without surface functionalization: Range III $BaSO_4$ NM-220: Range IV
	Biopersistence	t_{50} <40 days	Confirmation of MG1	CeO_2 NM-211 and NM-212: Decelerated clearance TiO_2 NM-105: Physiological clearance $BaSO_4$ NM-220: Accelerated clearance
Qualifier	Dustiness	None assigned	Indication of a substance's emission potential	
Supplementary criteria	Surface area	None assigned	Not primary grouping criteria	
	Surface chemistry	None assigned	Not primary grouping criteria	
	Surface charge	Positive: ζ >10 mV	Joint evaluation with "dispersibility" Pos. surface charge: Indication for MG4	CeO_2 NM-211 and NM-212: Positive surface charge
	Hydrophobicity	None assigned	Joint evaluation with "dispersibility"	

AAN, Average agglomeration number; a-SiO_2-susp, suspended amorphous SiO_2 (Levasil 200); GHS, globally harmonized system; MG, main group; NM, nanomaterial; NOAEC, no-observed adverse effect concentration; STIS, short-term inhalation study.

Based upon the outcome of the case studies, the following adaptations to the threshold values laid down in Arts et al. (2015) are made:

1. While the threshold values for water solubility and dissolution are adequate for NMs that release ions with GHS classification for systemic effects, they may have to be reconsidered for substances that dissolve into nontoxic components.
2. NMs may be assigned to MG2 on account of high aspect ratio, fibre diameter, and insolubility/low dissolution in water or biological media, even though their length does not meet the WHO criterion (>5 μm).
3. This threshold value applies for cytotoxicity tests performed with lung epithelial cells. For in vitro assays performed with alveolar macrophages, a particle surface-area based threshold value of 6000 mm^2/mL is laid down (Wiemann et al., 2016).

Adapted from Arts, J.H., Hadi, M., Irfan, M.A., Keene, A.M., Kreiling, R., Lyon, D., Maier, M., Michel, K., Petry, T., Sauer, U.G., Warheit, D., Wiench, K., Wohlleben, W., Landsiedel, R., 2015. A decision-making framework for the grouping and testing of nanomaterials (DF4nanoGrouping). Regul. Toxicol. Pharmacol. 71 (Suppl. 2), S1–S27; Arts, J.H., Irfan, M.A., Keene, A.M., Kreiling, R., Lyon, D., Maier, M., Michel, K., Neubauer, N., Petry, T., Sauer, U.G., Warheit, D., Wiench, K., Wohlleben, W., Landsiedel, R., 2016. Case studies putting the decision-making framework for the grouping and testing of nanomaterials (DF4nanoGrouping) into practice. Regul. Toxicol. Pharmacol. 2016;76, 234–261.

Recently the supplementary criterion "hydrophobicity" (assessed by "water contact angle") has been observed to correlate to the asphyxiation potential of otherwise inert compounds, such as organic pigments (Hofmann et al., 2016). In accordance with OECD TG 403, acute inhalation toxicity testing in rats requires very high aerosol concentration of 5 mg/L. At this concentration otherwise nontoxic particles may clog the airways thereby causing death by asphyxiation, which is both distressful to the animals and toxicologically irrelevant. Hofmann et al. (2016) suggest that this clogging could be anticipated by a material's hydrophobicity and may serve as a justification for lowering the highest testing concentration in acute inhalation testing. Accordingly, the status of the grouping criterion hydrophobicity as "supplementary" may warrant reconsideration.

Use (including manufacture), release, and route of exposure are applied as "qualifiers" within the DF4nanoGrouping to determine relevant exposure scenarios (Bos et al., 2015). If nanomaterials cannot be released from a product matrix in any way (Bräu et al., 2012; Wohlleben et al., 2011a), these qualifiers may serve to justify the waiving of testing (Arts et al., 2015). For each grouping criterion, Arts et al. (2015, 2016) suggest pragmatic, and often standardized, test methods (Wohlleben et al., 2013a). Additionally, specific threshold values for nanomaterial assignment to one of the MGs and benchmark materials, predominantly from the OECD sponsorship program (OECD, 2010), are suggested (Table 8.2). Benchmark materials are materials, which have been tested and evaluated according to standard criteria and to which new materials may reliably be compared for grouping (Kuempel et al., 2012). Accordingly, the availability of benchmark materials covering the range of potential effects for a given grouping criterion plays an important role for nanomaterial grouping.

Even though a concrete number of criteria were identified as essential for grouping, the DF4nanoGrouping does not imply fixed testing schemes for all nanomaterials in all applications. Instead, functionality-driven grouping is closely linked to (or even undistinguishable from) integrated approaches for the testing and assessment (IATAs) of nanomaterials. Both processes take into account the life cycle of a nanomaterial and its biological pathway, and both support a concern-driven stepwise collection and evaluation of information that is relevant for the given purpose (Oomen et al., 2014a; Stone et al., 2014). The collection of data is concluded as soon as the hazard and exposure potential and risk of a given nanomaterial can be assessed.

CONSIDERATION OF CELLULAR AND APICAL EFFECTS FOR NANOMATERIAL GROUPING

Taking into account that it is one of the main goals of grouping to reduce animal testing, this section further elucidates how in vitro assays may contribute to this goal. In the DF4nanoGrouping, assessment of cellular effects has been laid down as a Tier 2 grouping criterion. However, to date, standardized in vitro assays to evaluate nanomaterial cytotoxic potential, let alone regulatory guidance on how to incorporate in vitro assays into the hazard and risk assessment of nanomaterials, are unavailable (Kroll et al., 2011; Landsiedel et al., 2014b). For nanomaterial testing, the International Standardization Organization Technical Committee ISO TC 229 Nanotechnologies has drafted a large number of technical reports and technical specifications, but the actual number of published standards related to nanotechnologies is limited (ISO, 2016).

Nanomaterials may induce cellular effects by different mechanisms of toxicity, that is, (1) membrane damage including cationic phagolysosome damage that may ultimately lead to apoptosis and autophagy; (2) generation of reactive oxygen species (ROS), redox activities, and photocatalytic effects; (3) inflammasome activation and cytokine and chemokine production; (4) the shedding of toxic ions; (5) fiber effects; and (6) DNA damage (Nel et al., 2013b, 2015; Park et al., 2015; Stern et al., 2012). There is no indication that nanomaterials elicit new, hitherto unknown cellular effects.

The application of in vitro findings for nanomaterial grouping is restrained by the fact that many in vitro studies investigating nanomaterial pulmonary toxicity poorly correlate to in vivo inhalation studies (Landsiedel et al., 2014b). The outcome of in vitro assays may be adulterated when nanomaterials interfere with assay reagents (Monteiro-Riviere et al., 2009; Wohlleben et al., 2011b). Such interferences may be recognized and corrected for, for example, by including cell-free controls in the in vitro studies. Moreover, in many published in vitro studies, the effective dose (the particle mass reaching the cultured cells) was neither calculated nor measured, and in vitro doses were rarely correlated to aerosol concentrations or lung burdens in inhalation studies (Braakhuis et al., 2015a; Cohen et al., 2015). Even though many nanomaterials have been observed to induce some form of inflammatory and/or cytotoxic reaction in vitro, these effects were often only observed at concentrations that by far exceeded realistic in vivo exposure scenarios (Kroll et al., 2011; Landsiedel et al., 2014b).

Improved methods to estimate the in vitro effective dose of nanomaterials have been proposed (Cohen et al., 2015; DeLoid et al., 2014; Hinderliter et al., 2010; Teeguarden et al., 2007). If in vitro assays use standardized protocols and are conducted at concentrations reflecting effective in vivo dosages, they allow assessing mechanisms of nanomaterial toxicity (Horev-Azaria et al., 2013). This may provide valuable information for nanomaterial grouping.

Recently, the in vitro alveolar macrophage (AM) assay has proven highly predictive for in vivo respiratory tract effects (Wiemann et al., 2016), and it is recommended for the assessment of cellular effects in Tier 2 of the DF4nanoGrouping (Arts et al., 2015, 2016). The AM assay uses rat NR8383 cells which are similar to rat AMs in vivo that sequester most particles upon short-term inhalation. The in vitro NR8383 AM assay assesses four parameters: cellular release of lactate dehydrogenase, glucuronidase, tumor necrosis factor alpha, and ROS. Significant effects observed below the particle surface area-based threshold of $<6000\ mm^2/mL$ are interpreted as material-specific "noncellular overload" effects (Wiemann et al., 2016). Test materials are assessed as active if at least two of the four parameters undercut the threshold of $<6000\ mm^2/mL$, and they are assessed as passive if zero or one parameter is altered (Wiemann et al., 2016). Further, Westphal et al. (2015) reported that the supernatant from nanomaterial-exposed NR8383 cells affects the migration of differentiated human leukemia cells in a material- and dose-dependent manner. The usefulness of the NR8383 cells' potential to secrete neutrophil-attracting compounds as parameter for routine toxicological testing remains to be established. In addition, in vitro cultures composed of multiple cell types can now better mimic organ structures and allow particle exposure by air flow in the so-called air–liquid interface system for toxicity and translocation studies (Bachler et al., 2015).

Case studies assessing a broad spectrum of inorganic nanomaterials and nanosized organic pigments, and focusing on the inhalation route of exposure, confirmed the usefulness of the DF4nanoGrouping in identifying nanomaterial hazard potential. Importantly, nanomaterial hazard was already identified in the nonanimal Tiers 1 and 2 covering intrinsic and

system-dependent properties and cellular effects obtained in the in vitro NR8383 AM assay. For altogether 22 of the 25 case study materials, the Tier 1 and 2 MG assignment was consistent with the Tier 3 in vivo STIS-based MG assignment. The remaining three materials were identified as MG4 active in Tier 1 and 2, whereas they were assigned as MG3 passive in the Tier 3 in vivo STIS. Hence, hazard was never underpredicted in the nonanimal tiers (Table 8.1, Arts et al., 2016). While 90-day studies were not available for all test materials, the outcome of the available subchronic studies were in accordance with the MG assignments (Arts et al., 2016).

Consequently, the DF4nanoGrouping allows identifying MG4 active nanomaterials. These are the nanomaterials that merit in-depth investigations. To determine specific information needs for the in-depth investigations, the DF4nanoGrouping further provides a solid scientific rationale to subgroup active nanomaterials by STIS NOAEC range, by the reversibility or progression of effects and by their internal exposure, that is, their biopersistence, systemic uptake, and pattern of biodistribution (Arts et al., 2016).

NANOMATERIAL RISK ASSESSMENT AND RISK MANAGEMENT

As has been shown in the preceding sections of this chapter, the hazards of nanomaterials are dominated by the effects of released ions (MG1 of the DF4nanoGrouping) or by their fiber- or particle-like effects (MG2 and MG4, respectively). There is no evidence of "nano-specific" mechanisms of action or of a step-change in hazard for particles below 100 nm (Moreno-Horn and Gebel, 2014). Compared to nonnanosized substances, nanomaterials appear to induce relatively few toxic mechanisms or potential AOPs. By contrast, the external exposure to nanomaterials (e.g., the release of particles over the life cycle of the products and aerosol concentrations in the air, for the inhalation route of exposure) is generally more complex for nanomaterials than for nonnanosized substances. Humans are usually not exposed to a distinct nanoparticle, but to a population of particles, aggregates, and agglomerates of different sizes, shapes, and surface coatings. The same holds true for the internal exposure, even though systemic uptake and availability appears to be providentially limited for most nanomaterials both after inhalation and oral exposure and hence local effects in the primary exposed organ, that is, the lung and gastrointestinal tract, are of predominant concern. A schematic representation of the approaches for risk assessment with respect to using external exposure or internal exposure based on toxicokinetic data and modeling is provided in Fig. 8.2.

Risk-management measures include the setting of occupational exposure limits (OELs) or, in the context of the REACH Regulation, of derived-no-effect-levels (DNELs). When insufficient data are available, a hazard and control banding approach might be used for occupational settings as described by Kuempel et al. (2012) and ISO (2014). Different exposure limahave been proposed for specific types of nanomaterials reflecting the MGs of the DF4nanoGrouping. The German Federal Institute for Occupational Safety and Health (BAuA) recommended seeking air concentrations <10,000 fibers/m^3 for fibers (MG2) (BAuA, 2013). For GBPs (MG3), an "assessment criterion" of <0.5 mg/m^3 (considering an average agglomerate density of 2.0 g/cm^3 at the workplace) has been suggested, and for nonfibrous nanomaterials with specific toxicity (MG4), an "assessment criterion" of <0.1 mg/m^3 (BAuA, 2013).

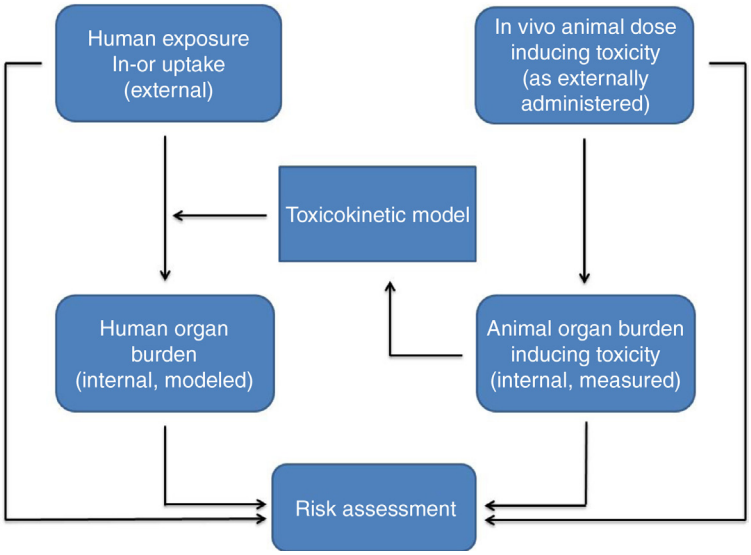


FIGURE 8.2 Risk assessment approach using either external or internal exposures and dose modeling. *Source: Adapted from Van Kesteren, P.C., Cubadda, F., Bouwmeester, H., Van Eijkeren, J.C., Dekkers, S., De Jong, W.H., Oomen, A.G., 2015. Novel insights into the risk assessment of the nanomaterial synthetic amorphous silica, additive E551, in food. Nanotoxicology 9, 442–452.*

Recently, the *German Research Association Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area* (MAK commission) recommended an OEL of 0.3 mg/m^3 for respirable GBPs given a substance density of 1 g/cm^3 (Hartwig, 2014). Specifically, the MAK commission concluded that all GBPs are carcinogenic to humans with a threshold effect. As discussed by Morfeld et al. (2015), the *Scientific Committee on Exposure Limits*, an advisory group to the EU Commission, is evaluating the potential use of the MAK recommendation for European Member States. If accepted, the OEL of 0.3 mg/m^3 would replace the previous general dust limit for the respirable fraction of 1.5 mg/m^3 (based on an average particle density of 2.5 g/cm^3 at the workplace) (BAuA, 2014). However, the MAK commission's justification for lowering the OEL for respirable GBPs has been challenged, just as its suggestion to classify all respirable GBPs as carcinogenic with a threshold effect (Morfeld et al., 2015, 2016). Nevertheless, for the risk assessment of nanomaterials, it is relevant that a threshold for tumor formation after inhalation of (nano-)particles in rats could be defined as these tumors do not seem to originate from primary genotoxic effects, but are most likely induced by chronic inflammation. Further investigations are necessary to determine the relevance of tumor formation in rats for humans, just as the biokinetics and dosimetry of such effects in rats need to be better understood. Eventually, the ongoing long-term inhalation study (Gebel and Landsiedel, 2013; Ma-Hock et al. 2014) will contribute to filling this knowledge gap.

Irrespective of ongoing discussions on the stipulation of the general dust OEL, the DF4nanoGrouping assignment to MG3 or MG4 may serve to determine whether a general dust OEL is applicable in managing long-term human exposure to nanomaterials, or not. For passive nanomaterials (MG3), a general dust OEL is sufficient, whereas active nanomaterials

(MG4) require specific, lower OELs and, accordingly, specific further investigations (Arts et al., 2015, 2016). For the inhalation route of exposure, the case studies applying the DF4nanoGrouping confirmed its efficiency in sorting out nanomaterials that could undergo hazard assessment without additional testing (Arts et al., 2016). These are soluble nanomaterials (MG1) whose further hazard assessment should rely on read-across to the dissolved materials; HAR nanomaterials (MG2) which may be assessed according to their potential fiber toxicity (Cassee et al., 2015); and passive nanomaterials (MG3) that only cause effects under pulmonary overload conditions (ECETOC, 2013; Kuempel et al., 2014). Nanomaterial assignment to one of the four MGs of the DF4nanoGrouping also provides preliminary information on the mode-of-action of nanomaterials. This may lay a foundation for the future development of AOPs, which may facilitate the identification of evidence-based in vitro models (Burden et al., 2016). The DF4nanoGrouping case studies may further form a scientific basis for the justification of read-across applications, for example, by using the benchmark materials as source nanomaterials.

While confirming grouping as a useful tool for risk assessment, Braakhuis et al. (2015a) caution that grouping frameworks, such as the DF4nanoGrouping, will most likely and for most cases not be sufficient to fully demonstrate safe use. As the DF4nanoGrouping case studies highlight (Arts et al., 2016), application of grouping for the risk assessment of nanomaterials requires the availability of benchmark materials with a comprehensive data set obtained in standardized tests against which new materials can be reliably compared. For a given toxicological endpoint, benchmark materials should be available that cover the spectrum of relevant effects. Accordingly, grouping nanomaterials by hazard potential upon inhalation exposure proved efficient already based upon the data obtained in the nonanimal tiers covering intrinsic and system-dependent properties and in vitro cellular effects (Arts et al., 2016). By comparison, for the REACH information requirements local toxicity or genotoxicity, the spectrum of nanomaterials for which comprehensive, reliable data sets are available do not reveal any positive findings. However, especially for genotoxicity contradicting results are reported in the literature for various nanomaterials including TiO_2 (Landsiedel et al., 2009, 2010b; Oesch and Landsiedel, 2012). For carcinogenicity, just as for reproductive toxicity, only single studies are available that again did not yield any positive findings. Accordingly, benchmark nanomaterials covering the corresponding modes-of-action of local toxicity, carcinogenicity, reproductive or developmental toxicity are currently unavailable. Nevertheless, if a nanomaterial is likely to have low or no bioavailability at a given target organ, assessment of the corresponding toxicological property may be of lower relevance. Eventually, the ongoing long-term inhalation study (Gebel and Landsiedel, 2013; Ma-Hock et al., 2014) will also help provide a basis for the general risk assessment of nanomaterials. In this respect, it will be important to apply read-across to other nanomaterials since only few (representative) nanomaterials are being tested in long-term inhalation studies.

The following brief examples highlight considerations that should be taken into account for nanomaterial risk assessment and risk management. Using publicly available data, Van Kesteren et al. (2015) and Heringa et al. (2016) performed classical risk assessments of different amorphous SiO_2 and TiO_2 nanomaterials, respectively, that are being used as food additives. In both studies, the risk assessment was based upon exposure estimation, hazard characterization (hazard identification and dose–response assessment), and risk characterization, and internal organ dose modeling based on experimental toxicokinetic data was applied.

Upon evaluation of all available data, a risk for humans arising from either amorphous SiO_2 or TiO_2 nanomaterial food additives could not be excluded. Both studies identified several sources of uncertainty that were caused by a lack of reliable information on exposure and a lack of properly performed toxicity studies. For instance, long-term studies were lacking, just as proper dose–response studies that included organ burden assessments. As Van Kesteren et al. (2015) and Heringa et al. (2016) conclude, better data are needed to enable a sound risk assessment. Such data should address the various forms of amorphous SiO_2 and TiO_2 nanomaterials, Si and Ti tissue levels should be determined in key toxicity studies, and SiO_2 and TiO_2 levels should be determined in food, and human uptake of SiO_2 and TiO_2 from the gastrointestinal tract and (when possible) organ levels in humans should be known. Hence, even for nanomaterials that have already been in use for prolonged periods of time, it is difficult to draw firm conclusions in the risk assessment.

Practical examples of risk assessment and risk management for MWCNTs include the estimation of acceptable exposure levels and the warranty of production processes with sufficiently low exposure (Schulte et al., 2010). In a subchronic inhalation study performed according to OECD TG 413, MWCNT Nanocyl NC 7000 (equivalent to MWCNT NM-400) was not observed to elicit systemic toxicity (Ma-Hock et al., 2009b). However, at 0.5 and 2.5 mg/m^3, this MWCNT did induce increased lung weight, pronounced multifocal granulomatous inflammation, diffuse histiocytic and neutrophilic inflammation, and lipoproteinosis in the lung and lung-associated lymph nodes. Also at 0.1 mg/m^3, minimal granulomatous inflammation was recorded so that a NOAEC could not be established (Ma-Hock et al., 2009b). A human working lifetime equivalent concentration of 0.0011 mg/m^3 was calculated from the LOAEC of this rat subchronic inhalation study (DHHS (CDC and NIOSH), 2013; OEHHA, 2008). This human working lifetime equivalent concentration was compared to MWCNT measurements at the production workplace (Kuhlbusch et al., 2013; Lee et al., 2010), and different release scenarios of composite materials with MWCNT nanofillers were studied (Hirth et al., 2013; Wohlleben et al., 2011a, 2013b). It was concluded, that *the test substance has low dust-forming potential, [...] but nonetheless strict industrial hygiene measures must be taken during handling and processing* (Ma-Hock et al., 2009b), and the production and compounding facilities were laid out accordingly.

Specific MWCNTs with a rigid structure and a certain length and aspect ratio were found to induce mesothelioma by intraperitoneal injection (Poland et al., 2009; Rittinghausen et al., 2014), while more tangled MWCNT did not (Muller et al., 2009). In this case, the risk management could be the choice of alternative MWCNT without critical material properties triggering the toxic effect. This approach is currently discussed as a "safety by design" approach (Donaldson et al., 2011).

Ultimately, risk assessments provide information on exposure levels which are considered to cause no or acceptable effects on human health, and the risk characterization will lead to a conclusion whether risk management is necessary. When the risk is concluded to be unacceptable, risk management has to be performed to control, reduce, or prevent occupational, consumer-related, or environmental exposure, as necessary (BAuA, 2015; Jähnel, 2015). Usually, this is achieved by reducing release of the nanomaterial during production and use, by personal protection or by limiting the use or banning the nanomaterial. The choice of the risk-management measures depends on the type of nanomaterial used and practicable possibilities for altering production and/or use circumstances.

CONCLUSIONS

Modern risk-assessment approaches integrate mechanistic data on system-dependent properties, biophysical interactions, and cellular effects with data on exposure, biokinetics, and hazard. Biokinetic data may be used to refine the risk assessment as it can provide the actual internal doses of the target organs. For nanomaterials that are already widely used data on human organ levels could be obtained and would increase the reliability of the risk assessments. In addition, by applying grouping concepts and IATAs, targeted data generation and assessment is ensured. This allows rapid material categorization according to risk potential, founded on scientifically justifiable categories, so that materials of high concern can be targeted for additional scrutiny, while material categories that pose the least risk can receive expedited review (Godwin et al., 2015). Modern approaches for the risk assessment of nanomaterials have been published by researchers from the *Duke University Center for the Environmental Implications of NanoTechnology* (Hendren et al., 2015) or as outcomes of the EU-funded projects ITS-NANO (Stone et al., 2014) and MARINA (Bos et al., 2015), of the ECETOC "Nano Task Force" (Arts et al., 2015, 2016) and of an international workshop jointly organized by the *University of California Center for Environmental Implications of Nanotechnology* and the *University of California Los Angeles Center for Nanobiology and Predictive Toxicology* (Godwin et al., 2015). They ensure that no studies are performed that do not provide crucial data, thereby allowing to save resources, while restricting animal testing to a last resort (Arts et al., 2014, 2015, 2016; Oomen et al., 2014a,b).

TAKE-HOME MESSAGES

- Traditional risk assessment involves sequential steps of exposure assessment, hazard identification, and hazard characterization, which are merged in risk characterization, and traditional hazard assessment largely relies on data on apical effects, that is, the observable outcome of substance exposure to test animals.
- While toxicity testing methods are generally applicable to nanomaterials (with adaptations), their application to comprehensively test all variants of nanomaterials is neither efficient nor compliant with animal welfare statutes.
- For nanomaterial testing, modern risk-assessment approaches are suggested that integrate mechanistic data on intrinsic material properties, system-dependent properties, biophysical interactions, and cellular effects with data on exposure, biokinetics, and hazard, utilizing animal testing as a last resort.
- Modern risk-assessment approaches ensure that testing is limited to the collection of such data that are truly necessary for the assessment, which warrants efficiency, as well as compliance with legal animal-welfare requirements.

References

Albanese, A., Tang, P.S., Chan, W.C.W., 2012. The effect of nanoparticle size, shape, and surface chemistry on biological systems. Annu. Rev. Biomed. Eng. 14, 1–16.

Ankley, G.T., Bennett, R.S., Erickson, R.J., Hoff, D.J., Hornung, M.W., Johnson, R.D., Mount, D.R., Nichols, J.W., Russom, C.L., Schmieder, P.K., Serrrano, J.A., Tietge, J.E., Villeneuve, D.L., 2010. Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. Environ. Toxicol. Chem. 29, 730–741.

Anzai, T., Kaminishi, M., Porth, A., Handley, J., Satoh, T., 2012. Worldwide trend in nanomaterial safety evaluation. Jpn. J. Environ. Toxicol. 15, 17–29.

Arts, J.H., Hadi, M., Irfan, M.A., Keene, A.M., Kreiling, R., Lyon, D., Maier, M., Michel, K., Petry, T., Sauer, U.G., Warheit, D., Wiench, K., Wohlleben, W., Landsiedel, R., 2015. A decision-making framework for the grouping and testing of nanomaterials (DF4nanoGrouping). Regul. Toxicol. Pharmacol. 71 (Suppl. 2), S1–S27.

Arts, J.H., Hadi, M., Keene, A.M., Kreiling, R., Lyon, D., Maier, M., Michel, K., Petry, T., Sauer, U.G., Warheit, D., Wiench, K., Landsiedel, R., 2014. A critical appraisal of existing concepts for the grouping of nanomaterials. Regul. Toxicol. Pharmacol. 70, 492–506.

Arts, J.H., Irfan, M.A., Keene, A.M., Kreiling, R., Lyon, D., Maier, M., Michel, K., Neubauer, N., Petry, T., Sauer, U.G., Warheit, D., Wiench, K., Wohlleben, W., Landsiedel, R., 2016. Case studies putting the decision-making framework for the grouping and testing of nanomaterials (DF4nanoGrouping) into practice. Regul. Toxicol. Pharmacol. 76, 234–261.

Arts, J.H., Muijser, H., Duistermaat, E., Junker, K., Kuper, C.F., 2007. Five-day inhalation toxicity study of three types of synthetic amorphous silicas in Wistar rats and post-exposure evaluations for up to 3 months. Food Chem. Toxicol. 45, 1856–1867.

Bachler, G., Losert, S., Umehara, Y., Von Goetz, N., Rodriguez-Lorenzo, L., Petri-Fink, A., Rothen-Rutishauser, B., Hungerbuehler, K., 2015. Translocation of gold nanoparticles across the lung epithelial tissue barrier: combining in vitro and in silico methods to substitute in vivo experiments. Part. Fibre Toxicol. 12, 18.

BAuA: Bundesanstalt für Arbeitsschutz und Arbeitsmedizin, 2013. Bekanntmachung zu Gefahrstoffen. Hergestellte Nanomaterialien. Federal Institute for Occupational Safety and Health. Announcement regarding hazardous substances. Manufactured Nanomaterials. BekGS 527. BMBl 25, 498–511.

BAuA, 2014. Bundesanstalt für Arbeitsschutz und Arbeitsmedizin. Begründung zum Allgemeinen Staubgrenzwert (2014/2001) in TRGS 900 Arbeitsplatzgrenzwerte, Nr. 12 GMBl 2014, 271, Bek. d. BMAS v. 14.2.2014. IIIb 3, 35125 – 5. In: Bekanntmachung von Technischen Regeln – Justification of the general dust limit in the technical rule on hazardous substances 900 (occupational exposure limits Nr. 12).

BAuA, 2015. Safe handling of nanomaterials and other advanced materials at workplace. NANOVALID—Developing reference methods for nanomaterials. Lead author and project monitoring: Baron, M., Federal Institute for Occupational Safety and Health.

Becker, H., Herzberg, F., Schulte, A., Kolossa-Gehring, M., 2011. The carcinogenic potential of nanomaterials, their release from products and options for regulating them. Int. J. Hyg. Environ. Health 214, 231–238.

Bellmann, B., 2011. 14-day nose-only inhalation toxicity study of Z-COTE HP1 in Wistar WU rats. 02 G 09 005 (draft report). Fraunhofer ITEM, Germany. Study owner: Cefic, Belgium.

Bermudez, E., Mangum, J.B., Wong, B.A., Asgharian, B., Hext, P.M., Warheit, D.B., Everitt, J.I., 2004. Pulmonary responses of mice, rats, and hamsters to subchronic inhalation of ultrafine titanium dioxide particles. Toxicol. Sci. 77, 347–357.

Borm, P., Cassee, F.R., Oberdörster, G., 2015. Lung particle overload: old school—new insights? Part. Fibre Toxicol. 12, 10.

Bos, P.M., Gottardo, S., Scott-Fordsmand, J.J., Van Tongeren, M., Semenzin, E., Fernandes, T.F., Hristozov, D., Hund-Rinke, K., Hunt, N., Irfan, M.A., Landsiedel, R., Peijnenburg, W.J., Sánchez Jiménez, A., Van Kesteren, P.C., Oomen, A.G., 2015. The MARINA Risk Assessment Strategy: a flexible strategy for efficient information collection and risk assessment of nanomaterials. Int. J. Environ. Res. Public Health 12, 15007–15021.

Boverhof, D.R., Bramante, C.M., Butala, J.H., Clancy, S.F., Lafranconi, M., West, J., Gordon, S.C., 2015. Comparative assessment of nanomaterial definitions and safety evaluation considerations. Regul. Toxicol. Pharmacol. 73, 137–150.

Braakhuis, H.M., Cassee, F.R., Fokkens, P.H.B., De La Fonteyne, L.J.J., Oomen, A.G., Krystek, P., De Jong, W.H., Van Loveren, H., Park, M.V.D.Z., 2015b. Identification of the appropriate dose metric for pulmonary inflammation of silver nanoparticles in an inhalation toxicity study. Nanotoxicology 23, 1–11.

Braakhuis, H.M., Oomen, A.G., Cassee, F.R., 2015a. Grouping nanomaterials to predict their potential to induce pulmonary inflammation. Toxicol. Appl. Pharmacol. 299, 3–7.

Bräu, M., Ma-Hock, L., Hesse, C., Nicoleau, L., Strauss, V., Treumann, S., Wiench, K., Landsiedel, R., Wohlleben, W., 2012. Nanostructured calcium silicate hydrate seeds accelerate concrete hardening: a combined assessment of benefits and risks. Arch. Toxicol. 86, 1077–1087.

Buesen, R., Landsiedel, R., Sauer, U.G., Wohlleben, W., Strauss, V., Groeters, S., Kamp, H.G., Van Ravenzwaay, B., 2014. Effects of SiO_2, ZrO_2, and $BaSO_4$ nanomaterials with or without surface functionalization upon 28-day oral exposure to rats. Arch. Toxicol. 88, 1881–1906.

Burden, N., Aschberger, K., Chaudhry, Q., Dlift, M.J.D., Doak, S.H., Fowler, P., Johnston, H., Landsiedel, R., Rowland, J., Stone, V., 2016. Aligning nanotoxicology with the 3Rs: what is needed to realise the short, medium and long-term opportunities? Nano Today. (Epub ahead of print).

Butz, T., Reinert, T., Pinheiro, T., Moretto, P., Pallon, J., Kiss, A.Z., Stachura, J., Dabros, W., Stachura, Z., Lekki, J., Lekka, M., Hunyadi, J., Biro, T., Sticherling, M., Van Vaeck, L., Van Royen, P., Surlève-Bazeille, J.E., 2007. NANODERM. Quality of skin as a barrier to ultra-fine particles. Final Report. QLK4-CT-2002-02678.

Cassee, F.R., Kreyling, W., Aitken, R., Poland, C., 2015. Dosimetry and toxicology of nanosized particles and fibres. In: Viana, M., (Ed.), Indoor and Outdoor Nanoparticles: Determinants of Release and Exposure Scenarios, The Handbook of Environmental Chemistry. Springer. doi 10.1007/698_2015_415.

Cohen, J.M., DeLoid, G.M., Demokritou, P., 2015. A critical review of in vitro dosimetry for engineered nanomaterials. Nanomed epub ahead of print September 30, 2015.

Cordelli, E., Keller, J., Eleuteri, P., Villani, P., Ma-Hock, L., Schulz, M., Landsiedel, R., Pacchierotti, F., 2016. No genotoxicity in rat blood cells upon 3- or 6-month inhalation exposure to CeO_2 or $BaSO_4$ nanomaterials. Mutagenesis, pii:gew005. (Epub ahead of print).

De Jong, W.H., Hagens, W.I., Krystek, P., Burger, M.C., Sips, A.J., Geertsma, R.E., 2008. Particle size-dependent organ distribution of gold nanoparticles after intravenous administration. Biomaterials 29, 1912–1919.

De Jong, W.H., Van Der Ven, L.T., Sleijffers, A., Park, M.V., Jansen, E.H., Van Loveren, H., Vandebriel, R.J., 2013. Systemic and immunotoxicity of silver nanoparticles in an intravenous 28 days repeated dose toxicity study in rats. Biomaterials 34, 8333–8343.

DeLoid, G., Cohen, J.M., Darrah, T., Pyrgiotakis, G., Wohlleben, W., Demokritou, P., 2014. Estimating effective density of engineered nanomaterials for accurate in vitro dosimetry. ACS Nano 5, 3514.

DHHS (CDC and NIOSH), 2013. Department of Health and Human Services (Center for Disease Control Prevention and National Institute for Occupational Safety and Health): Occupational exposure to carbon nanotubes and nanofibers. Current Intelligence Bulletin 65. Publication No. 2013-145.

DHHS (NIOSH), 2012. Department of Health and Human Services (National Institute for Occupational Safety and Health). General safe practices for working with engineered nanomaterials in research laboratories. Publication No. 2012-147.

Dix, D.J., Houck, K.A., Martin, M.T., Richard, A.M., Setzer, R.W., Kavlock, R.J., 2007. The ToxCast program for prioritizing toxicity testing of environmental chemicals. Toxicol. Sci. 95, 5–12.

Doak, S.H., Manshian, B., Jenkins, G.J.S., Singh, N., 2012. In vitro genotoxicity testing strategy for nanomaterials and the adaptation of current OECD guidelines. Mutat. Res. 745, 104–111.

Dobrovolskaia, M.A., Aggarwal, P., Hall, J.B., McNeil, S.E., 2008. Preclinical studies to understand nanoparticle interaction with the immune system and its potential effects on nanoparticle biodistribution. Mol. Pharm. 58, 487–495.

Donaldson, K., Murphy, F., Schinwald, A., Duffin, R., Poland, C.A., 2011. Identifying the pulmonary hazard of high aspect ratio nanoparticles to enable their safety-by-design. Nanomedicine 6, 143–156.

Donaldson, K., Tran, L., Jimenez, L.A., Duffin, R., Newby, D.E., Mills, N., MacNee, W., Stone, V., 2005. Combustion-derived nanoparticles: a review of their toxicology following inhalation exposure. Part. Fibre Toxicol. 2, 10.

ECETOC, 2013. European Centre for the Ecotoxicology and Toxicology of Chemicals. Technical Report 122. Poorly soluble particles. Lung overload.

ECHA, 2012. European Chemicals Agency. Guidance on information requirements and chemical safety assessment. Appendix R7-1 Recommendations for nanomaterials applicable to Chapter R7a Endpoint specific guidance. ECHA-12-G-03-EN.

ECHA, 2013. European Chemicals Agency. Grouping of substances and read-across approach. Part I. Introductory note. ECHA-13-R-02-EN.

ECHA, 2014. European Chemicals Agency. Human health and environmental exposure assessment and risk characterization of nanomaterials. Best practice for REACH registrants. Third GAARN meeting—Helsinki, September 30, 2013. ECHA-14-R-10-EN.

EFSA: European Food Safety Authority, 2009. Scientific opinion on the potential risks arising from nanoscience and nanotechnologies on food and feed safety. EFSA J. 958, 1–39.

EFSA: European Food Safety Authority, 2011. Guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain. EFSA J. 9, 2140.

Elder, A., Oberdörster, G., 2006. Translocation and effects of ultrafine particles outside of the lung. Clin. Occup. Environ Med. 5, 785–796.

Elder, A., Gelein, R., Finkelstein, J.N., Driscoll, K.E., Harkema, J., Oberdörster, G., 2005. Effects of subchronically inhaled carbon black in three species. I. Retention kinetics, lung inflammation, and histopathology. Toxicol. Sci. 88, 614–629.

EPA, 2015. Environmental Protection Agency. Proposed rule—chemical substances when manufactured or processed as nanoscale materials; TSCA reporting and recordkeeping requirements. FRL: 9920-90. Docket No. EPA-HQ-OPPT-2010-0572. Prepublication copy notice, March 20, 2015.

EU Commission, 2011. Recommendation 2011/696/EU on the definition of nanomaterial. OJ L 275:38, October 18, 2011.

Fabian, E., Landsiedel, R., Ma-Hock, L., Wiench, K., Wohlleben, W., Van Ravenzwaay, B., 2008. Tissue distribution and toxicity of intravenously administered titanium dioxide nanoparticles in rats. Arch. Toxicol. 82, 151–157.

Gamer, A.O., Leibold, E., Van Ravenzwaay, B., 2006. The in vitro absorption of microfine zinc oxide and titanium dioxide through porcine skin. Toxicol In Vitro 20, 301–307.

Gebel, T., 2012. Small difference in carcinogenic potency between GBP nanomaterials and GBP micromaterials. Arch. Toxicol. 86, 995–1007.

Gebel, T., Foth, H., Damm, G., Feyberger, A., Kramer, P.J., Lilienblum, W., Röhl, C., Schupp, T., Weiss, C., Wollin, K.M., Hengstler, J.G., 2014. Manufactured nanomaterials: categorization and approaches to hazard assessment. Arch. Toxicol. 88, 2191–2211.

Gebel, T., Landsiedel, R., 2013. Inhalte der Sicherheitsforschung: Langzeitwirkungen biobeständiger Nanostäube. Gefahrstoffe Reinhaltung der Luft 10, 414.

Geraets, L., Oomen, A.G., Krystek, P., Jacobsen, N.R., Wallin, H., Laurentie, M., Verharen, H.W., Brandon, E.F., De Jong, W.H., 2014. Tissue distribution and elimination after oral and intravenous administration of different titanium dioxide nanoparticles in rats. Part. Fibre Toxicol. 11, 30.

Geraets, L., Oomen, A.G., Schroeter, J.D., Coleman, V.A., Cassee, F.R., 2012. Tissue distribution of inhaled micro- and nano-sized cerium oxide particles in rats: results from a 28-day exposure study. Toxicol. Sci. 127, 463–473.

Godwin, H., Nameth, C., Avery, D., Bergeson, L.L., Bernard, D., Beryt, E., Boyes, W., Brown, S., Clippinger, A.J., Cohen, Y., Doa, M., Hendren, C.O., Holden, P., Houck, K., Kane, A.B., Klaessig, F., Kodas, T., Landsiedel, R., Lynch, I., Malloy, T., Miller, M.B., Muller, J., Oberdörster, G., Petersen, E.J., Pleus, R.C., Sayre, P., Stone, V., Sullivan, K.M., Tentschert, J., Wallis, P., Nel, A.E., 2015. Nanomaterial categorization for assessing risk potential to facilitate regulatory decision-making. ACS Nano 9, 3409–3417.

Gosens, I., Bokkers, B.G.H., Zanella, M., Manodori, L., Hristozov, D., De Jong, W.H., Stone, V., Cassee, F.R., 2015. Pulmonary effects of nano copper oxide in a short-term inhalation study (STIS). Poster No. 2348 at the 54th Annual Meeting of the Society of Toxicology (SOT), San Diego, CA, USA, March 22–26, 2015.

Greim, H., Ziegler-Skylakakis, K., 2007. Risk assessment for biopersistent granular particles. Inhal. Toxicol. 19 (Suppl. 1), 199–204.

Hankin, S.M., Peters, S.A.K., Poland, C.A., Foss Hansen, S., Holmqvist, J., Ross, B.L., Varet, J., Aitken, R.J., 2011. Specific advice on fulfilling information requirements for nanomaterials under REACH (RIP-oN 2)–final project report. REACH-NANO consultation. RNC/RIP-oN2/FPR/1/FINAL.

Hartwig, A., 2014. General Threshold Limit Value for Dust (R Fraction) (Biopersistent Granular Dusts). Wiley-VCH, Available from: http://onlinelibrary.wiley.com/doi/10.1002/3527600418.

Heinrich, U., Fuhst, R., Rittinghausen, S., Creutzenberg, O., Bellmann, B., Koch, M., Levsen, K., 1995. Chronic inhalation exposure of Wistar rats and two different strains of mice to diesel engine exhaust, carbon black, and titanium dioxide. Inhal. Toxicol. 7, 533–556.

Henderson, R.F., Driscoll, K.E., Harkema, J.R., Lindenschmidt, R.C., Chang, I.Y., Maples, K.R., Barr, E.B., 1995. A comparison of the inflammatory response of the lung to inhaled versus instilled particles in F344 rats. Fundam. Appl. Toxicol. 24, 183–197.

Hendren, C.O., Lowry, G.V., Unrine, J.M., Wiesner, M.R., 2015. A functional assay-based strategy for nanomaterial risk forecasting. Sci. Total Environ. 536, 1029–1037.

Heringa, M.B., Geraets, L., Van Eijkeren, J.C.H., Vandebriel, R.J., De Jong, W.H., Oomen, A.G., 2016. Risk assessment of titanium dioxide nanoparticles through oral exposure, including toxicokinetic considerations. Nanotoxicology 10, 1515–1525.

Hinderliter, P.M., Minard, K.R., Orr, G., Chrisler, W.B., Thrall, B.D., Pounds, J.G., Teeguarden, J.G., 2010. ISDD: A computational model of particle sedimentation, diffusion and target cell dosimetry for in vitro toxicity studies. Part. Fibre Toxicol. 7, 36.

Hirth, S., Cena, L., Cox, G., Tomovic´, Z., Peters, T., Wohlleben, W., 2013. Scenarios and methods that induce protruding or released CNTs after degradation of nanocomposite materials. J Nanopart Res 15, 1504.

Hofmann, T., Ma-Hock, L., Strauss, V., Treumann, S., Rey-Moreno, M., Neubauer, N., Wohlleben, W., Gröters, S., Wiench, K., Van Ravenzwaay, B., Landsiedel, R., 2016. Comparative short term inhalation toxicity of five DPP pigments and two iron-oxide based pigments. Manuscript in preparation.

Hofmann, T., Schneider, S., Wolterbeek, A., Van De Sandt, H., Landsiedel, R., Van Ravenzwaay, B., 2015. Prenatal toxicity of synthetic amorphous silica nanomaterial in rats. Reprod. Toxicol. 56, 141–146.

Horev-Azaria, L., Baldi, G., Beno, D., Bonacchi, D., Golla-Schindler, U., Kirkpatrick, J.C., Kolle, S., Landsiedel, R., Maimon, O., Marche, P.N., Ponti, J., Romano, R., Rossi, F., Sommer, D., Uboldi, C., Unger, R.E., Villiers, C., Korenstein, R., 2013. Predictive toxicology of cobalt ferrite nanoparticles: comparative in-vitro study of different cellular models using methods of knowledge discovery from data. Part. Fibre Toxicol. 10, 32.

Hougaard, K.S., Campagnolo, L., Chavatte-Palmer, P., Tarrade, A., Rousseau-Ralliard, D., Valentino, S., Park, M.V., De Jong, W.H., Wolterink, G., Piersma, A.H., Ross, B.L., Hutchison, G.R., Hansen, J.S., Vogel, U., Jackson, P., Slama, R., Pietroiusti, A., Cassee, F.R., 2015. A perspective on the developmental toxicity of inhaled nanoparticles. Reprod. Toxicol. 56, 118–140.

Husain, M., Kyjovska, Z.O., Bourdon-Lacombe, J., Saber, A.T., Jensen, K.A., Jacobsen, N.R., Williams, A., Wallin, H., Halappanavar, S., Vogel, U., Yauka, C.L., 2015. Carbon black nanoparticles induce biphasic gene expression changes associated with inflammatory responses in the lungs of C57BL/6 mice following a single intratracheal instillation. Toxicol. Appl. Pharmacol. 289, 573–588.

IARC, 2010. International Agency for Research on Cancer monographs on the evaluation of carcinogenic risks to humans, IARC Lyon, France 93, 166–171.

ISO, 2014. International Standardization Organization. Technical specification. Nanotechnologies—occupational risk management applied to engineered nanomaterials—Part 2 Use of the control banding approach, first ed. ISO/TS 12901-2.

ISO, 2016. International Standardization Organization. Website of the Technical Committee ISO TC 229 Nanotechnologies, Available from: http://www.iso.org/iso/iso_technical_committee?commid=381983

Jähnel, J., 2015. Conceptual questions and challenges associated with the traditional risk assessment paradigm for nanomaterials. Nanoethics 9, 261–276.

Keller, J., Wohlleben, W., Ma-Hock, L., Groeters, S., Küttler, K., Strauss, V., Wiench, K.E., Herden, C., Oberdörster, G., Van Ravenzwaay, B., Landsiedel, R., 2014. Time-course of lung retention and toxicity of inhaled particles: short-term exposure to nano-Ceria. Arch. Toxicol. 88, 2033–2059.

Kim, Y.S., Kim, J.S., Cho, H.S., Rha, D.S., Kim, J.M., Park, J.D., Choi, B.S., Lim, R., Chang, H.K., Chung, Y.H., Kwon, I.H., Jeong, J., Han, B.S., Yu, I.J., 2008. Twenty-eight-day oral toxicity, genotoxicity, and gender-related tissue distribution of silver nanoparticles in Sprague-Dawley rats. Inhal. Toxicol. 20, 575–583.

Kishore, A.S., Surekha, P., Murthy, P.B., 2009. Assessment of the dermal and ocular irritation potential of multi-walled carbon nanotubes by using in vitro and in vivo methods. Toxicol. Lett. 191, 268–274.

Klein, C.L., Wiench, K., Wiemann, M., Ma-Hock, L., Van Ravenzwaay, B., Landsiedel, R., 2012. Hazard identification of inhaled nanomaterials: making use of short-term inhalation studies. Arch. Toxicol. 86, 1137–1151.

Kolle, S.N., Sauer, U.G., Rey-Moreno, M.C., Teubner, W., Wohlleben, W., Landsiedel, R., 2016. Eye irritation testing of nanomaterials using the EpiOcular™ eye irritation test and the Bovine Corneal Opacity and Permeability assay. Part. Fibre Toxicol. 13, 18.

Konduru, N., Keller, J., Ma-Hock, L., Groeters, S., Landsiedel, R., Donaghey, T.C., Brain, J.D., Wohlleben, W., Molina, R.M., 2014. Biokinetics and effects of barium sulfate nanoparticles. Part. Fibre Toxicol. 11, 55.

Krewski, D., Andersen, M.E., Mantus, E., Zeise, L., 2009. Toxicity testing in the 21st century: implications for human health risk assessment. Risk Anal. 29, 474–479.

Kreyling, W.G., Semmler-Behnke, M., Seitz, J., Scymczak, W., Wenk, A., Mayer, P., Takenaka, S., Oberdörster, G., 2009. Size dependence of the translocation of inhaled iridium and carbon nanoparticle aggregates from the lung of rats to the blood and secondary target organs. Inhal. Toxicol. 21 (Suppl. 1), 55–60.

Kroll, A., Dierker, C., Rommel, C., Hahn, D., Wohlleben, W., Schulze-Isfort, C., Göbbert, C., Voetz, M., Hardinghaus, F., Schnekenburger, J., 2011. Cytotoxicity screening of 23 engineered nanomaterials using a test matrix of ten cell lines and three different assays. Part. Fibre Toxicol. 8, 9.

Küchler, S., Radowski, M.R., Blaschke, T., Dathe, M., Plendl, J., Haag, R., Schäfer-Korting, M., Kramer, K.D., 2009. Nanoparticles for skin penetration enhancement —a comparison of a dendritic core-multishell-nanotransporter and solid lipid nanoparticles. Eur. J. Pharm. Biopharm. 71, 243–250.

Kuempel, E.D., Attfield, M.D., Stayner, L.T., Castranova, V., 2014. Human and animal evidence supports lower occupational exposure limits for poorly-soluble respirable particles: Letter to the editor re: 'Low-toxicity dusts: Current exposure guidelines are not sufficiently protective' by Cherrie, Brosseau, Hay and Donaldson. Ann. Occup. Hyg. 8, 1205–1208.

Kuempel, E.D., Castranova, V., Geraci, C.L., Schulte, P.A., 2012. Development of risk-based nanomaterial groups for occupational exposure control. J. Nanopart. Res. 14, 1029.

Kuempel, E.D., Sweeney, L.M., Morris, J.B., Jarabek, A.M., 2015. Advances in inhalation dosimetry models and methods for occupational risk assessment and exposure limit derivation. J. Occup. Environ. Hyg. 12 (Suppl. 1), S18–S40.
Kuhlbusch, T.A.J., Asbach, C., Fissan, H., Göhler, D., Stintz, M., 2013. Nanoparticle exposure at nanotechnology workplaces: a review. Part. Fibre Toxicol. 8, 22.
Kyjovska, Z.O., Jacobsen, N.R., Saber, A.T., Bengtson, S., Jackson, P., Wallin, H., Vogel, U., 2015. DNA strand breaks, acute phase response and inflammation following pulmonary exposure by instillation to the diesel exhaust particle NIST1650b in mice. Mutagenesis 30, 499–507.
Lademann, J., Richter, H., Teichmann, A., Otberg, N., Blume-Peytavi, U., Luengo, J., Weiss, B., Schaefer, U.F., Lehr, C.M., Wepf, R., Sterry, W., 2007. Nanoparticles–an efficient carrier for drug delivery into the hair follicles. Eur. J. Pharmaceut. Biopharmaceut. 66, 159–164.
Lai, D.Y., 2012. Toward toxicity testing of nanomaterials in the 21st century: a paradigm for moving forward. Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol. 4, 1–15.
Landsiedel, R., 2015. Concern-driven integrated approaches for the grouping, testing and assessment of nanomaterials. Environ. Pollut. 218, 1376–1380.
Landsiedel, R., Fabian, E., Ma-Hock, L., Wohlleben, W., Wiench, K., Oesch, F., Van Ravenzwaay, B., 2012b. Toxico-/biokinetics of nanomaterials. Arch. Toxicol. 86, 1021–1060.
Landsiedel, R., Kapp, M.D., Schulz, M., Wiench, K., Oesch, F., 2009. Genotoxicity investigations on nanomaterials: methods, preparation and characterization of test material, potential artifacts and limitations—many questions, some answers. Mutat. Res. 681, 241–258.
Landsiedel, R., Ma-Hock, L., Kroll, A., Hahn, D., Schnekenburger, J., Wiench, K., Wohlleben, W., 2010a. Testing metal oxide nanomaterials for human safety. Adv. Mater. 22, 2601–2627.
Landsiedel, R., Ma-Hock, L., Haussmann, H.J., Van Ravenzwaay, B., Kayser, M., Wiench, K., 2012a. Inhalation studies for the safety assessment of nanomaterials: status quo and the way forward. Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol. 4, 399–413.
Landsiedel, R., Ma-Hock, L., Hofmann, T., Wiemann, M., Strauss, V., Treumann, S., Wohlleben, W., Groeters, S., Wiench, K., Van Ravenzwaay, B., 2014a. Application of short-term inhalation studies to assess the inhalation toxicity of nanomaterials. Part. Fibre Toxicol. 11, 16.
Landsiedel, R., Ma-Hock, L., Van Ravenzwaay, B., Schulz, M., Wiench, K., Champ, S., Schulte, S., Wohlleben, W., Oesch, F., 2010b. Gene toxicity studies on titanium dioxide and zinc oxide nanomaterials used for UV-protection in cosmetic formulations. Nanotoxicology 4, 364–381.
Landsiedel, R., Sauer, U.G., Ma-Hock, L., Schnekenburger, J., Wiemann, M., 2014b. Pulmonary toxicity of nanomaterials: a critical comparison of published in vitro assays with in vivo inhalation or instillation studies. Nanomedicine 9, 2557–2585.
Lee, J.H., Lee, S.B., Bae, G.N., Jeon, K.S., Yoon, J.U., Ji, J.H., Sung, J.H., Lee, B.G., Lee, J.H., Yang, J.S., Kim, H.Y., Kang, C.S., Yu, I.J., 2010. Exposure assessment of carbon nanotube manufacturing workplaces. Inhal. Toxicol. 22, 369–381.
Lucarelli, M., Gatti, A.M., Savarino, G., Quattroni, P., Martinelli, L., Monari, E., Boraschi, D., 2004. Innate defence functions of macrophages can be biased by nano-sized ceramic and metallic particles. Eur. Cytokine Netw. 15, 339–346.
Lynch, I., Weiss, C., Valsami-Jones, E., 2014. A strategy for grouping of nanomaterials based on key physico-chemical descriptors as a basis for safer-by-design nanomaterials. Nano Today 9, 266–270.
Ma-Hock, L., Burkhardt, S., Strauss, V., Gamer, A.O., Wiench, K., Van Ravenzwaay, B., Landsiedel, R., 2009a. Development of a short-term inhalation test in the rat using nano-titanium dioxide as a model substance. Inhal. Toxicol. 21, 102–118.
Ma-Hock, L., Gamer, A.O., Landsiedel, R., Leibold, E., Frechen, T., Sens, B., Linsenbuehler, M., Van Ravenzwaay, B., 2007. Generation and characterization of test atmospheres with nanomaterials. Inhal. Toxicol. 19, 833–848.
Ma-Hock L, Keller J, Groeters S, Strauss V, Van Ravenzwaay B, Landsiedel R., 2014. A life-time carcinogenicity study with two nanomaterials. Abstracts of the 43rd Annual Meeting of the European Environmental Mutagen Society Lancaster University, UK, July 6–10, 2014. Mutagen 29, 530.
Ma-Hock, L., Landsiedel, R., Wiench, K., Geiger, D., Strauss, V., Gröters, S., Van Ravenzwaay, B., Gerst, M., Wohlleben, W., Scherer, G., 2012. Short-term rat inhalation study with aerosols of acrylic ester-based polymer dispersions containing a fraction of nanoparticles. Int. J. Toxicol. 31, 46–57.
Ma-Hock, L., Strauss, V., Treumann, S., Kuttler, K., Wohlleben, W., Hofmann, T., Groeters, S., Wiench, K., Van Ravenzwaay, B., Landsiedel, R., 2013. Comparative inhalation toxicity of multi-wall carbon nanotubes, graphene, graphite nanoplatelets and low surface carbon black. Part. Fibre Toxicol. 10, 23.

Ma-Hock, L., Treumann, S., Strauss, V., Brill, S., Luizi, F., Mertler, M., Wiench, K., Gamer, A.O., Van Ravenzwaay, B., Landsiedel, R., 2009b. Inhalation toxicity of multiwall carbon nanotubes in rats exposed for 3 months. Toxicol. Sci. 112, 468–481.

Mao, L., Hu, M., Pan, B., Xie, Y., Petersen, E.J, 2016. Biodistribution and toxicity of radio-labeled few layer graphene in mice after intratracheal instillation. Part. Fibre Toxicol. 13, 7.

MAK, 2013. Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area. Nanomaterials—Report. Deutsche Forschungsgemeinschaft—German Research Foundation (Ed.). ISBN 978-3-527-33571-8. Wiley-VCH, 2013.

Maser, E., Schulz, M., Sauer, U.G., Wiemann, M., Ma-Hock, M., Wohlleben, W., Hartwig, A., Landsiedel, R., 2015. In vitro and in vivo genotoxicity investigations of differently sized amorphous SiO_2 nanomaterials. Mutat. Res. 794, 57–74.

Meek, M.E., Boobis, A.R., Crofton, K.M., Heinemeyer, G., Van Raaij, M., Vickers, C., 2011. Risk assessment of combined exposure to multiple chemicals: a WHO/IPCS framework. Regul. Toxicol. Pharmacol. 60 (Suppl. 1), S1–S14.

Mercer, T.T., 1967. On the role of particle size in the dissolution of lung burdens. Health Phys. 13, 1211–1222.

Mohamud, R., Xiang, S.D., Selomulya, C., Rolland, J.M., O'Hehir, R.E., Hardy, C.L., Plebanski, M., 2014. The effects of engineered nanoparticles on pulmonary immune homeostasis. Drug Metab. Rev. 46, 176–190.

Molina, R.M., Konduru, N.V., Jimenez, R.J., Wohlleben, W., Brain, J.D., 2014. Bioavailability, distribution and clearance of tracheally instilled, gavaged or injected cerium dioxide nanoparticles and ionic cerium. Environ. Sci. Nano 1, 561.

Monteiro-Riviere, N.A., 2016. Safety of nanoparticle skin penetration. In: Dragicevic, N., Maibach, H.I. (Eds.), Percutaneous Penetration Enhancers—Chemical Methods in Penetration Enhancement: Nanocarriers. Springer, Berlin, Heidelberg.

Monteiro-Riviere, N.A., Inman, A.O., Zhang, L.W., 2009. Limitations and relative utility of screening assays to assess engineered nanoparticle toxicity in a human cell line. Toxicol. Appl. Pharmacol. 234, 222–235.

Monteiro-Riviere, N.A., Wiench, K., Landsiedel, R., Schulte, S., Inman, A.O., Riviere, J.E., 2011. Safety evaluation of sunscreen formulations containing titanium dioxide and zinc oxide nanoparticles in UVB sunburned skin: an in vitro and in vivo study. Toxicol. Sci. 123, 264–280.

Moreno-Horn, M., Gebel, T., 2014. Granular biodurable nanomaterials: no convincing evidence for systemic toxicity. Crit. Rev. Toxicol. 44, 849–875.

Morfeld, P., Bruch, J., Levy, L., Ngiewih, Y., Chaudhuri, I., Muranko, H.J., Myerson, R., McCunney, R.J., 2015. Translational toxicology in setting occupational exposure limits for dusts and hazard classification—a critical evaluation of a recent approach to translate dust overload findings from rats to humans. Part. Fibre Toxicol. 12, 3.

Morfeld, P., Bruch, J., Levy, L., Ngiewih, Y., Chaudhuri, I., Muranko, H.J., Myerson, R., McCunney, R.J., 2016. Response to the Reply on behalf of the 'Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area' (MAK Commission) by A. Hartwig (KIT). Part. Fibre Toxicol. 13, 1.

Morfeld, P., Treumann, S., Ma-Hock, L., Bruch, J., Landsiedel, R., 2012. Deposition behavior of inhaled nanostructured TiO_2 in rats: fractions of particle diameter below 100 nm (nanoscale) and the slicing bias of transmission electron microscopy. Inhal. Toxicol. 24, 939–951, Erratum in: Inhal. Toxicol. 25, 307, 2013.

Morimoto, Y., Izumi, H., Yoshiura, Y., Tomonaga, T., Lee, B.W., Okada, T., Oyabu, T., Myojo, T., Kawai, K., Yatera, K., Shimada, M., Kubo, M., Yamamoto, K., Kitajima, S., Kuroda, E., Horie, M., Kawaguchi, K., Sasaki, T., 2015. Comparison of pulmonary inflammatory responses following intratracheal instillation and inhalation of nanoparticles. Nanotoxicology 11, 1–12.

Morrow, P.E., 1988. Possible mechanisms to explain dust overloading of the lungs. Fundam. Appl. Toxicol. 10, 369–384.

Mortensen, L.J., Oberdörster, G., Pentland, A.P., Delouise, L.A., 2008. In vivo skin penetration of quantum dot nanoparticles in the murine model: the effect of UVR. Nano Lett. 8, 2779–2787.

Muller, J., Delos, M., Panin, N., Rabolli, V., Huaux, F., Lison, D., 2009. Absence of carcinogenic response to multiwall carbon nanotubes in a 2-year bioassay in the peritoneal cavity of the rat. Toxicol. Sci. 110, 442–448.

Nel, A.E., Nasser, E., Godwin, H., Avery, D., Bahadori, T., Bergeson, L., Beryt, E., Bonner, J.C., Boverhof, D., Carter, J., Castranova, V., Deshazo, J.R., Hussain, S.M., Kane, A.B., Klaessig, F., Kuempel, E., Lafranconi, M., Landsiedel, R., Malloy, T., Miller, M.B., Morris, J., Moss, K., Oberdörster, G., Pinkerton, K., Pleus, R.C., Shatkin, J.A., Thomas, R., Tolaymat, T., Wang, A., Wong, J., 2013a. A multi-stakeholder perspective on the use of alternative test strategies for nanomaterial safety assessment. ACS Nano 7, 6422–6433.

Nel, A.E., Parak, W.J., Chan, W.C., Xia, T., Hersam, M.C., Brinker, C.J., Zink, J.I., Pinkerton, K.E., Baer, D.R., Weiss, P.S., 2015. Where are we heading in nanotechnology environmental health and safety and materials characterization? ACS Nano 9, 5627–5630.

Nel, A.E., Xia, T., Meng, H., Wang, X., Lin, S., Ji, Z., Zhang, H., 2013b. Nanomaterial toxicity testing in the 21st century: use of a predictive toxicological approach and high-throughput screening. Acc. Chem. Res. 46, 607–621.

Nikula, K.J., Snipes, M.B., Barr, E.B., Griffith, W.C., Henderson, R.F., Mauderly, J.L., 1995. Comparative pulmonary toxicities and carcinogenicities of chronically inhaled diesel exhaust and carbon black in F344 rats. Fundam. Appl. Toxicol. 25, 80–94.

Oberdörster, G., Ferin, J., Lehnert, B.E., 1994. Correlation between particle size, in vivo particle persistence, and lung injury. Environ. Health Perspect. 102 (Suppl. 5), 173.

Oberdörster, G., Oberdörster, E., Oberdörster, J., 2005. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environ. Health Perspect. 113, 823–839.

OECD, 2010. List of manufactured nanomaterials and list of endpoints for phase one of the sponsorship programme for the testing of manufactured nanomaterials: Revision. Series on the safety of manufactured nanomaterials. ENV/JM/MONO(2010)46. December 1, 2010.

OECD, 2012a. Important issues on risk assessment of manufactured nanomaterials. Series on the safety of manufactured nanomaterials. No. 33. ENV/JM/MONO(2012)8. March 28, 2012.

OECD, 2012b. Appendix I. Collection of working definitions. Available from: http://www.oecd.org/chemicalsafety/testing/49963576.pdf

OEHHA, 2008. Office of Environmental Health Hazard Assessment of the California Environmental Protection Agency (EPA). Draft technical support document (TSD) for noncancer reference exposure levels (RELs), SRP2. Appendix F: Estimating human equivalent concentrations using the US EPA default approach.

Oesch, F., Landsiedel, R., 2012. Genotoxicity investigations on nanomaterials. Arch. Toxicol. 86, 985–994.

Oomen, A.G., Bleeker, E.A., Bos, P.M., Van Broekhuizen, F., Gottardo, S., Groenewold, M., Hristozov, D., Hund-Rinke, K., Irfan, M.A., Marcomini, A., Peijnenburg, W.J., Rasmussen, K., Jiménez, A.S., Scott-Fordsmand, J.J., Van Tongeren, M., Wiench, K., Wohlleben, W., Landsiedel, R., 2015. Grouping and read-across approaches for risk assessment of nanomaterials. Int. J. Environ. Res. Public Health 12, 13415–13434.

Oomen, A.G., Bos, P.M.J., Fernandes, T.F., Hund-Rinke, K., Boraschi, D., Byrne, H.J., Aschberger, K., Gottardo, S., Van Der Kammer, F., Kühnel, D., Hristozov, D., Marcomini, A., Migliore, L., Scott-Fordsmand, J., Wick, P., Landsiedel, R., 2014a. Concern-driven integrated approaches to nanomaterial testing and assessment—Report of the NanoSafety Cluster Working Group 10. Nanotoxicology 8, 334–348.

Oomen, A., Bos, P., Landsiedel, R., 2014b. Concern-driven safety assessment of nanomaterials: an integrated approach using material properties, hazard, biokinetic and exposure data and considerations on grouping and read-across. In: Wohlleben, W., Kuhlbusch, T., Lehr, C.-M., Schnekenburger, J. (Eds.), Safety of Nanomaterials Along Their Lifecycle: Release, Exposure and Human Hazards. Taylor & Francis, (Chapter 16).

Packroff, R., Gebel, T., 2014. A strategic approach on testing and information requirements for nanomaterials. Poster at the Topical Scientific Workshop on Regulatory Challenges in Risk Assessment of Nanomaterials, Helsinki, Finland, October 23, 2014.

Park, E.J., Lee, G.H., Han, B.S., Lee, B.S., Lee, S., Cho, M.H., Kim, J.H., Kim, D.W., 2015. Toxic response of graphene nanoplatelets in vivo and in vitro. Arch. Toxicol. 89, 1557–1568.

Pauluhn, J., 2014. Derivation of occupational exposure levels (OELs) of low-toxicity isometric biopersistent particles: how can the kinetic lung overload paradigm be used for improved inhalation toxicity study design and OEL-derivation? Part. Fibre Toxicol. 11, 72.

Pfuhler, S., Elespuru, R., Aardema, M.J., Doak, S.H., Donner, M., Honma, M., Kirsch-Volders, M., Landsiedel, R., Manjanatha, M., Singer, T., Kim, J.H., 2013. Genotoxicity of nanomaterials: refining strategies and tests for hazard identification. Environ. Mol. Mutagen. 54, 229–239.

Poland, C.A., Duffin, R., Donaldson, K., 2009. High aspect nanoparticles and the fibre pathogenicity paradigm. In: Sahu, S.C., Casciano, D.A. (Eds.), Nanotoxicity: From In Vivo and In Vitro Models to Health Risks. Wiley-VCH Verlag GmbH, Germany, (Chapter 4).

Potthoff, A., Weil, M., Meißner, T., Kühnel, D., 2015. Towards sensible toxicity testing for nanomaterials: proposal for the specification of test design. Sci. Technol. Adv. Mater. 16, 065006.

Prabhakar, U., Maeda, H., Jain, R.K., Sevick-Muraca, E.M., Zamboni, W., Farokhzad, O.C., Barry, S.T., Gabizon, A., Grodzinski, P., Blakey, D.C., 2013. Challenges and key considerations of the enhanced permeability and retention effect for nanomedicine drug delivery in oncology. Cancer Res. 73, 2412–2417.

Rittinghausen, S., Hackbarth, A., Creutzenberg, O., Ernst, H., Heinrich, U., Leonhardt, A., Schaudien, D., 2014. The carcinogenic effect of various multi-walled carbon nanotubes (MWCNTs) after intraperitoneal injection in rats. Part. Fibre Toxicol. 11, 59.

Rovida, C., Alépée, N., Api, A.M., Basketter, D.A., Bois, F.Y., Caloni, F., Corsini, E., Daneshian, M., Eskes, C., Ezendam, J., Fuchs, H., Hayden, P., Hegele-Hartung, C., Hoffmann, S., Hubesch, B., Jacobs, M.N., Jaworska, J., Kleensang, A., Kleinstreuer, N., Lalko, J., Landsiedel, R., Lebreux, F., Luechtefeld, T., Locatelli, M., Mehling, A., Natsch, A., Pitchford, J.W., Prater, D., Prieto, P., Schepky, A., Schüürmann, G., Smirnova, L., Toole, C., Van Vliet, E., Weisensee, D., Hartung, T., 2015. Integrated testing strategies (ITS) for safety assessment. ALTEX 32, 25–40.

Rudén, C., Hansson, S.O., 2010. Registration, evaluation, and authorization of chemicals (REACH) is but the first step-how far will it take us? Six further steps to improve the European chemicals legislation. Environ. Health Perspect. 118, 6–10.

Russell, W.M.S., Burch, R.L., 1959. The Principles of Humane Experimental Technique. Methuen, London, UK.

Samberg, M.E., Oldenburg, S.J., Monteiro-Riviere, N.A., 2010. Evaluation of silver nanoparticle toxicity in skin in vivo and keratinocytes in vitro. Environ. Health Perspect. 118, 407–413.

Sauer, U.G., Aumann, A., Ma-Hock, L., Landsiedel, R., Wohlleben, W., 2014. Influence of dispersive agent on nanomaterial agglomeration and implications for biological effects in vivo or in vitro. Toxicol. In Vitro 29, 182–186.

SCCS, 2012. Scientific Committee on Consumer Safety. Guidance on the safety assessment of nanomaterials in cosmetics. Opinion SCCS/1484/12 adopted at the 15th plenary meeting of the SCCS, June 26–27, 2012.

SCENIHR, 2006. Scientific Committee on Emerging and Newly Identified Health Risks. The appropriateness of existing methodologies to assess the potential risks associated with engineered and adventitious products of nanotechnologies, March 10, 2006.

SCENIHR, 2007. The Scientific Committee on Emerging and Newly Identified Health Risks. The appropriateness of the risk assessment methodology in accordance with the technical guidance documents for new and existing substances for assessing the risks of nanomaterials, June 21–22, 2007.

SCENIHR, 2009. The Scientific Committee on Emerging and Newly Identified Health Risks. Opinion on risk assessment of products of nanotechnologies, January 19, 2009.

SCENIHR, 2015. The Scientific Committee on Emerging and Newly Identified Health Risks. Opinion on the guidance on the determination of potential health effects of nanomaterials used in medical devices, January 6, 2015.

Schäfer-Korting, M., Romero, E.L., 2016. Core-shell nanotransporters for the skin. In: Dragicevic, N., Maibach, H.I. (Eds.), Percutaneous Penetration Enhancers—Chemical Methods in Penetration Enhancement: Nanocarriers. Springer, Berlin, Heidelberg.

SCHER, SCENIHR, SCCS, 2013. Scientific Committee on Health and Environmental Risks, Scientific Committee on Emerging and Newly Identified Health Risks, Scientific Committee on Consumer Safety. Addressing the new challenges for risk assessment, Opinion adopted via written procedure, March 2013.

Schulte, P.A., Murashov, V., Zumwalde, R., Kuempel, E.D., Geraci, C.L., 2010. Occupational exposure limits for nanomaterials: state of the art. J. Nanopart. Res. 12, 1971–1987.

Schulz, M., Ma-Hock, L., Brill, S., Strauss, V., Treumann, S., Gröters, S., Van Ravenzwaay, B., Landsiedel, R., 2012. Investigation on the genotoxicity of different sizes of gold nanoparticles. Mutat. Res. 745, 51–57.

Sellers, K., Deleebeeck, N.M.E., Messiaen, M., Jackson, M., Bleeker, E.A.J., Sijm, D.T.H.M., Van Broekhuizen, F.A., 2015. Grouping nanomaterials. A strategy towards grouping and read-across. National Institute for Public Health and the Environment (RIVM) Report 2015-0061.

Slob, W., 2002. Dose-response modeling of continuous endpoints. Toxicol. Sci. 66, 298–312.

Stark, W.J., Stoessel, P.R., Wohlleben, W., Hafner, A., 2015. Industrial applications of nanoparticles. Chem. Soc. Rev. 44, 5793–5805.

Stern, S.T., Adiseshaiah, P.P., Crist, R.M., 2012. Autophagy and lysosomal dysfunction as emerging mechanisms of nanomaterial toxicity. Part. Fibre Toxicol. 9, 20.

Stone, V., Pozzi-Mucelli, S., Tran, L., Aschberger, K., Sabella, S., Vogel, U., Poland, C., Balharry, D., Fernandes, T., Gottardo, S., Hankin, S., Hartl, M.G., Hartmann, N., Hristozov, D., Hund-Rinke, K., Johnston, H., Marcomini, A., Panzer, O., Roncato, D., Saber, A.T., Wallin, H., Scott-Fordsmand, J.J., 2014. ITS-NANO—prioritising nanosafety research to develop a stakeholder driven intelligent testing strategy. Part. Fibre Toxicol. 11, 9.

Teeguarden, J.G., Hinderliter, P.M., Orr, G., Thrall, B.D., Pounds, J.G., 2007. Particokinetics in vitro: dosimetry considerations for in vitro nanoparticle toxicity assessments. Toxicol. Sci. 95, 300–312.

Tice, R.R., Austin, C.P., Kavlock, R.J., Bucher, J.R., 2013. Improving the human hazard characterization of chemicals: a Tox21 update. Environ. Health Perspect. 121, 756–765.

Toyokuni, S., 2013. Genotoxicity and carcinogenicity risk of carbon nanotubes. Adv. Drug Deliv. Rev. 65, 2098–2110.

Treumann, S., Ma-Hock, L., Groeters, S., Landsiedel, R., Van Ravenzwaay, B., 2013. Additional histopathologic examination of the lungs from a 3-month inhalation toxicity study with multiwall carbon nanotubes in rats. Toxicol. Sci. 134, 103–110.

UBA, 2014. Umweltbundesamt—Federal Environmental Agency. Carcinogenicity and mutagenicity of nanoparticles—assessment of current knowledge as basis for regulation. Project No. (FKZ) 3709 61 220 Report No. (UBA-FB) 001725/E. Submitted by: Schröder, K., Pohlenz-Michel, C., Simetska, N., Voss, J.U., Escher, S., Mangelsdorf I, Fraunhofer ITEM, Hannover. Texte 50.

Van Der Zande, M., Vandebriel, R.J., Groot, M.J., Kramer, E., Rivera, Z.H., Rasmussen, K., Ossenkoppele, J.S., Tromp, P., Gremmer, E.R., Peters, R.J.B., Hendriksen, P.J., Marvin, H.J.P., Peijnenburg, A.A.C.M., Bouwmeester, H., 2014. Sub-chronic toxicity study in rats orally exposed to nanostructured silica. Part. Fibre Toxicol. 11, 8.

Van Der Zande, M., Vandebriel, R.J., Van Doren, E., Kramer, E., Herrera Rivera, Z., Serrano-Rojero, C.S., Gremmer, E.R., Mast, J., Peters, R.J.B., Hollman, P.C.H., Hendriksen, P.J.M., Marvin, H.J.P., Peijnenburg, A.A.C.M., Bouwmeester, H., 2012. Distribution, elimination, and toxicity of silver nanoparticles and silver ions in rats after 28-day oral exposure. ACS Nano 6, 7427–7442.

Van Kesteren, P.C., Cubadda, F., Bouwmeester, H., Van Eijkeren, J.C., Dekkers, S., De Jong, W.H., Oomen, A.G., 2015. Novel insights into the risk assessment of the nanomaterial synthetic amorphous silica, additive E551, in food. Nanotoxicology 9, 442–452.

Vandebriel, R.J., Tonk, E.C., De La Fonteyne-Blankestijn, L.J., Gremmer, E.R., Verharen, H.W., Van Der Ven, L.T., Van Loveren, H., De Jong, W.H., 2014. Immunotoxicity of silver nanoparticles in an intravenous 28-day repeated-dose toxicity study in rats. Part. Fibre Toxicol. 11, 21.

Vinas, R., Nagy, A., Pradeep, P., Merril, S.J., Brown, R.P., Goering, P.L., 2015. Derivative of a provisional tolerable intake for intravenous exposure to silver nanoparticles. Toxicol. Suppl. Toxicol. Sci. 144, Abstr.197.

Wang, X.Z., Yang, Y., Li, R., McGuinnes, C., Adamson, J., Megson, I.L., Donaldson, K., 2014. Principal component and causal analysis of structural and acute in vitro toxicity data for nanoparticles. Nanotoxicology 8, 465–476.

Westphal, G.A., Schremmer, I., Rostek, A., Loza, K., Rosenkranz, N., Brüning, T., Epple, M., Bünger, J., 2015. Particle-induced cell migration assay (PICMA): a new in vitro assay for inflammatory particle effects based on permanent cell lines. Toxicol. In Vitro 29, 997–1005.

Wiemann, M., Vennemann, A., Sauer, U.G., Wiench, K., Ma-Hock, L., Landsiedel, R., 2016. An alveolar macrophage assay for predicting the short-term inhalation toxicity of nanomaterials. J. Nanobiotechnol. 14, 16.

Wijnhoven, S.W.P., Peijnenburg, W.J.G.M., Herberts, C.A., Hagens, W.I., Oomen, A.G., Heugens, E.H.W., Roszek, B., Bisschops, J., Gosens, I., Van De Meent, D., Dekkers, S., De Jong, W.H., Van Zijverden, M., Sips, A.J.A.M., Geertsma, R.E., 2009. Nano-silver—a review of available data and knowledge gaps in human and environmental risk assessment. Nanotoxicology 3, 109–138.

Wills, J.W., Johnson, G.E., Doak, S.H., Soeteman-Hernández, L.G., Slob, W., White, P.A., 2015. Empirical analysis of BMD metrics in genetic toxicology part I: In vitro analyses to provide robust potency rankings and support MOA determinations. Mutagen 31, 255–263.

Wohlleben, W., Brill, S., Meier, M.W., Mertler, M., Cox, G., Hirth, S., Von Vacano, B., Strauss, V., Treumann, S., Wiench, K., Ma-Hock, L., Landsiedel, R., 2011a. On the lifecycle of nanocomposites: comparing released fragments and their in vivo hazards from three release mechanisms and four nanocomposites. Small 7, 2384–2395.

Wohlleben, W., Driessen, M.D., Raesch, S., Schaefer, U.F., Schulze, C., Von Vacano, B., Vennemann, A., Wiemann, M., Ruge, C.A., Platsch, H., Mues, S., Ossig, R., Tomm, J.M., Schnekenburger, J., Kuhlbusch, T.A.J., Luch, A., Lehr, C.-M., Haase, H., 2016. Influence of agglomeration and specific lung lining lipid/protein interaction on short-term inhalation toxicity. Nanotoxicology 10, 970–980.

Wohlleben, W., Kolle, S.N., Hasenkamp, L.C., Böser, A., Vogel, S., Von Vacano, B., Van Ravenzwaay, B., Landsiedel, R., 2011b. Artifacts by marker enzyme adsorption on nanomaterials in cytotoxicity assays with tissue cultures. J. Phys. Conf. Ser. 304, 012061.

Wohlleben, W., Ma-Hock, L., Boyko, V., Cox, G., Egenolf, H., Freiberger, H., Hinrichsen, B., Hirth, S., Landsiedel, R., 2013a. Nanospecific guidance in REACH: a comparative physicalchemical characterization of 15 materials with methodical correlations. J. Ceramic Sci. Technol. 4, 93–104.

Wohlleben, W., Meier, M.W., Vogel, S., Landsiedel, R., Cox, G., Hirth, S., Tomovic´, Ž., 2013b. Elastic CNT-polyurethane nanocomposite: synthesis, performance and assessment of fragments released during use. Nanoscale 5, 369–380.

Wolterbeek, A., Oosterwijk, T., Schneider, S., Landsiedel, R., De Groot, D., Van Ee, R., Wouters, M., Van De Sandt, H., 2015. Oral two-generation reproduction toxicity study with NM-200 synthetic amorphous silica in Wistar rats. Reprod. Toxicol. 56, 147–154.

Xia, X.R., Monteiro-Riviere, N.A., Riviere, J.E., 2010. Skin penetration and kinetics of pristine fullerenes (C60) topically dosed in industrial organic solvents. Toxicol. Appl. Pharmacol. 242, 29–37.

Yokel, R.A., Hussain, S., Garantziotis, S., Demokritou, P., Castranova, V., Cassee, F.R., 2014. The Yin: an adverse health perspective of nanoceria: uptake, distribution, accumulation, and mechanisms of its toxicity. Environ. Sci. Nano 1, 406–428.

Zhang, L.W., Monteiro-Riviere, N.A., 2008. Assessment of quantum dot penetration into intact, tape-stripped, abraded and flexed rat skin. Skin Pharmacol. Physiol. 21, 166–180.

CHAPTER

9

Respiratory System, Part One: Basic Mechanisms

Craig A. Poland[*,***], *Ken Donaldson*[**]

***Institute of Occupational Medicine, Edinburgh, United Kingdom; **MRC/University of Edinburgh Centre for Inflammation Research, Queen's Medical Research Institute, Edinburgh, United Kingdom**

OUTLINE

Introduction 225

Nanoparticle Deposition and Clearance From the Lungs 226
Aerodynamic Equivalent Diameter 227

Pulmonary Responses 230
Inflammation 230
Fibrosis 231
Genotoxicity and Carcinogenesis 231

Processes and Mechanisms Underlying Particle-Induced Effects in the Pleuro-Pulmonary System 233
Cellular Stress 233
Biopersistence 237

Conclusions 238

Acknowledgments 239

References 239

INTRODUCTION

When considering the risks associated with nanomaterials, exposure is the key factor in defining risk with hazard being a key variable. Exposures can occur at various points along the value chain from research and development of a new engineered nanomaterial (ENM), its production at scale, its incorporation into products, such as composites, cosmetics etc., release from the application or use of a product (e.g., wear and tear) and at the end of life, for example, during recycling or incineration. When we consider exposure, important factors are routes (e.g., oral, dermal, inhalation) levels (e.g., mass or particle number), duration and frequency. In relation to levels, duration and frequency, occupational exposure provides the

Adverse Effects of Engineered Nanomaterials. http://dx.doi.org/10.1016/B978-0-12-809199-9.00009-4
Copyright © 2017 Elsevier Inc. All rights reserved.

greatest level of concern due to the potential for successive exposures as part of the working day/week etc. In relation to routes of exposure, while all offer potential points of ingress into the body, the lungs due to the sensitivity and ability to accumulate an internal dose perhaps cause the greatest concern. We know that nanoparticles can be inhaled and penetrate the lung with efficiency, depositing beyond the ciliated airways in the respiratory zone. From here they may move further into the lung parenchyma or beyond, translocating from their original site of deposition to the pleural space, lymphatic system or vasculature. While the body is well adapted to constant exposure to particles from a variety of sources (primarily anthropogenic) with effective clearance and immunological defense mechanisms, exposure can also lead to disease and a real concern is the effect that new ENM may have on the lung.

In this chapter, we describe the routes of ENM into the body via the lung and factors affecting this as well as possible pathological reactions ranging from inflammation to carcinogenicity.

NANOPARTICLE DEPOSITION AND CLEARANCE FROM THE LUNGS

The concept of dose and response are the fundamental processes in toxicology and in the case of inhalation exposure of the lung to particles, the dose is delivered by deposition to various parts of the lung dependent on their aerodynamic diameter of (Kreyling et al., 2007). Larger particles deposit higher up in the nose and upper respiratory tract, while there is an ever-greater elutriation of particles as the branches of the bronchial tree are penetrated and flow of air slows down, resulting in only the smaller size fractions being deposited in the more peripheral bronchioles and proximal alveolar region. In the air, nanoparticles undergo dynamic interactions with each other, resulting in aggregation so that the size distribution of the nanoparticles is variable over time (Dennekamp et al., 2001). Particle deposition is affected by three different mechanisms: (1) sedimentation, (2) impaction, and (3) diffusion. Sedimentation affects particles >0.5 μm in aerodynamic diameter, while impaction acts on particles >1.5 μm aerodynamic diameter, and diffusion regulates diffusion of particles <0.5 μm in thermodynamic diameter (Forchhammer et al., 2010). These mechanisms operate in different parts of the bronchial tree resulting in hotspot deposition at airway bifurcations because of turbulent flow leading to increased impaction; there is also large-scale deposition by Brownian motion around the proximal alveolar/terminal bronchiolar region where the net movement of air becomes zero and there is time for particles to encounter the airspace walls by diffusion. Following deposition in these different regions of the lung, the clearance mechanisms that are normally operative can act on the particles to remove them:

Airways: In the airways, ciliated and mucus-producing cells combine together to form the mucociliary escalator which propels particles trapped in the mucus upward toward the throat for swallowing. This system becomes ever more depleted peripherally until, by the time terminal bronchioles are encountered, mucociliary clearance is much slower (Schlesinger et al., 1997).

The Proximal Alveolar Region: Beyond the ciliated airways macrophage action is the principle mechanism of clearance of deposited particles. This site of interest has been defined as the proximal alveolar region (PAR) and comprises the terminal bronchiole and the first 400–600 μm beyond the bronchiolar-alveolar duct junction (Pinkerton et al., 2004). This same region is also referred to as centri-acinar, that is, the opening into the pulmonary acinus, the unit of gas exchange, defined as the composite set of alveoli arising from a single terminal bronchiole, also known as the ventilatory unit of the lungs (Mercer and Crapo, 1987). The

PAR is the transitional zone between the terminal airways and the alveoli where gas exchange occurs and is the prime site for particle deposition and accumulation (Donaldson et al., 2008). Macrophages are important cells in integrating the immunological and inflammatory responses and so any modulating effect of phagocytosed particles on macrophage function can have a profound effect on initiation of immuno/inflammatory responses. The epithelial cells in the airways, terminal bronchioles, and in the alveolar region are also involved in immunoinflammatory response by their ability to produce chemokines and other inflammatory mediators. Therefore, the interaction between deposited particles in the fixed epithelial cells and the mobile alveolar macrophages are important in dictating the local responses to particle deposition during clearance and retention. Particles have been observed to clear through the epithelium in and around the terminal bronchioles and enter the interstitium where they can lodge over a protracted period of time, for example, coal macules in coal miners. Nanoparticles are also considered to cross cellular and tissue barriers (Geiser et al., 2005) where they can accumulate in the interstitium (Ferin et al., 1992) and reach the blood where they can translocate to other organs (not dealt with here). Once across the airspace epithelial barrier they may also enter the sumps of the lymphoid system which terminate around the terminal bronchioles. The particles are then be swept cranially in the lymph to the mediastinal lymph nodes which are well known to be loaded with dust following any kind of dust exposure.

The Pleura: In addition it has become apparent that particles deposit peripherally and in alveoli that are subpleural can be cleared to the pleural space (reviewed in Donaldson et al., 2010a). Normally only very small particles reach this region due to the elutriating effect of negotiating the airways in a bifurcating airstream that becomes progressively slower as it moves into the lungs. However, because of the unusual aerodynamics of fiber-shaped particles, long fibers can also reach this site (see later). Particles that deposit subpleurally appear to be able to migrate through the subpleural tissues and enter the pleural space (Mercer et al., 2010). Particles entering the pleural space are cleared via the outflow through the stomata in the parietal pleura to the mediastinal lymph nodes. However, long fibers unable to negotiate the stomata, which are size-restricted to $<\sim 10\ \mu m$, will be trapped at parietal pleura where protracted interaction with the mesothelial cells will occur along with elicitation of an inflammatory response. The pleura is thus only a target for fiber-shaped particles.

The Lymph Nodes: The consequences of accumulation of particles in the lymph nodes is not known, but particle-loaded lymph nodes are common in all industrial-related lung diseases, with little evidence of lymph adenopathy or immunopathological sequelae.

Therefore, the sites of particle retention where the dose is concentrated over a period of time include alveolar macrophages, epithelial cells, the interstitium, the lymph nodes and in the case of HARN, around the stomata on the parietal pleura. In these sites where the dose of nanoparticles (NP) is delivered over a protracted time there is potential for adverse effects via oxidative stress and inflammation, if the particles are sufficiently reactive or are long fibers. There is also clearance of a small fraction of deposited nanoparticles to the blood and these particles may circulate around the body for deposition in other target organs, but this is not dealt with here.

Aerodynamic Equivalent Diameter

Size is a key factor when considering exposure to nanomaterials as it can have a substantial impact on the ability to enter the body and in turn further distribute (translocation). When considering the lungs as a route of entry, aerosolized particles are drawn in during inspiration

where they navigate the convoluted airways of the respiratory tract in the airflow and eventually deposit in the lung. This passage along the respiratory tract and region of deposition is dictated by the particles size but as the particle is being carried within airflow, it is the particles *aerodynamic equivalent diameter* (D_{ae}) which is of most interest. The D_{ae} can differ from the physical diameter and is analogous to the diameter of an idealized sphere of unit density (1 g/cm^3) with the same settling speed as the particle of interest (Sturm and Hofmann, 2009). Were a particle to be a perfect sphere and the same density as above, then its D_{ae} would match its physical diameter but to the most part, this is not the case. Particles may be irregularly shaped (e.g., fractal or elongate), aggregated, or differing in densities, all of which influence the settling speed of particles, meaning that a particle's physical diameter is often not truly reflective of its aerodynamic diameter.

The convoluted and progressively narrowing nature of the airways means that lungs have an elutriating effect whereby, to the most part, larger particles (>20 μm D_{ae}) are excluded from the respiratory system (depositing instead in the head airways). The thoracic fraction is those particles small enough to pass beyond the larynx while the respirable fraction is the fraction of inhaled particles small enough to pass beyond the ciliated airways. More recently, Brown et al. (2013) used a combination of experimental data and mathematical models to provide estimates of the thoracic and respirable fractions for adults. They found that while current thinking suggests a 50% cut-size for the thoracic fraction at an aerodynamic diameter of around 10 μm in adults, there studies showed a much lower cut-off at around 3 μm. Therefore, overestimating the mass of particles generally expected to penetrate into the lower respiratory tract. The particles reaching the nonciliated alveolar region tend to be small (<4.25 μm D_{ae}) (BSI, EN 481, 1993) and to the most part can be dealt with by alveolar macrophages. However, while the elutriating effect of the lung means that large particles cannot penetrate to the respiratory zone, there are some exceptions and these are particles which display a small D_{ae} while at the same time, present a large physical dimension with fibers being the most well-known example.

Fibers

Fibers which are far longer than particles generally found in the respirable fraction are known to be able to penetrate and deposit in the alveolar region of the lung (Barbieri et al., 2012; Dodson et al., 1990; Dufresne et al., 1995; Hesterberg et al., 1999). This is because a fiber's aerodynamic diameter is not dictated by its length but instead, by its diameter (Jones, 1993) meaning that while its length may be well in excess of the respirable size, its diameter is small (high aspect ratio) and as such, its aerodynamic diameter is relatively small compared to its length. This is reflected in the World Health Organization (WHO) definition of a fiber which dictates that to be classified as a fiber it must have a length greater than 5 μm, a diameter less than 3 μm (which allows respirability), and an aspect ratio of greater than 3:1 (WHO, 1997).

Platelets

Platelet shaped particles, such as graphene family nanomaterials (GFN) can also possess a high aspect ratio as they possess a large diameter yet are very thin. GFN platelets can also present a very small aerodynamic diameter yet still possess a substantial lateral dimension. This has been shown in two recent publications (Sanchez et al., 2012; Schinwald et al., 2012a), which calculated the aerodynamic diameter based upon different platelet diameters and thicknesses (layers). To demonstrate this, Table 9.1 shows the calculated D_{ae} in microns for various hypothetical lateral dimension/thickness combinations. In addition, the

TABLE 9.1 Projected Area Diameter Versus Estimated Aerodynamic Diameter and Percentage Alveolar Deposition (Brackets) for Circular Plate-Like Particles

		Lateral dimension (μm)								
		1	**5**	**10**	**15**	**20**	**40**	**60**	**80**	**100**
Thickness (μm)	0.01	0.19 (12.7%)	0.42 (8.0%)	0.59 (7.9%)	0.73 (7.7%)	0.84 (8.0%)	1.19 (9.3%)	1.46 (10.3%)	1.68 (10.9%)	1.88 (11.3%)
	0.02	0.27 (10.0%)	0.59 (7.9%)	0.84 (8.0%)	1.03 (8.6%)	1.19 (9.3%)	1.68 (10.9%)	2.06 (11.6%)	2.38 (11.8%)	2.66 (11.7%)
	0.04	0.38 (8.3%)	0.84 (8.0%)	1.19 (9.3%)	1.46 (10.3%)	1.68 (10.9%)	2.38 (11.8%)	2.91 (11.5%)	3.36 (10.9%)	3.76 (10.2%)
	0.06	0.46 (7.8%)	1.03 (8.6%)	1.46 (10.3%)	1.78 (11.1%)	2.06 (11.6%)	2.91 (11.5%)	3.57 (10.6%)	4.12 (9.5%)	4.60 (8.6%)
	0.08	0.53 (7.6%)	1.19 (9.3%)	1.68 (10.9%)	2.06 (11.6%)	2.38 (11.8%)	3.36 (10.9%)	4.12 (9.5%)	4.75 (8.3%)	5.32 (7.2%)
	0.10	0.59 (7.5%)	1.33 (9.8%)	1.88 (11.3%)	2.30 (11.8%)	2.66 (11.7%)	3.76 (10.2%)	4.60 (8.6%)	5.32 (7.2%)	5.94 (6.1%)
	0.25	0.94 (8.3%)	2.10 (11.6%)	2.97 (11.5%)	3.64 (10.4%)	4.20 (9.4%)	5.94 (6.1%)	7.28 (4.2%)	8.41 (3.0%)	9.40 (2.2%)
	0.50	1.33 (9.8%)	2.97 (11.5%)	4.20 (9.4%)	5.15 (7.5%)	5.94 (6.1%)	8.41 (3.0%)	10.29 (1.7%)	11.89 (1.1%)	13.29 (0.69%)
	1.00	1.88 (11.3%)	4.20 (9.4%)	5.94 (6.1%)	7.28 (4.2%)	8.41 (3.0%)	11.89 (1.1%)	14.56 (0.48%)	16.81 (0.26%)	18.79 (0.15%)

associated calculated percentage deposition in the alveolar region in humans was calculated using multiple-path particle dosimetry model (MPPD v 2.11) (Anjilvel and Asgharian, 1995; MPPDM, 2002) and the percentage deposition shown in brackets. These calculations demonstrate that for the majority of combinations, deposition in the alveolar region is relatively high irrespective of the lateral dimension or thickness. This suggests that, despite having large physical diameters, the platelets will be respirable, and are likely to deposit in the alveolar region. Indeed, it is only where we have increase in both lateral dimension and thickness that the percentage alveolar deposition drops below 3% of the total inhaled dose.

The only available experimental evidence providing a point of comparison to the calculations in Table 9.1 and those published by Sanchez et al. (2012) and Schinwald et al. (2012a), were provided by Ma-Hock et al. (2013) who as part of a rodent inhalation experiment, aerosolized graphene-like platelets and monitored the aerosol. They noted that platelets with a maximal lateral dimension of 10 μm possessed an aerodynamic diameter of less than 0.4 μm which is commensurate with the results given.

PULMONARY RESPONSES

Deposition within the lung brings with it a host of possible responses where a particle interacts with the local tissues, including inflammation, fibrosis, and genotoxicity and cancer, as summarized below (and see Chapter 10 for a more detailed discussion on allergic responses and asthma).

Inflammation

Normal lung defense involves the uptake of particles into macrophages which are also part of the innate immune system, which are capable of mediating inflammation and immune responses. For completely phagocytosable particles and for particles that do not exert stress on the macrophage, uptake has little effect on macrophage secretion or motility and so is followed by translocation of the macrophage to the foot of the mucociliary escalator and eventually its clearance up the mucociliary escalator. However, if there is damage or stress to the macrophage, it can undergo differentiation and produce cytokines, chemokines, and expression of other NF-κB-dependent genes and production of lipid mediators leading to inflammation. A number of nanoparticle types have been shown to induce inflammation in the rat lung (Cho et al., 2013; Crouzier et al., 2010; Morimoto et al., 2015; Rushton et al., 2010; Shvedova et al., 2008) and for the same material there is greater inflammation produced by an equal mass of nanoparticles compared to micron-sized particles of the same materials (Brown et al., 2001). This is because on the whole for low toxicity, low solubility materials, such as compact carbon, titanium dioxide, and polystyrene beads, the surface area is the metric that dictates inflammation (Duffin et al., 2007). Surface area as a metric driving inflammation received considerable support, in the form of studies which took place late in the last century using bulk manufactured nanoparticles, such as titanium dioxide, photocopier toner, and diesel soot (Oberdorster et al., 1994). These studies showed that surface area of low toxicity materials was the driver for onset of inflammation in rat lung overload. In the case of fiber shaped particles, inflammation is induced by long particles via their ability to cause

frustrated phagocytosis in macrophages which cannot fully enclose the fiber, resulting in an unstable, stressed macrophage. This paradigm (Donaldson et al., 2013) has been shown to operate for long carbon nanotubes (Poland et al., 2008) as well as other fibrous nanomaterials (Murphy et al., 2012a; Schinwald et al., 2012b). Such "frustrated" macrophages are proinflammatory in terms of their cytokine profile and via activation of transcriptional factors, such as NF-κB (Hamilton et al., 2009; Ye et al., 1999).

Fibrosis

Up until relatively recently a clear link has been drawn between persistent inflammation in the lungs and fibrosis. In many models, including conventional particles, there is a distinct relationship between continuing inflammation following exposure to persistent pathogenic particles in the lungs and the development of fibrosis. Fibrosis is characterized by the accumulation of collagen and fibronectin matrix, fibroblast proliferation and myofibroblast transformation. This occurs along with an increased level of growth factors and cytokines expressed at the site of the developing lesion. More recently, however, the link between inflammation and fibrosis has been questioned and some kinds of pulmonary fibrosis, such as idiopathic pulmonary fibrosis (Stramer et al., 2007). In the case of carbon nanotubes there is evidence that they can cause fibrosis in the absence of inflammation (Warheit et al., 2004), but the dominant paradigms still suggest that inflammation, and inflamed tissue in general, is a fertile ground for fibrosis. However, the type of inflammation might be sufficiently subtle not to be easily detectable, for example, interstitial and not detected by BAL or macrophage mediated rather than neutrophilic. The extensive studies of Brody et al. using high levels of asbestos in a single exposure show fibrosis developing in and around their site of deposition around the first alveolar duct bifurcations without neutrophilic response (Brody et al., 1989, 1992; Yin et al., 2007). However, the ability of nanoparticles to interstialize close to the sites where the fibroblasts proliferate during fibrosis suggests that some kinds of nanoparticles may have direct effects on fibroblasts. This has been supported by studies with carbon nanotubes (Wang et al., 2010). In these studies single wall carbon nanotubes were added to cultures of lung fibroblasts and this was found to stimulate fibroblast proliferation and collagen production. MMP9, which is involved in lung fibrosis, was also increased in the cultures treated with the single wall carbon nanotubes. These data show that in the absence of leukocytes or any kind of inflammatory response there can be direct nanoparticle induced stimulation of fibroblasts indicative of effects which in vivo would lead to fibrosis. It is not yet known whether other kinds of nanoparticles can directly active fibroblasts.

Genotoxicity and Carcinogenesis

For particles there are two models for genotoxic action—primary and secondary genotoxicity (Chapter 18). Primary genotoxicity involves direct interaction between the nanoparticle and the target cell, whereas secondary genotoxicity acts through inflammation to cause ROS from to damage DNA (Donaldson et al., 2010b). Primary article induced genotoxicity may be direct where the nanoparticle enters the nucleus and directly interacts with the DNA or indirect by release free radicals that directly damage the DNA or obstruct chromosome segregation during mitosis leading to aneuploidy. It might also be indirect where the particles deplete

antioxidants so increasing the steady state of oxidative damage within the cell or increase oxidative damage that arises from mitochondrial activity or inhibition of DNA repair. Many in vitro studies have shown the ability of nanoparticles to cause direct genotoxicity (Landsiedel et al., 2009). There is much less data to demonstrate the mechanism of action or to demonstrate whether in vivo primary or secondary genotoxicity is the effective mechanism. There is reason to believe that the mechanism might differ between different nanoparticle types. Although none of the new engineered nanoparticles have been tested for carcinogenicity in full term animal studies, in the 1980s bulk manufactured nanoparticles, such as carbon black, titanium dioxide, and diesel oil soot, were tested for their pathogenicity in rats over lifetime studies. These studies demonstrated that, for all ultrafine materials which were low toxicity/low solubility, at very high lung burden following a protracted period to high exposure, there was carcinogenic and fibrogenic response. This phenomenon, which appears to be confined only to rats, was named "rat lung overload" and has questionable relevance for human risk assessment. It seems possible that high enough exposure to any nanoparticle that is reactive leading to inflammation and oxidative stress.

More recently, there has been considerable interest in the genotoxic and carcinogenic effects of carbon nanotubes, promoted by the concern that given their structure, they may present an asbestos-like (i.e., carcinogenic) toxicity. It has been shown in several studies by Sargent et al. (2009, 2012) that coincubation of SWCNT airway epithelial cells can cause aneuploidy, mitotic disruption. Indeed, Sargent et al. (2012) and Siegrist et al. (2014) exposed airway epithelial cells to the equivalent of 34 years of exposure at the NIOSH recommended exposure limit (REL) of 1 $\mu g/m^3$. This resulted in exposure at 0.024, 0.24, 2.4, and 24 $\mu g/cm^2$ to either SWCNT or MWCNT and showed the formation of abnormal mitotic spindles, dose related disruption of centrosomes, aneuploid chromosome as well as 3D reconstructions showing CNT integrated with microtubles, and DNA (Siegrist et al., 2014).

Indeed, genotoxic and cell-transformational effects of CNT have been shown in a number of studies in lung epithelial cells (Lindberg et al., 2009; Vales et al., 2016) including the observation of the induction of DNA strand breaks at lower doses (5 and 10 $\mu g/cm^2$) for straight MWCNT as opposed to tangled MWCNT which required much higher doses (200 $\mu g/cm^2$) to cause such an effect (Catalan et al., 2016). Comparative studies have also shown mesothelial cells (the cells lining the pleural space and base of mesothelioma) appear more sensitive to CNT induced DNA damage when compared to lung epithelial cells (BEAS 2B cells) (Lindberg et al., 2013).

There has also been increasing in vivo evidence of the potential for CNT to cause tumor formation. This was first raised by Takagi et al. (2008) who noted the induction of mesothelioma in mice after intraperitoneal injection of 3 mg of MWCNT into P53 heterozygous mice. A study which was criticized heavily for the dose used. A follow-up study dose response study, using the P53(+/−) mice used either high 300 μg, middle 30 μg, or low 3 μg dose injected intraperitoneally. The results showed an incidence of mesothelioma in the groups of mice of 19/20, 17/20, and 5/20 for the high medium and low treatment groups, respectively (Takagi et al., 2012). In wild-type animals Sakamoto et al. (2009) have shown the induction of intraperitoneally disseminated mesothelioma after a single intrascrotal injection of MWCNT (1 mg/kg) in rats. Most recently, Rittinghausen et al. (2014) published the results of an intraperitoneal instillation study in rats using four different MWCNT samples which were injected based on numbers of WHO fibers (10^9 or 5×10^9 WHO carbon nanotubes). They

found that MWCNT induced tumors, including mesotheliomas, in all dose groups although the incidences and times to tumor differed between groups. Linking the response to physicochemical properties, the authors observed the highest frequencies and earliest appearances after treatment with straight MWCNT. Later in the 2-year study, rats treated with the most curved type of MWCNT showed evidence of tumor formation indicating a delayed, albeit positive response. In comparing the results to asbestos, they noted tumors from CNT and asbestos treated animals were histopathologically and immunohistochemically similar but crucially they were also similar to those mesotheliomas in man, suggesting similar pathogenesis.

PROCESSES AND MECHANISMS UNDERLYING PARTICLE-INDUCED EFFECTS IN THE PLEURO-PULMONARY SYSTEM

Cellular Stress

The ability of NP to induce cellular stress in epithelial cells and macrophages following uptake can be seen as a key switching event in determining whether a NP is likely to cause inflammation. This section outlines the potential pathways and mechanisms for NP to cause cellular stress. These pathways are summarized in Fig. 9.1.

Oxidative Stress

Oxidative stress is considered to be a major process in the pathobiological effects of particles and indeed is the dominant hypothesis for the adverse effects of NP at the cellular level (Donaldson et al., 2009). The ability of NP to generate oxidative stress has formed the basis of

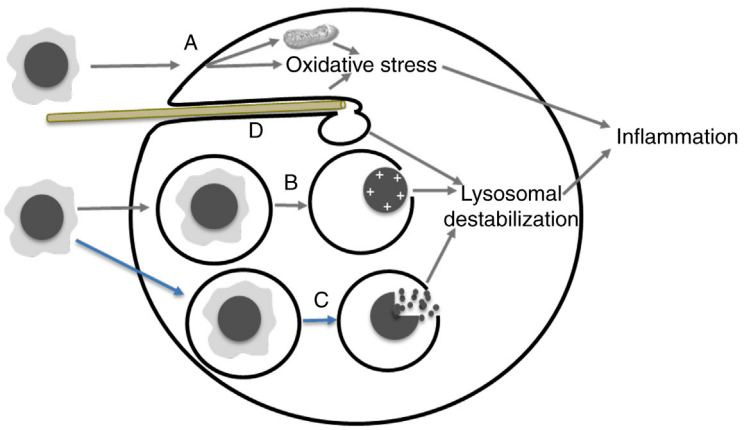


FIGURE 9.1 Pathways for NP-induced cellular stress. The compact nanoparticle is shown surrounded by its corona. *A*, Particle delivers oxidative stress directly or indirectly via disruption of normal redox function in the cell (e.g., mitochondrial effects); *B*, Positively charged NP with a high positive zeta potential is stripped of its corona in the lysosome and the positively charge surface that is revealed interacts with the lysosomal membrane from within, destabilizing it; *C*, An NP that is soluble at acid pH delivers a large intralysosomal dose of ions which destabilizes the lysosome; *D*, A long thin HARN induces frustrated phagocytosis with subsequent destabilization of the phagolysosomal process and chronic stimulation of NADPH oxidase leading to oxidative stress.

a hypothetical structure/toxicity relationship for NP (Rushton et al., 2010). Oxidative stress is defined as an imbalance between oxidants and antioxidants inside cells, in lung lining fluid or tissue fluid, such that there is more oxidation. Reactive oxygen species (ROS) are produced by particles themselves from intrinsic chemical reactions, such as the Fenton reaction (Akhtar et al., 2010), and by cells as part of normal respiration. Inflammatory cells are particularly active at producing free radicals as part of their microbicidal arsenal and so during inflammatory responses these cells can contribute considerably to oxidative stress. The ROS released from phagocytes are thought to be a major source of cell and tissue damage associated with chronic inflammation and the development of disease. Therefore the effects of deposited particles and the oxidative leukocytic response to them can be important in causing oxidative stress that leads to further inflammation and pathogenic change. The redox sensitive transcription factor, such as NF-κB, are important in the inflammatory effects following particle deposition (Schins and Donaldson, 2000). When these transcription factors are activated they lead to transcription of proinflammatory genes, such as cytokines, chemokines, and adhesion molecules. Antioxidants are found in two broad categories—enzymatic and nonenzymatic. The nonenzymatic antioxidants are important in lung lining fluid and include uric acid, ascorbic acid, and alpha-tocopherol (vitamin E). Together these antioxidants can break the oxidation chain in the aqueous and lipid phases of the cell. Antioxidant enzymes include superoxide dismutase, which catalyses the dismutation of superoxide to hydrogen peroxide and oxygen, and catalase which scavenges H_2O_2 to water. Glutathione (GSH) is a tripeptide which incorporates two thiol or sulfhydryl groups which on oxidation are converted to a sulfhydryl bridge to make glutathione disulfide. GSH is a major antioxidant in the lungs and is found at 100 times more concentration in the epithelial lining fluid of the lungs than in the plasma and intercellular levels of GSH are also very high.

When the antioxidant defenses are overwhelmed oxidative stress occurs. Oxidative stress is considered to be a major process in the pathobiological effects of particles and indeed is the dominant hypothesis for the adverse effects of NP at the cellular level. Nel et al. (2006) have described a tiered response to differing degrees of nanoparticle-induced oxidative stress which may partly explain the pleiotropic effects of oxidative stress. The adverse effects can be seen only to occur at moderate level of oxidative stress when inflammation occurs and a mild oxidative stress is dealt with homeostatically and is not harmful. The challenge of in vitro studies where oxidative stress is suggested to be predictive of pathogenic potential is to contextualize the extent of the stress to the kind of graded response seen in the diagram. Merely inducing oxidative stress does not indicate that there is necessarily a pathogenic outcome if the homeostatic antioxidant defenses can deal with the stress (Donaldson et al., 2009).

As stated earlier, ROS are produced by particles themselves from intrinsic chemical reactivity, such as the Fenton reaction (Akhtar et al., 2010). Additionally cells themselves produce oxidative stress from a number of sources but especially as part of normal respiration, as mitochondria leak electron from the respiratory chain. Particles could increase this by directly affecting mitochondria or by depleting the normal antioxidant defense of the cell that protects against this endogenous source of oxidative stress (Li et al., 2007). Additionally, phagocytic cells which ingest particles undergo an oxidative burst which produces ROS and also reactive nitrogen species (RNS). This is part of the evolved microbicidal function of phagocytes but when multiple sterile phagocytic events occur with particle exposure over time, there can be pathogenic consequences of continued oxidative burst activity and its subsequent oxidative stress.

Direct induction of cellular oxidative stress by particles has formed the basis of a hypothetical structure/toxicity relationship (Rushton et al., 2010) although other studies have failed to show a clear relationship between intrinsic free radical generating activity and ability to cause inflammation in the lungs (Lu et al., 2009). Studies with particles and some with nanoparticles have shown that there is upregulation of antioxidant enzymes (Park et al., 2008) and depletion of nonenzymatic antioxidants following exposure to nanoparticles (Akhtar et al., 2010). The induction of oxidative stress is directly linked to other key pathogenic processes namely inflammation, fibrosis, genotoxicity, and carcinogenesis. This pathway for production of cellular stress is outlined in Fig. 9.1—Pathway A.

Inflammasome Activation

The surface charge of NP, measured by zeta potential (ZP) has been suggested to be an important factor in the ability to cause cellular injury. Nel et al. (2009) described high positive ZP of particle as a factor in destabilizing the phagolysosome after uptake. The positive ZP on NP is immediately neutralized on formation of a corona after deposition in lung lining fluid but inside the lysosomes, the acid and protease/lipase activity may be able to remove this, revealing the charged surface. This can the interact with the lysosomal membrane leading to rupture and release of lysosomal contents with concomitant activation of the NALP3 inflammasome, as has been shown for quartz particles (Hornung et al., 2008). Another hypothetical mechanism whereby positively charge NP might destabilize membranes implicates accumulation of chloride ions and water as a consequence of chronic stimulation of the lysosomal membrane proton pump (Nel et al., 2009). Fig. 9.1—Pathway B outlines this mechanism.

For NP that are sparingly soluble at pH 7.0 but acid soluble there is potential for dissolution in the acid milieu of the lysosomes after the have been taken up into this compartment. ZnO NP are exemplars of this mechanism as suggested by Nel et al. (2009). In this case the key structural attributes are that the NP must be acid—solubility and when such NP are in the acid milieu of the lysosomes they will undergo dissolution; the local high ion concentrations in the lysosomes then cause destabilization. This is demonstrated in Fig. 9.1—Pathway C.

Frustrated Phagocytosis

The fiber pathogenicity paradigm is a model that predicts fiber pathogenicity and this has been found to be applicable to nanofibers including carbon nanotubes (Agrawal et al., 2013; Brown et al., 2007; Murphy et al., 2011; Poland et al., 2008), nickel oxide nanowires (Poland et al., 2011), silver nanowires (Schinwald et al., 2012b,c) TiO_2 nanofibers (Allegri et al., 2016; Hamilton et al., 2009) etc. During frustrated phagocytosis, which occurs with long fibers, the phagosomes fails to close and phagolysosomal formation is affected leading to lysosomal destabilization and NALP3 inflammasome activation (Hamilton et al., 2009).

Recently, Schinwald et al. (2012c) performed a series of experiments to define the threshold length for fiber induced toxicity. Using a panel of different nanofibers differing in both composition (silver, nickel, and carbon nanotubes) and length, they performed intrapleural instillations and assessed the inflammatory response. They found a clear threshold effect whereby fibers beyond 4 μm in length resulted in an inflammatory and granulomatous response in the pleural space. The mechanism behind this relates in part to the size selective nature of the points of egress from the pleural space. Here, pore-like stomata in the parietal pleura connect the pleural space to the underlying lymphatic system, draining particulates etc. to

mediastinal lymph nodes (Murphy et al., 2011, 2012b). These stomata are approximately 2–5 μm in diameter and so facilitate the removal of small particulates but hinder the passage of larger material, such as fibers.

In a later study, Schinwald and colleagues also sought to identify the threshold lengths for fiber induced toxicity in the lung, again using a panel of different length nanofibers aspirated into the lung as well as directly incubated with macrophages to determine hindrance of macrophage mobility. They found that aspiration of silver nanowires resulted in a length dependent inflammatory response in the lungs with threshold at a fiber length of 14 μm (Schinwald et al., 2012b). However, shorter fibers (3, 5, and 10 μm) caused no significant inflammation in the lung. Within their in vivo studies, they found that macrophage locomotion was also restricted in a length dependent manner which occurred for fibers ≥5 μm. The difference in threshold between the lung and the pleura reflects the different mechanisms of clearance. Both are size dependent, but in the pleura it is defined the pleural stomata which imposes a cut-off around 4 μm while in the lungs it is imposed by the macrophages and their ability to fully enclose fibers and remove them from the lung.

Another form of nanoparticle for which size may be a contributing factor to toxicity is GFN platelets. As noted previously in this chapter, by virtue of their extremely thin nature, GFN can present a very small aerodynamic diameter despite relatively large lateral diameter. Both Schinwald et al. (2012a) and Sasidharan et al. (2012) have shown that the unusual size of GFN in the alveolar region of the lung, can cause frustrated phagocytosis which may trigger cell death as well as oxidative stress and inflammation. By measuring the release of proinflammatory cytokine (IL-1β), Schinwald et al. (2012a) also showed that an inflammatory response was induced. They further demonstrated that particle uptake was required to initiate the inflammatory response and that secretion of IL-1β was mediated by the inflammasome which is a multiprotein complex whose activity has been implicated in physiological and pathological inflammation (Gross, 2012). Similarly, Russier et al. (2013) confirmed that the lateral dimensions of GFN can impact the level of oxidative damage and inflammation in vitro. The authors compared three different sizes of graphene oxide (GO) and demonstrated that a smaller sheet size induces more intracellular reactive oxygen species (ROS) than larger sheets. The smaller sheets are also more efficient in inducing cellular activation and cytokine release, which is suggested to be because the smaller GO sheets were more effectively taken-up by the macrophages resulting in a higher cell dose. Russier et al. (2013) also highlighted that the mouse macrophage cells were significantly more responsive than the human macrophage cells used in their study, emphasizing the need to choose appropriate models for investigating material toxicity and hazard, both in vitro and in vivo. Further evidence was provided by Akhavan and colleagues, who also demonstrated that the lateral dimensions of GFN can influence toxicity, by exposing human mesenchymal stem cells to reduced GO nanoplatelets (rGONP) in vitro. Cell viability was significantly reduced even at low doses of rGONP, in comparison to rGO sheets, which exhibited a significant cytotoxic effect only at high concentrations. Oxidative stress and cell wall membrane damage were determined as the main mechanism involved in the cytotoxicity and the authors also state that the interaction of graphene sheets with stem cells and probably other biological systems, such as organisms and tissues strongly depends on the lateral size of the sheets (Akhavan et al., 2012).

These studies demonstrate that GFN can elicit frustrated phagocytosis in vitro, leading to oxidative stress, and expression of proinflammatory cytokines which may cause an

inflammatory response in vivo. The mechanisms of this response in vivo were investigated by Schinwald and colleagues, who carried out a study aspirating graphene-based platelets (GP) and carbon black (CB) into the lungs of mice. Twenty four hours after exposure to GP, the total number of cells washed from the lungs was significantly increased as were the numbers of inflammatory cells, demonstrating acute inflammation. This was supported by a significantly increased marker of cellular toxicity (LDH) and significant increases in various cytokines associated with active inflammation. Histological analysis of tissue sections removed 24 h after aspiration of particles showed that the CB particles had been successfully removed and showed clear airways. Although some black particulate accumulation can be seen, the morphology of the bronchiole was similar to the vehicle control. In contrast, GP aspiration showed a large accumulation of GP associated with inflammatory cells (mainly macrophages) in the airway, partially blocking it. This cell/GP structure is termed a granuloma and is a typical response to a foreign body which cannot be easily removed (Schinwald et al., 2012a).

Biopersistence

The ability of nanoparticles to persist in the lung is an important factor in sustaining the dose. This paradigm originates from fiber studies where the biopersistence of fiber is a major factor in determining their long-term pathogenic effects. Non-biopersistent fibers break down and are removed from the lung, whereas biopersistent fibers retain their shape and deliver long fiber dose to the lung over an extended period of time. In the case of nanoparticles, those which are able to retain their morphology, continue to deliver dose in the compartment in which they are retained. There is a more complex aspect to biopersistence where the soluble products of the dissolution may be toxic, as in the case of soluble transition metal ions or zinc ions which are important in the toxicity of zinc (Cho et al., 2011, 2012).

In relation to the digestion of fibrous nanomaterials, specifically carbon nanotubes, there have been a range of studies looking at the ability of CNT to be degraded. Thus far, studies have investigated the biodegradation of SWCNT by myeloperoxidase (MPO) which is predominantly expressed in the granules of neutrophils and eosinophil peroxidase, a major oxidant producing enzyme during inflammation (Andon et al., 2013; Kotchey et al., 2013). The degradation of CNT by neutrophils and/or eosinophils is relevant within an inflamed lung; however, could be said to be less so in a healthy noninflamed lung where neutrophils and eosinophils form only a subfraction of the cell population in the lung, accounting for 1.3% and 0.3%, respectively as compared to macrophages which comprise 95% of the overall population (Heron et al., 2012). Therefore, when considering fiber/cell interactions, it is the macrophage fiber interaction that is perhaps the most relevant to repeated, low level exposure (not resulting in acute inflammation) that is most reflective of occupational exposures.

Still considering CNT, a very recent study investigated the ability of lung macrophages to degrade SWCNT. It has previously been demonstrated that SWCNT can be degraded by free radicals, such as the highly reactive hydroxyl radicals ($^{\cdot}OH$) produced via Fenton homolytic cleavage of H_2O_2 (Allen et al., 2009; Kagan et al., 2014) as well as oxidized through the action of enzymes, such as MPO and horse-radish peroxidase (HRP). However, the focus of this study was to look at macrophage derived oxidants and their role in degradation of SWCNT. Central to this is the enzyme, nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) which is a major generator of superoxide radicals which through the action

of superoxide dismutase (SOD) can also form H_2O_2. In addition, the production of nitric oxide (NO) by macrophages in a prooxidant environment rich in superoxide via inducible nitric oxide synthase (iNOS) pathway can also lead to the formation of peroxynitrite ($ONOO^-$) which is an oxidant noted to be of comparable potency to MPO and hypochlorous acid generated by neutrophils (Kagan et al., 2014).

The induction of NADPH oxidase can occur after appropriate stimulation of the cell by stimuli, such as phagocytosis (Robinson, 2008) and so consideration of the contribution such oxidants may have on fiber degradation is certainly justified. Kagan et al. sought to directly assess the ability of macrophages to utilize the superoxide/NO → peroxynitrite oxidative system in the degradation of SWCNT using activated THP-1 cells as a model macrophage system. Using a fluorescent probe to detect peroxynitrite anions and hydroxyl radicals, the authors noted that upon cellular activated there were increased levels of peroxynitrite anions and hydroxyl radicals reacting with the probe. However, upon coincubation with SWCNT, this decreased which the authors suggested was evidence that the radicals were interacting with the SWCNT rather than the probe. In addition, shortening of the SWCNT was also observed after 96 h incubation as was degradation of the CNT structure as detected by Raman spectroscopy. To further investigate this, the authors used chemical models to generate peroxynitrite and noted that degradation of SWCNTs was detectable visually as the initially optically dense SWCNT suspensions were becoming more translucent over time (Kagan et al., 2014). This study demonstrated that relatively robust SWCNT could be degraded by oxidants produced by macrophages as well as acellular analogs of a peroxynitrite rich environment. Given such oxidants are produced during normal clearance activities by macrophages as well as during situations of frustrated phagocytosis, these may play a role in cellular degradation of nanomaterials.

While there have been considerable efforts in defining the degradation of CNT as a form of HARN, there have been relatively few studies addressing graphene platelets. Recently, Schinwald et al. (2014) investigated the medium-term effects of graphene in lung tissue which included an assessment of their biopersistence in the lung tissue using Raman spectroscopy as well as examination of degradation in vitro after HRP treatment up to 1 week. Mice were exposed by pharyngeal aspiration of unoxidized, multilayered GPs. They noted that no degradation GP occurred during the 6 weeks in the lung despite large deposits being present. The Raman analysis of GP in tissue showed minimal oxidation and this was supported by in vitro observations of enzymatic oxidation of GP via HRP and H_2O_2 which showed minimal changes in ID/IG ratio and the appearance of the Raman D′ band at 1620 cm^{-1} (surrogates of graphene oxidation) (Schinwald et al., 2014).

CONCLUSIONS

There is strong evidence that certain ENP can pose a risk to the pleuro-pulmonary system ranging from minor reversible inflammation, to more detrimental and chronic effects including allergic airways disease, fibrosis, and even cancer. The evidence base for many of these effects primarily relates to in vivo and in vitro studies with few human studies (e.g., case studies) showing evidence of serious effects in humans resulting from exposure ENM. However, currently exposures are relatively limited and also relatively new. This latter point is an

important consideration as some diseases such certain forms of cancer can take many years to become apparent and so a lack of reported effects at the moment does not mean effects will not present in the future. As such, it is imperative that risks be identified and where possible exposure limits determined and adhered to in order to prevent the emergence of respiratory disease arising from the use of ENM.

When considering the underlying pathological processes that nanoparticles elicit including more defined adverse outcome pathways (AOPs) that can be linked to long-term pathology, these have been shown for some nanoparticles, but not for others. In other words, nanoparticles like other particles show differences and heterogeneity in their pathogenicity. A key area of research has been the identification of physicochemical attributes, such as shape, reactivity, size etc. that contribute or dictate toxicity. Such approaches, when linked to AOPs can be enormously beneficial not only in understanding the basis of particle mediated disease and risk assessment but also in helping to prevent toxicity issues by designing out such properties, thereby making nanomaterials (and subsequent products) safe-by-design.

Acknowledgments

CAP was funded by the H2020 project PolyGraph (Grant No. 604143) funded by the European Commission.

References

Agrawal, B., et al., 2013. Detection of norfloxacin and monitoring its effect on caffeine catabolism in urine samples. Biosens. Bioelectron. 47, 307–312.

Akhavan, O., Ghaderi, E., Akhavan, A., 2012. Size-dependent genotoxicity of graphene nanoplatelets in human stem cells. Biomaterials 33 (32), 8017–8025.

Akhtar, M.J., et al., 2010. Nanotoxicity of pure silica mediated through oxidant generation rather than glutathione depletion in human lung epithelial cells. Toxicology 276 (2), 95–102.

Allegri, M., et al., 2016. Shape-related toxicity of titanium dioxide nanofibres. PLoS One 11 (3), e0151365.

Allen, B.L., et al., 2009. Mechanistic investigations of horseradish peroxidase-catalyzed degradation of single-walled carbon nanotubes. J. Am. Chem. Soc. 131 (47), 17194–17205.

Andon, F.T., et al., 2013. Biodegradation of single-walled carbon nanotubes by eosinophil peroxidase. Small 9 (16), 2721–2729, 2720.

Anjilvel, S., Asgharian, B., 1995. A multiple-path model of particle deposition in the rat lung. Fundam. Appl. Toxicol. 28 (1), 41–50.

Barbieri, P.G., et al., 2012. Asbestos fibre burden in the lungs of patients with mesothelioma who lived near asbestos-cement factories. Ann. Occup. Hyg. 56 (6), 660–670.

Brody, A.R., McGavran, P.D., Overby, L.H., 1989. Brief inhalation of chrysotile asbestos induces rapid proliferation of bronchiolar-alveolar epithelial and interstitial cells. IARC Sci. Publ. (90), 93–99.

Brody, A.R., et al., 1992. Interstitial pulmonary macrophages produce platelet-derived growth factor that stimulates rat lung fibroblast proliferation in vitro 1. J. Leukoc. Biol. 51 (6), 640–648.

Brown, D.M., et al., 2001. Size-dependent proinflammatory effects of ultrafine polystyrene particles: a role for surface area and oxidative stress in the enhanced activity of ultrafines. Toxicol. Appl. Pharmacol. 175 (3), 191–199.

Brown, D., et al., 2007. An in vitro study of the potential of carbon nanotubes and nanofibres to induce inflammatory mediators and frustrated phagocytosis. Carbon 45 (9), 1743–1756.

Brown, J.S., et al., 2013. Thoracic and respirable particle definitions for human health risk assessment. Part. Fibre Toxicol. 10, 10–12.

BSI, EN 481, 1993. Workplace atmospheres. Size fraction definitions for measurement of airborne particles.

Catalan, J., et al., 2016. In vitro and in vivo genotoxic effects of straight versus tangled multi-walled carbon nanotubes. Nanotoxicology 10 (6), 794–806.

Cho, W.S., et al., 2011. Progressive severe lung injury by zinc oxide nanoparticles; the role of Zn^{2+} dissolution inside lysosomes. Part. Fibre Toxicol. 8, 27.
Cho, W.S., et al., 2012. Zeta potential and solubility to toxic ions as mechanisms of lung inflammation caused by metal/metal-oxide nanoparticles. Toxicol. Sci. 126 (2), 469–477.
Cho, W.S., et al., 2013. Predictive value of in vitro assays depends on the mechanism of toxicity of metal oxide nanoparticles. Part. Fibre Toxicol. 10 (1), 55.
Crouzier, D., et al., 2010. Carbon nanotubes induce inflammation but decrease the production of reactive oxygen species in lung. Toxicology 272 (1–3), 39–45.
Dennekamp, M., et al., 2001. Ultrafine particles and nitrogen oxides generated by gas and electric cooking. Occup. Environ. Med. 58 (8), 511–516.
Dodson, R.F., et al., 1990. Asbestos content of lung tissue, lymph nodes, and pleural plaques from former shipyard workers. Am. Rev. Resp. Dis. 142 (4), 843–847.
Donaldson, K., et al., 2008. Concordance between in vitro and in vivo dosimetry in the proinflammatory effects of low-toxicity, low-solubility particles: the key role of the proximal alveolar region. Inhal. Toxicol. 20, 53–62.
Donaldson, K., et al., 2009. The limits of testing particle-mediated oxidative stress in vitro in predicting diverse pathologies; relevance for testing of nanoparticles. Part. Fibre Toxicol. 6, 13.
Donaldson, K., et al., 2010a. Asbestos, carbon nanotubes and the pleural mesothelium: a review of the hypothesis regarding the role of long fibre retention in the parietal pleura, inflammation and mesothelioma. Part. Fibre Toxicol. 7 (5), 5.
Donaldson, K., Poland, C.A., Schins, R.P., 2010b. Possible genotoxic mechanisms of nanoparticles: criteria for improved test strategies. Nanotoxicology 4, 414–420.
Donaldson, K., et al., 2013. Pulmonary toxicity of carbon nanotubes and asbestos—Similarities and differences. Adv. Drug Deliv. Rev. 65 (15), 2078–2086.
Duffin, R., et al., 2007. Proinflammogenic effects of low-toxicity and metal nanoparticles in vivo and in vitro: highlighting the role of particle surface area and surface reactivity. Inhal. Toxicol 19, 849–856.
Dufresne, A., et al., 1995. Fibers in lung tissues of mesothelioma cases among miners and millers of the township of Asbestos, Quebec. Am. J. Ind. Med. 27 (4), 581–592.
Ferin, J., Oberdorster, G., Penney, D.P., 1992. Pulmonary retention of ultrafine and fine particles in rats. Am. J. Respir. Cell Mol. Biol. 6 (5), 535–542.
Forchhammer, L., et al., 2010. Variation in the measurement of DNA damage by comet assay measured by the ECVAG inter-laboratory validation trial. Mutagenesis 25 (2), 113–123.
Geiser, M., 2005. Ultrafine particles cross cellular membranes by non-phagocytic mechanisms in lungs and in cultured cells. Environ. Health Perspect. 113 (11), 1555–1560.
Gross, O., 2012. Measuring the inflammasome. Methods Mol. Biol. 844, 199–222.
Hamilton, R.F., et al., 2009. Particle length-dependent titanium dioxide nanomaterials toxicity and bioactivity. Part. Fibre Toxicol. 6, 35.
Heron, M., et al., 2012. Bronchoalveolar lavage cell pattern from healthy human lung. Clin. Exp. Immunol. 167 (3), 523–531.
Hesterberg, T.W., et al., 1999. Studies on the inhalation toxicology of two fiberglasses and amosite asbestos in the syrian golden hamster. Part I. Results of a subchronic study and dose selection for a chronic study. Inhal. Toxicol. 11 (9), 747–784.
Hornung, V., et al., 2008. Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. Nat. Immunol. 9 (8), 847–856.
Jones, A.D., 1993. Respiable industrial fibres: deposition, clearance and dissolution in animal models. Ann. Occup. Hyg. 37 (2), 211–226.
Kagan, V.E., et al., 2014. Lung macrophages "digest" carbon nanotubes using a superoxide/peroxynitrite oxidative pathway. ACS Nano 8 (6), 5610–5621.
Kotchey, G.P., et al., 2013. Peroxidase-mediated biodegradation of carbon nanotubes in vitro and in vivo. Adv. Drug Deliv. Rev. 65 (15), 1921–1932.
Kreyling, W., et al., 2007. Particle dosimetry: deposition and clearance from the respiratory tract and translocation towards extrapulmonary sites. In: Donaldson, K., Borm, P. (Eds.), Particle Toxicology. CRC Press.
Landsiedel, R., et al., 2009. Genotoxicity investigations on nanomaterials: methods, preparation and characterization of test material, potential artifacts and limitations--many questions, some answers. Mutat. Res. 681 (2–3), 241–258.

Li, H., et al., 2007. Inhibition of the mitochondrial respiratory chain function abrogates quartz induced DNA damage in lung epithelial cells. Mutat. Res. 617 (1–2), 46–57.
Lindberg, H.K., et al., 2009. Genotoxicity of nanomaterials: DNA damage and micronuclei induced by carbon nanotubes and graphite nanofibres in human bronchial epithelial cells in vitro. Toxicol. Lett. 186 (3), 166–173.
Lindberg, H.K., et al., 2013. Genotoxicity of short single-wall and multi-wall carbon nanotubes in human bronchial epithelial and mesothelial cells in vitro. Toxicology 313 (1), 24–37.
Lu, S., et al., 2009. Efficacy of simple short-term in vitro assays for predicting the potential of metal oxide nanoparticles to cause pulmonary inflammation. Environ. Health Perspect. 117 (2), 241–247.
Ma-Hock, L., et al., 2013. Comparative inhalation toxicity of multi-wall carbon nanotubes, graphene, graphite nanoplatelets and low surface carbon black. Part. Fibre Toxicol. 10 (1), 23.
Mercer, R.R., Crapo, J.D., 1987. Three-dimensional reconstruction of the rat acinus. J. Appl. Physiol. 63 (2), 785–794.
Mercer, R.R., et al., 2010. Distribution and persistence of pleural penetrations by multi-walled carbon nanotubes. Part. Fibre Toxicol. 7, 28.
Morimoto, Y., et al., 2015. Comparison of pulmonary inflammatory responses following intratracheal instillation and inhalation of nanoparticles. Nanotoxicology 10 (5), 607–618.
Multiple Path Particle Dosimetry Model (MPPD v 1.0), 2002. A model for human and rat airway particle dosimetry. Bilthoven, The Netherlands.
Murphy, F., et al., 2011. Length-dependent retention of carbon nanotubes in the pleural space of mice initiates sustained inflammation and progressive fibrosis on the parietal pleura. Am. J. Pathol. 178, 2587–2600.
Murphy, F.A., et al., 2012a. Length-dependent pleural inflammation and parietal pleural responses after deposition of carbon nanotubes in the pulmonary airspaces of mice. Nanotoxicology 7 (6), 1157–1167.
Murphy, F.A., et al., 2012b. The mechanism of pleural inflammation by long carbon nanotubes: interaction of long fibres with macrophages stimulates them to amplify pro-inflammatory responses in mesothelial cells. Part. Fibre Toxicol. 9, 8.
Nel, A., et al., 2006. Toxic potential of materials at the nanolevel. Science 311 (5761), 622–627.
Nel, A.E., et al., 2009. Understanding biophysicochemical interactions at the nano-bio interface. Nat. Mater. 8 (7), 543–557.
Oberdorster, G., Ferin, J., Lehnert, B.E., 1994. Correlation between particle-size, in-vivo particle persistence, and lung injury. Environ. Health Perspect. 102, 173–179.
Park, E.J., et al., 2008. Oxidative stress and apoptosis induced by titanium dioxide nanoparticles in cultured BEAS-2B cells. Toxicol. Lett. 180 (3), 222–229.
Pinkerton, K.E., et al., 2004. Reduced lung cell proliferation following short-term exposure to ultrafine soot and iron particles in neonatal rats: key to impaired lung growth? Inhal. Toxicol. 16 (Suppl. 1), 73–81.
Poland, C.A., et al., 2008. Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study. Nat. Nanotechnol. 3 (7), 423–428.
Poland, C., et al., 2011. Length-dependent pathogenic effects of nickel nanowires in the lungs and the peritoneal cavity. Nanotoxicology 6, 899–911.
Rittinghausen, S., et al., 2014. The carcinogenic effect of various multi-walled carbon nanotubes (MWCNTs) after intraperitoneal injection in rats. Part. Fibre Toxicol. 11 (1), 59.
Robinson, J.M., 2008. Reactive oxygen species in phagocytic leukocytes. Histochem. Cell Biol. 130 (2), 281–297.
Rushton, E.K., et al., 2010. Concept of assessing nanoparticle hazards considering nanoparticle dosemetric and chemical/biological response metrics. J. Toxicol. Environ. Health 73 (5), 445–461.
Russier, J., et al., 2013. Evidencing the mask effect of graphene oxide: a comparative study on primary human and murine phagocytic cells. Nanoscale 5 (22), 11234–11247.
Sakamoto, Y., et al., 2009. Induction of mesothelioma by a single intrascrotal administration of multi-wall carbon nanotube in intact male Fischer 344 rats. J. Toxicol. Sci. 34 (1), 65–76.
Sanchez, V.C., et al., 2012. Biological interactions of graphene-family nanomaterials: an interdisciplinary review. Chem. Res. Toxicol. 25 (1), 15–34.
Sargent, L.M., et al., 2009. Induction of aneuploidy by single-walled carbon nanotubes. Environ. Mol. Mutagen 50 (8), 708–717.
Sargent, L.M., et al., 2012. Single-walled carbon nanotube-induced mitotic disruption. Mutat. Res. 745 (1–2), 28–37.
Sasidharan, A., et al., 2012. Hemocompatibility and macrophage response of pristine and functionalized graphene. Small 8 (8), 1251–1263.

Schins, R.P.F., Donaldson, K., 2000. Nuclear factor kappa B activation by particles and fibres. Inhal. Toxicol. 12 (Suppl. 3), 317–326.
Schinwald, A., et al., 2012a. Graphene-based nanoplatelets: a new risk to the respiratory system as a consequence of their unusual aerodynamic properties. ACS Nano 6 (1), 736–746.
Schinwald, A., Chernova, T., Donaldson, K., 2012b. Use of silver nanowires to determine thresholds for fibre length-dependent pulmonary inflammation and inhibition of macrophage migration in vitro. Part. Fibre Toxicol. 9 (1), 47.
Schinwald, A., et al., 2012c. The threshold length for fiber-induced acute pleural inflammation: shedding light on the early events in asbestos-induced mesothelioma. Toxicol. Sci. 128, 461–470.
Schinwald, A., et al., 2014. Minimal oxidation and inflammogenicity of pristine graphene with residence in the lung. Nanotoxicology 8 (8), 824–832.
Schlesinger, R.B., et al., 1997. Disposition of inhaled toxicicants. In: Massaro, E.J. (Ed.), Handbook of Human Toxicology. CRC Press, Boca Raton, pp. 493–550.
Shvedova, A.A., et al., 2008. Inhalation vs. aspiration of single-walled carbon nanotubes in C57BL/6 mice: inflammation, fibrosis, oxidative stress, and mutagenesis. Am. J. Physiol. 295 (4), L552–L565.
Siegrist, K.J., et al., 2014. Genotoxicity of multi-walled carbon nanotubes at occupationally relevant doses. Part. Fibre Toxicol. 11, 6.
Stramer, B.M., Mori, R., Martin, P., 2007. The inflammation-fibrosis link? A Jekyll and Hyde role for blood cells during wound repair. J. Invest. Dermatol. 127 (5), 1009–1017.
Sturm, R., Hofmann, W., 2009. A theoretical approach to the deposition and clearance of fibers with variable size in the human respiratory tract. J. Hazard. Mater. 170 (1), 210–218.
Takagi, A., et al., 2008. Induction of mesothelioma in p53+/- mouse by intraperitoneal application of multi-wall carbon nanotube. J Toxicol. Sci. 33 (1), 105–116.
Takagi, A., et al., 2012. Dose-dependent mesothelioma induction by intraperitoneal administration of multi-wall carbon nanotubes in p53 heterozygous mice. Cancer Sci. 103 (8), 1440–1444.
Vales, G., Rubio, L., Marcos, R., 2016. Genotoxic and cell-transformation effects of multi-walled carbon nanotubes (MWCNT) following in vitro sub-chronic exposures. J. Hazard. Mater. 306, 193–202.
Wang, L., et al., 2010. Direct fibrogenic effects of dispersed single-walled carbon nanotubes on human lung fibroblasts. J. Toxicol. Environ. Health 73 (5), 410–422.
Warheit, D.B., et al., 2004. Comparative pulmonary toxicity assessment of single-wall carbon nanotubes in rats. Toxicol. Sci. 77 (1), 117–125.
WHO, 1997. Determination of Airborne Fibre Number Concentrations. A Recommended Method, by Phase-Contrast Optical Microscopy Membrane Filter Method. http://www.who.int/occupational_health/publications/airfibre/en/
Ye, J., et al., 1999. Critical role of glass fiber length in TNF-alpha production and transcription factor activation in macrophages. Am. J. Physiol. 276 (3 Pt. 1), L426–L434.
Yin, Q., Brody, A.R., Sullivan, D.E., 2007. Laser capture microdissection reveals dose-response of gene expression in situ consequent to asbestos exposure. Int. J. Exp. Pathol. 88 (6), 415–425.

CHAPTER

10

Respiratory System, Part Two: Allergy and Asthma

Harri Alenius[*,**], *Michael R. Shurin*[†], *Galina V. Shurin*[†], *Don Beezhold*[‡], *Anna A. Shvedova*[‡]

*University of Helsinki, Helsinki, Finland; **Systems Toxicology Unit, Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden; †University of Pittsburgh Medical Center, Pittsburgh, PA, United States; ‡National Institute for Occupational and Safety Health, Morgantown, WV, United States

OUTLINE

Allergic Reactions and Asthma 243
Engineered Nanoparticles and Allergy 244
Carbon Nanotubes and Allergic Pulmonary Inflammation 246
Modulation of Allergen-Induced Airway Inflammation by CNTs 248
Mechanisms of CNT-Induced/Mediated Allergic Responses 249
Conclusions 250
Take-Home Messages 251
Acknowledgments 251
Disclaimer 251
References 251

ALLERGIC REACTIONS AND ASTHMA

Allergy is defined as disease resulting from an exaggerated response by the immune system to specific but otherwise innocuous antigens, called allergens. Allergic reactions are commonly divided into four classes of hypersensitivity reactions according to the specific immunologic mechanisms mediating the response. The term hypersensitivity describes

Adverse Effects of Engineered Nanomaterials. http://dx.doi.org/10.1016/B978-0-12-809199-9.00010-0
Copyright © 2017 Elsevier Inc. All rights reserved.

objective and reproducible symptoms initiated by exposure to a defined allergen at a dose tolerated by normal persons. Hypersensitivity can be antibody-mediated or cell-mediated. Antibody-mediated hypersensitivity involves IgE-induced release of inflammatory mediators from mast cells (Type I), IgG or IgM mediated cytotoxic reactions (Type II), and immune complex mediated reactions (Type III) (Murphy et al., 2012). Cell-mediated or delayed type reactions (Type IV) are mediated primarily by T cells but monocytes/macrophages, NK, and granulocytes can also participate. The allergic response can be divided into early events, called early phase reactions, which are mediated by short-lived agents, such as histamine. Late phase reactions involve leukotrienes, cytokines, chemokines, and the recruitment and activation of eosinophils, basophils, and antigen specific T cells. Typically, proteins and other high molecular weight allergens elicit the immediate type I allergic reactions while low molecular weight chemicals induce the delayed type IV hypersensitivity reactions.

Asthma is a term that describes a group of clinical symptoms of generally reversible acute obstructive airway disease. Asthma is often characterized by expiratory airflow limitation due to bronchial hyperresponsiveness. The pathogenesis of asthma is not completely understood, but asthma is defined as "a heterogeneous disease, usually characterized by chronic airway inflammation" (Fanta, 2009). The most common form of asthma, allergic asthma, is triggered by allergen-induced activation of submucosal mast cells in the lower airways leading to immediate bronchial constriction and amplified secretion of mucus by the airway epithelium. Allergic asthma is typically characterized by chronic airway inflammation in the presence of Th2 lymphocytes, eosinophils, neutrophils, and other leukocytes. These cells work together to cause remodeling of the airways, accompanied by augmented mucus production. Th2 cytokines, such as IL-4, IL-5, and IL-13, may directly affect airway epithelial cells and cause the induction of goblet-cell metaplasia and secretion of mucus (Lloyd and Hessel, 2010). Persistent inflammation can also lead to irreversible fibrotic airway remodeling induced by improper activation of smooth muscle cells and lung fibroblasts. In addition to Th2 lymphocytes, mast cells are important contributors in the development, maintenance, and augmentation of asthma with release of acute-phase mediators and also producing variety of inflammatory cytokines (Galli et al., 2008).

There is strong evidence showing an imbalance of reducing and oxidizing equivalents in the lungs, produces an environment conducive to acceleration of oxidative stress in asthma. Endogenous and exogenous reactive oxygen species, such as superoxide anion, hydroxyl radical, hypohalite radical, and hydrogen peroxide, and reactive nitrogen species, such as nitric oxide, peroxynitrite, and nitrite, play a major role in the airway inflammation that is a key element of asthma severity outcomes (Sahiner et al., 2011).

ENGINEERED NANOPARTICLES AND ALLERGY

Engineered nanoparticles (ENP) are well known to interact with immune cells, including the alteration of phagocytosis and activation or inhibition of immune cells (Dobrovolskaia et al., 2015) (and see Chapter 13). Studies on inhalation of nanoparticles consistently demonstrates them to be potent inducers of sterile inflammation, a response to noxious nonviable stimuli orchestrated by damage-associated molecular patterns (DAMPS) and cytokines

of the IL-1 family. Nanoparticles can also "trigger" hypersensitivity reactions since patients with bronchial asthma are highly sensitive to inhaled substances including particulate matter. Unfortunately, the mechanism of allergy to nanostructures as well as a possible modulation of allergen effects by binding to nanomaterials is generally unknown. In this regard, the concept of pseudoallergy, a response that resembles a true allergic reaction, but lacks a specific allergen, should be introduced. The mechanisms of pseudoallergy are not known, but may involve nonspecific complement activation, inflammatory responses, or alterations in histamine metabolism.

Acute exposure to different types of silica nanoparticles (SNP) alone or in conjunction with ovalbumin, a high molecular weight protein allergen, were shown to induce strong airway inflammation and airway hyperresponsiveness. In ovalbumin-SNP asthma models, ovalbumin (OVA) with SNP-treated mice displayed substantial airway inflammation, which was significantly higher than in mice treated with OVA without SNP (Park et al., 2015). Furthermore, three types of metal oxide nanoparticles, TiO_2, ZnO, and SiO_2, were shown to increase serum concentrations of total IgE, OVA-specific IgG1, and OVA-specific IgE (ZnO only) following pharyngeal aspiration 24 h prior to sensitization with inhaled ovalbumin (Horie et al., 2015), suggesting that they have the potential to aggravate allergic reactions. Interestingly, the rapid onset of serious nickel allergy in a nanonickel occupationally exposed worker was recently reported (Journeay and Goldman, 2014).

Accumulating data suggests that when allergen is bound to ENP, IgE can still recognize the allergen. Comparative analysis of basophil activation from allergic patients in response to free allergen or gold nanoparticles conjugated with the major allergens of birch pollen, timothy grass pollen, and house dust mite revealed that depending on the allergen, different effects were observed after binding to ENP, including enhanced allergic recognition of some of the tested allergens (Radauer-Preiml et al., 2016). These observations may help in the development of more sensitive allergen testing or cell specific delivery of pharmaceuticals for prevention of allergic responses. Several studies reported different effects of another ENP, C_{60} fullerenes, a potent free radical scavenger, on mast cells and basophils. Using human skin and lung mast cells and peripheral blood basophils, Ryan et al. (2007) demonstrated a marked inhibition of IgE-dependent mediator release when cells were preincubated with C_{60} fullerenes. Furthermore, in the mouse model of mast cell-dependent anaphylaxis, fullerenes prevented the in vivo release of histamine. In addition to possible stabilization of FcεRI-mediated mast cell and basophil responses, fullerenes and specifically engineered fullerene derivatives may stabilize these responses through non-IgE-mediated mechanisms related to the potent free radical scavenging activity of fullerenes, including the absorption of induced cellular reactive oxygen species, calcium and prostaglandin D2 resulting in reduced activation of ERK1/ERK2 signaling (Dellinger et al., 2013).

In spite of the potential for sensitization to certain airborne ENPs (nickel), can still be used in the development of better immunotherapy for patients with allergy. Polyethylene glycol modified nanoparticles can envelop an allergen and deliver it to specific cells serving as nanocarrier that degrades when it encounters the acidic part of these cells, simultaneously releasing the allergen and removing the packaging (Pohlit et al., 2015). Another interesting study focusing on the development of oral dispersible tablets containing prednisolone-loaded chitosan nanoparticles opens a new avenue for the better management of asthma in pediatric patients (Chen et al., 2015). Interestingly, the use of magnetic core-shell nanoparticles as a

microchip for allergy diagnosis has increased capture kinetics by a 1000-fold by quantifying IgE (Teste et al., 2011).

Although the pulmonary delivery of locally-acting drugs encapsulated in nanocarriers provides several advantages for the treatment of respiratory diseases, including asthma, potential health effects of ENP are of great importance and should be carefully evaluated with the growing number of their commercial applications and demonstrated potential for pulmonary exposure in the workplace. Further studies using well-characterized ENP, well controlled protocols and appropriate routes of exposure are required in order to clarify differential allergic effects and provide information suitable for risk assessments of ENP. Moreover, it seems impossible to assess the risks associated with the use of ENP without considering alterations and transformation with life cycle of the particles, both in the environment and inside of the human body or experimental model systems.

CARBON NANOTUBES AND ALLERGIC PULMONARY INFLAMMATION

Among ENPs, single and multiwalled carbon nanotubes (SWCNT and MWCNT), are some of the most promising nanomaterials that have been manufactured broadly during the last decade. Because of their fiber-like morphology, much attention is paid to the health outcomes of pulmonary exposure to these nanoparticles, and in several animal studies, carbon nanotubes (CNTs) have been reported to cause asthma-like responses.

Park et al. (2009) found pulmonary inflammation and enhanced humoral immune responses after intratracheal instillation of MWCNT in mice. Proinflammatory, Th1-type and Th2-type cytokines were increased by the immune cells in both BAL fluid and in the blood. The elevated numbers of B cells, IL-10, and total IgE were observed after a single intratracheal instillation. The authors concluded that this combination of responses was an allergic reaction in the mice treated with MWCNTs (Park et al., 2009). However, the reported pulmonary response was not typical of allergic inflammation since many characteristic features of allergic airway inflammation, such as eosinophilia and goblet cell hyperplasia were not observed (Fanta, 2009). In a study by Hsieh et al. (2012) , a single intratracheal instillation of SWCNT induced airway hyperreactivity and chronic airway obstruction in mice and also lead to persistent lung parenchymal injury. Global gene expression analysis revealed that exposure to SWCNT upregulated proteinases and metalloproteinases, chemokines and macrophage receptors. Results suggest that exposure to SWCNT may cause irreversible obstructive airway disease via sterile inflammation although classical features of allergic airway inflammation were not found (Hsieh et al., 2012).

Inhalation exposure is the best method to mimic real-life scenarios, however, the number of such studies is still limited. In the study by Rydman et al. (2014), healthy mice were exposed by inhalation to two types of MWCNT, rigid rod-like and flexible tangled CNT. Short-term inhalation (4 days) of rigid rod-shaped CNT (rCNT), but not of tangled CNT, induced all the signs of allergic airway inflammation (Fig.10.1A). Exploration of the early events by transcriptomics analysis revealed that a 4 h exposure to rCNT causes dramatic up-regulation of genes involved in innate immunity and cytokine/chemokine pathways. Early proinflammatory cytokines as well as proallergic cytokines and chemokines were synthetized by rCNT-activated

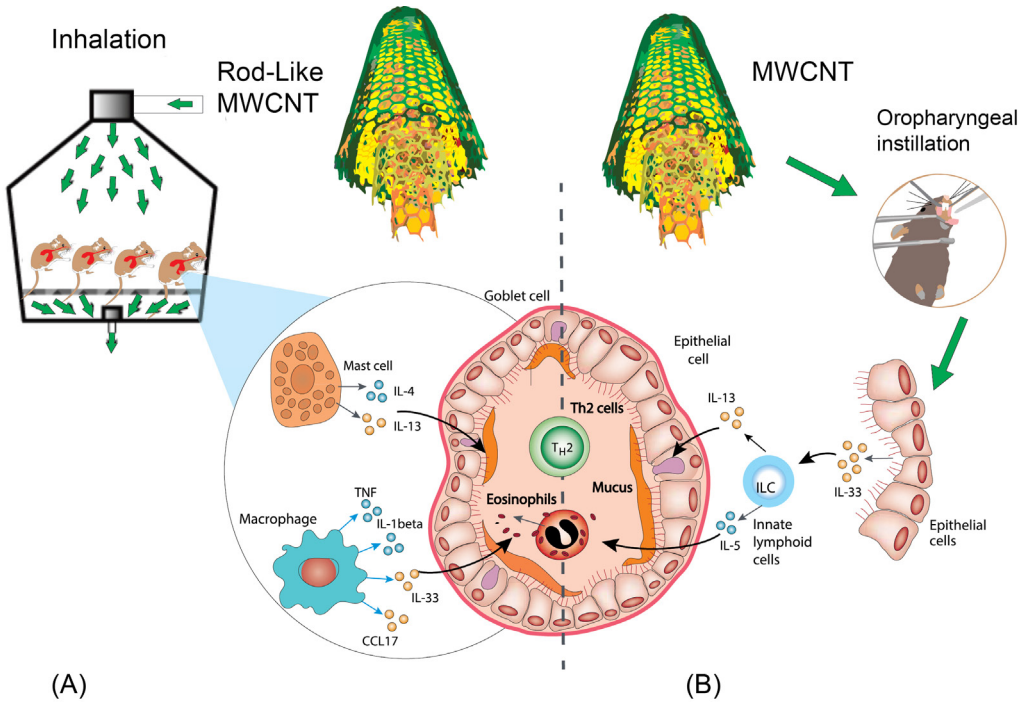


FIGURE 10.1 Effects of carbon nanotubes on allergic airway inflammation. (A) Rydman et al. (2014) reported that inhalation exposure to rod-like MWCNT induces fast and early production of Th2 cytokines (IL-4 and IL-13) from lung mast cells and proinflammatory (TNF and IL-1β) and proallergic cytokines (IL-33 and CCL17) from alveolar macrophages. Repeated exposure to rod-like MWCNT finally leads to establishment of allergic airway inflammation with recruitment of eosinophils and Th2 lymphocytes, mucus hypersecretion, and airway hyperreactivity. (B) In contrast, Beamer et al. (2013) concluded that oropharyngeal instillation with MWCNT induces airway epithelial damage and secretion of IL-33. This, in turn, results in activation of so-called innate lymphoid cells to produce Th2 cytokines (IL-5 and IL-13) which induces mucus secretion, pulmonary eosinophilia, and airway hyperreactivity.

alveolar macrophages, while mast cells were likely an early source of rCNT induced Il-4 and Il-13 (Fig. 10.1A). These observations emphasize the diverse abilities of different type of CNTs to impact the immune system, and they also suggest that inhalation exposure to rCNT may cause asthma-like allergic airway inflammation without the conventional specific protein allergen or treatment with artificial adjuvant substances (Rydman et al., 2014).

Rydman et al. (2015) investigated the airway effects of two different type CNT (long tangled, and long rod-like CNT) and crocidolite asbestos in mice after a single pharyngeal aspiration exposure. Results demonstrated that pulmonary inflammation progresses over time; starting from neutrophilic infiltration at 4–16 h and then changing to eosinophilia, peaking at 7 days after the rod-like CNT exposure. In line with pulmonary eosinophilia, Charcot-Leyden-like crystals were seen in acidophilic macrophages 28 days after the exposure to rod-like CNT. To explore the functional role of IL-1β in CNT induced pulmonary inflammation, they utilized pharmacological TNF and IL-1 receptor antagonists as well as IL1R knock-out mice. The lack of IL-1β was able to prevent acute neutrophilic infiltration but it has no effect on Th2 type

inflammation, see 1 month after the exposure to rod-like CNT (Rydman et al., 2015). These results demonstrate that IL-1R interaction mainly regulates neutrophilic inflammation in the acute inflammatory response, but this interaction is not critical for Th2 dominated chronic type response. In line with this, Køobler et al. (2015) reported that the longer and thicker CNT resulted in more severe pulmonary eosinophilia and induction of crystalline bodies in alveolar macrophages compared to shorter and thinner CNT. Moreover, it has been shown that eosinophil peroxidase (EPO) derived from in vitro and ex vivo activated eosinophils is able to promote SWCNTs biodegradation (Andón et al., 2013) and that pulmonary exposure to MWCNTs mediates Th2 type inflammation with EPO release into the pulmonary inflammation foci (Girtsman et al., 2014).

MODULATION OF ALLERGEN-INDUCED AIRWAY INFLAMMATION BY CNTs

Ovalbumin (OVA) induced experimental asthma is one of the most widely studied allergic asthma model worldwide. There are several variations of the model in which sensitization is done by repeated intranasal or intratracheal administration with OVA, but without adjuvant, or repeated intraperitoneal injection with adjuvant. Additionally, house dust mite extract (HDM) and other purified allergens have been used to induce Th2 type allergic immune responses in mice.

Several studies have examined the risk of exposure to a combination of CNTs with allergen. For instance, Inoue et al. (2009, 2010) examined the effects of MWCNT and SWCNT by repeated intranasal exposure with and without OVA allergen. Exposure to both SWCNT and MWCNT augmented OVA-induced pulmonary inflammation. Exposure to MWCNT also exhibited adjuvant activity for allergen-specific IgG1 and IgE, augmented lung T helper cytokines and chemokines (Inoue et al., 2009). Similarly, exposure to a combination of MWCNT and ovalbumin allergen induced an increase in airway resistance, airway inflammation, goblet cell hyperplasia, and the production of antigen-specific antibodies (Inoue et al., 2010).

Interestingly, exacerbations of allergen-induced airway inflammation and mucus-cell metaplasia by MWCNT could be enhanced by deficiencies in COX-2, and was associated with the activation of a mixed Th1/Th2/Th17 immune response (Sayers et al., 2013). A similar protective role for STAT1 in chronic allergen-induced airway inflammation and exacerbation of lung remodeling caused by aspiration of MWCNT has been recently suggested when MWCNT were reported to further increase ovalbumin-induced goblet cell hyperplasia, airway fibrosis and subepithelial apoptosis in Stat1−/− mice compared with WT mice (Thompson et al., 2015).

Nygaard et al. (2009) reported that both SWCNT and MWCNT together with OVA strongly augmented serum levels of OVA-specific IgE, the number of eosinophils in BAL, and the secretion of Th2-associated cytokines in the draining lymph nodes in mice. In line with this, Li et al. (2014), showed in Wistar rats that exposure to SWCNT exacerbated OVA-induced allergic asthma. Interestingly, SWCNT induced allergic asthma exacerbation was prevented by concurrent administration of vitamin E suggesting that the relief of several asthma related symptoms was explained by antagonistic effects of vitamin E (Li et al., 2014). In the study by Ryman-Rasmussen et al. (2009), significant airway fibrosis

was observed at 14 days in mice that received a combination of ovalbumin and MWCNT, when compared to mice that received OVA or MWCNT only. Further analysis suggested that airway fibrosis resulting from combined OVA sensitization and MWCNT inhalation requires platelet-derived growth factor (PDGF), a potent fibroblast mitogen, and TGF-β1, which stimulates collagen production (Ryman-Rasmussen et al., 2009). These findings propose that individuals with preexisting allergic inflammation may be susceptible to airway fibrosis from inhaled MWCNT.

Individuals can also be sensitized to various chemicals especially in the occupational setting. Staal et al. (2014) studied MWCNT responses by using high IgE-responding Brown Norway rats with trimellitic anhydride (TMA)-induced respiratory allergy. Large MWCNT agglomerates were found in granulomas especially in the allergic rats, suggesting decreased macrophage clearance. Moreover, exposure to MWCNT decreased serum IgE levels and the number of lymphocytes in bronchoalveolar lavage suggesting that some allergy-associated immune responses were modulated by MWCNT exposure (Staal et al., 2014).

MECHANISMS OF CNT-INDUCED/MEDIATED ALLERGIC RESPONSES

IL-33 (IL-1F11) is a cytokine that belongs to the IL-1 family and is linked to Th2-type immune responses (Liew et al., 2010). Beamer et al. (2013) showed that MWCNT exposure results in elevated levels of IL-33 in the lavage fluid, enhanced AHR, eosinophil recruitment, and production of Th2-associated cytokines and chemokines. Exposure to MWCNTs elicited also infiltration of innate lymphoid cells into the airways which in turn provoked IL-33-dependent airway inflammation (Fig. 10.1B). They also demonstrated these MWCNT regulated events were dependent on IL-13 signaling and the IL-33/ST2 axis, but independent of T and B cells (Beamer et al., 2013).

Ronzani et al. (2014) investigated the effect of MWCNT using HDM allergen induced asthma model. HDM extract induced levels of HDM-specific IgG1, pulmonary eosinophilia and neutrophilia, production of collagen and mucus, as well as expression of Th2 cytokines and chemokines, were dose-dependently increased in mice exposed to HDM and MWCNT compared to HDM alone. These effects were associated with an increased production of allergy promoting cytokines TSLP, IL-25, IL-33, and GM-CSF in the airways. Data also suggest a role for airway epithelium and innate cytokines in these effects (Ronzani et al., 2014). Under conditions of increased reactive oxygen species (ROS) production both from the ENPs and by immune system components (e.g., neutrophils), the antioxidant system is rapidly depleted. Several studies have shown that ROS overproduction is in direct correlation with airway tract hyperresponsiveness upon methacholine challenge (Sahiner et al., 2011). It was also found that individuals lacking antioxidant defense are more susceptible to asthma (McCunney, 2005) and accumulation of oxidatively modified proteins, discovered in a variety of airway cells, such as chitinase-like Ym1/Ym2, FIZZ1 and SP-D, could play an important role in the pathogenesis of asthma (Zhang et al., 2009).

The study by Kapralov et al. (2012) provided the evidence of the surfactant-derived lipid-protein coating on SWCNTs in mice upon pulmonary exposure, including SP-D. In another study, two different types of MWCNT (Mitsui-7 and NM-401) induced eosinophil influx with

significant increases in *Chi3L3* (encoding for Ym1) mRNA levels (Køhler et al., 2015). These studies suggest that a weakened antioxidant defense is associated with an elevated inflammatory response.

Several systemic immune effects of CNT-induced inflammation have been recognized in recent studies. Mice that inhaled MWCNT had suppressed systemic immune function, as inhaled MWNTs can activate the release of TGF-β in the lung. TGF-β has a direct effect on prostaglandin production by spleen cells, leading to potent immune suppression (Mitchell et al., 2009). Analysis of SWCNTs on allergic airway inflammation also suggests that SWCNT can exacerbate murine allergic airway inflammation via enhanced activation of T helper immunity and increased oxidative stress (Inoue et al., 2010). Some effects of SWCNT can be explained by the direct effect of SWCNT on DC and altered ability of DC to activate proliferation of T cells (Tkach et al., 2011).

Upregulation of myeloid-derived suppressor cells (MDSC), reported by Shvedova et al. (2013) in SWCNT-exposed mice may also trigger the activation of allergic response in lungs, as MDSC are phenotypically similar myeloid derived regulatory cells, that play major role in both pulmonary inflammatory response and hyperresponsiveness (Deshane et al., 2011).

The ability to alter the allergen-induced airway inflammation was confirmed for graphene oxide (GO). Hence, exposure to GO has been shown to modulate the allergic pulmonary response in a murine model of ovalbumin-induced asthma: GO amplified airway hyper-responsiveness, airway remodeling (goblet cell hyperplasia and smooth muscle hypertrophy), production of OVA-specific IgG2a, macrophages recruitment in broncho-alveolar lavage (BAL) fluid and macrophage production of the mammalian chitinases, CHI3L1 and AMCase, whose expression is associated with asthma. However, at the same time, exposure to GO reduced BAL IL-4, IL-5, and IL-13 and decreased the level of eosinophils in BAL fluid (Shurin et al., 2014).

CONCLUSIONS

It is evident that inhalation of ENM can elicit asthma and allergy-like pulmonary inflammation in murine models. Although mechanisms are not completely known, it is likely that the ENMs induce sterile inflammation through damage-associated molecular patterns (DAMPS) and secretion of the IL-1 family members (including IL-33) and thereby play an important role in allergic inflammation. Evidence exits that rod-like multiwalled carbon nanotubes are especially adept at inducing innate immune-mediated asthma-like reactions. Not unexpectedly, airway exposure to ENPs also exacerbates Th2 inflammation in mouse models suggesting that subjects with underlying allergies may be more susceptible to inhalation of ENP compared to healthy subjects. Importantly, new evidence demonstrates that airborne ENMs may also alter the functional activity of antigen-presenting cells, such as dendritic cells, in the lungs and systemically. In line with this, strong evidence exits that patients with bronchial asthma are highly sensitive to inhaled substances including particulate matter. Despite the growing evidence of the involvement of ENPs in inflammatory reactions, the mechanism by which nanostructures possibly modulate allergic responses are still poorly known. The evidence suggests that both innate and adaptive immune responses may be involved in the modulation of asthmatic reactions after exposure to ENMs. Understanding the interplay

between sterile and allergen induced airway inflammation will be important in discerning the influence of nanoparticles on allergic disease.

TAKE-HOME MESSAGES

- Inhalation of ENPs has been shown to influence both innate and adaptive immunity mediated asthma-like reactions in mouse models.
- Airway exposure to ENPs can exacerbate preexisting allergic pulmonary inflammation in mice.
- Although the influence of ENP on allergic diseases is still poorly known, the evidence derived from mouse studies suggest that ENPs may also have an important impact on allergic diseases in humans.

Acknowledgments

This work was supported by EC-FP7-NANOSOLUTIONS (grant no. 309329), NTRC 939011K, and RO1 CA154369. We would like to thank Kimberly S. Clough-Thomas for figure preparation.

Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

References

Andón, F.T., Kapralov, A.A., Yanamala, N., et al., 2013. Biodegradation of single-walled carbon nanotubes by eosinophil peroxidase. Small 9 (16), 2721–2729.

Beamer, C.A., Girtsman, T.A., Seaver, B.P., Finsaas, K.J., Migliaccio, C.T., Perry, V.K., Rottman, J.B., Smith, D.E., Holian, A., 2013. IL-33 mediates multi-walled carbon nanotube (MWCNT)-induced airway hyper-reactivity via the mobilization of innate helper cells in the lung. Nanotoxicology 7, 1070–1081.

Chen, Y.D., Liang, Z.Y., Cen, Y.Y., Zhang, H., Han, M.G., Tian, Y.Q., et al., 2015. Development of oral dispersible tablets containing prednisolone nanoparticles for the management of pediatric asthma. Drug. Des. Devel. Ther. 9, 5815–5825.

Dellinger, A., Brooks, D., Plunkett, B., Vonakis, B., Sandros, M., Zhou, X., et al., 2013. Effects of novel nanomaterials on allergic mediator release from human mast cells and basophils through non-IgE mediated pathways. J. Nanomed. Nanotechol. 3 (8), 153.

Deshane, J., Zmijewski, J.W., Luther, R., et al., 2011. Free radical-producing myeloid-derived regulatory cells: potent activators and suppressors of lung inflammation and airway hyperresponsiveness. Mucosal Immunol. 4 (5), 503–518.

Dobrovolskaia, M.A., Shurin, M., Shvedova, A.A., 2015. Current understanding of interactions between nanoparticles and the immune system. Toxicol. Appl. Pharmacol. 299, 78–89.

Fanta, C.H., 2009. Asthma. N. Engl. J. Med. 360, 1002–1014.

Galli, S.J., Tsai, M., Piliponsky, A.M., 2008. The development of allergic inflammation. Nature 454, 445–454.

Girtsman, T.A., Beamer, C.A., Wu, N., Buford, M., Holian, A., 2014. IL-1R signaling is critical for regulation of multi-walled carbon nanotubes-induced acute lung inflammation in C57Bl/6 mice. Nanotoxicology 8 (1), 17–27.

Horie, M., Stowe, M., Tabei, M., Kuroda, E., 2015. Pharyngeal aspiration of metal oxide nanoparticles showed potential of allergy aggravation effect to inhaled ovalbumin. Inhal. Toxicol. 27 (3), 181–190.

Hsieh, W.Y., Chou, C.C., Ho, C.C., Yu, S.L., Chen, H.Y., Chou, H.Y., Chen, J.J., Chen, H.W., Yang, P.C., 2012. Single-walled carbon nanotubes induce airway hyperreactivity and parenchymal injury in mice. Am. J. Respir. Cell Mol. Biol. 46, 257–267.

Inoue, K., Koike, E., Yanagisawa, R., Hirano, S., Nishikawa, M., Takano, H., 2009. Effects of multi-walled carbon nanotubes on a murine allergic airway inflammation model. Toxicol. Appl. Pharmacol. 237, 306–316.

Inoue, K., Yanagisawa, R., Koike, E., Nishikawa, M., Takano, H., 2010. Repeated pulmonary exposure to single-walled carbon nanotubes exacerbates allergic inflammation of the airway: possible role of oxidative stress. Free Radic. Biol. Med. 48, 924–934.

Journeay, W.S., Goldman, R.H., 2014. Occupational handling of nickel nanoparticles: a case report. Am. J. Ind. Med. 57 (9), 1073–1076.

Kapralov, A.A., Feng, W.H., Amoscato, A.A., et al., 2012. Adsorption of surfactant lipids by single-walled carbon nanotubes in mouse lung upon pharyngeal aspiration: role in uptake by macrophages. ACS Nano 6 (5), 4147–4156.

Køobler, C., Poulsen, S.S., Saber, A.T., Jacobsen, N.R., Wallin, H., Yauk, C.L., Halappanavar, S., Vogel, U., Qvortrup, K., Molhave, K., 2015. Time-dependent subcellular distribution and effects of carbon nanotubes in lungs of mice. PLoS One 10, e0116481.

Li, J., Li, L., Chen, H., Chang, Q., Liu, X., Wu, Y., Wei, C., Li, R., Kwan, J.K., Yeung, K.L., Xi, Z., Lu, Z., Yang, X., 2014. Application of vitamin E to antagonize SWCNTs-induced exacerbation of allergic asthma. Sci. Rep. 4, 4275.

Liew, F.Y., Pitman, N.I., McInnes, I.B., 2010. Disease-associated functions of IL-33: the new kid in the IL-1 family. Nat. Rev. Immunol. 10, 103–110.

Lloyd, C.M., Hessel, E.M., 2010. Functions of T cells in asthma: more than just T(H)2 cells. Nat. Rev. Immunol. 10, 838–848.

McCunney, R.J., 2005. Asthma, genes, and air pollution. J. Occup. Environ. Med. 47 (12), 1285–1291.

Mitchell, L.A., Lauer, F.T., Burchiel, S.W., McDonald, J.D., 2009. Mechanisms for how inhaled multiwalled carbon nanotubes suppress systemic immune function in mice. Nat. Nanotechnol. 4 (7), 451–456.

Murphy, K., Travers, P., Walport, M., Janeway, C., 2012. Janeway's Immunobiology, eighth ed. Garland Science, New York.

Nygaard, U.C., Hansen, J.S., Samuelsen, M., Alberg, T., Marioara, C.D., Lovik, M., 2009. Single-walled and multi-walled carbon nanotubes promote allergic immune responses in mice. Toxicol. Sci. 109, 113–123.

Park, E.J., Cho, W.S., Jeong, J., Yi, J., Choi, K., Park, K., 2009. Pro-inflammatory and potential allergic responses resulting from B cell activation in mice treated with multi-walled carbon nanotubes by intratracheal instillation. Toxicology 259, 113–121.

Park, H.J., Sohn, J.H., Kim, Y.J., Park, Y.H., Han, H., Park, K.H., et al., 2015. Acute exposure to silica nanoparticles aggravate airway inflammation: different effects according to surface characteristics. Exp. Mol. Med. 47, e173.

Pohlit, H., Bellinghausen, I., Schomer, M., Heydenreich, B., Saloga, J., Frey, H., 2015. Biodegradable pH-sensitive poly(ethylene glycol) nanocarriers for allergen encapsulation and controlled release. Biomacromolecules 16 (10), 3103–3111.

Radauer-Preiml, I., Andosch, A., Hawranek, T., Luetz-Meindl, U., Wiederstein, M., Horejs-Hoeck, J., et al., 2016. Nanoparticle-allergen interactions mediate human allergic responses: protein corona characterization and cellular responses. Part. Fibre Toxicol. 13 (1), 3.

Ronzani, C., Casset, A., Pons, F., 2014. Exposure to multi-walled carbon nanotubes results in aggravation of airway inflammation and remodeling and in increased production of epithelium-derived innate cytokines in a mouse model of asthma. Arch. Toxicol. 88, 489–499.

Ryan, J.J., Bateman, H.R., Stover, A., Gomez, G., Norton, S.K., Zhao, W., et al., 2007. Fullerene nanomaterials inhibit the allergic response. J. Immunol. 179 (1), 665–672.

Rydman, E.M., Ilves, M., Koivisto, A.J., Kinaret, P.A., Fortino, V., Savinko, T.S., Lehto, M.T., Pulkkinen, V., Vippola, M., Hameri, K.J., Matikainen, S., Wolff, H., Savolainen, K.M., Greco, D., Alenius, H., 2014. Inhalation of rod-like carbon nanotubes causes unconventional allergic airway inflammation. Part. Fibre Toxicol. 11, 48.

Rydman, E.M., Ilves, M., Vanhala, E., Vippola, M., Lehto, M., Kinaret, P.A., Pylkkanen, L., Happo, M., Hirvonen, M.R., Greco, D., Savolainen, K., Wolff, H., Alenius, H., 2015. A single aspiration of rod-like carbon nanotubes induces asbestos-like pulmonary inflammation mediated in part by the IL-1R. Toxicol. Sci. 147, 140–155.

Ryman-Rasmussen, J.P., Tewksbury, E.W., Moss, O.R., Cesta, M.F., Wong, B.A., Bonner, J.C., 2009. Inhaled multi-walled carbon nanotubes potentiate airway fibrosis in murine allergic asthma. Am. J. Respir. Cell Mol. Biol. 40, 349–358.

Sahiner, U.M., Birben, E., Erzurum, S., Sackesen, C., Kalayci, O., 2011. Oxidative stress in asthma. World Allergy Organ. J. 4 (10), 151–158.

Sayers, B.C., Taylor, A.J., Glista-Baker, E.E., Shipley-Phillips, J.K., Dackor, R.T., Edin, M.L., et al., 2013. Role of cyclooxygenase-2 in exacerbation of allergen-induced airway remodeling by multiwalled carbon nanotubes. Am. J. Respir. Cell Mol. Biol. 49 (4), 525–535.

Shurin, M.R., Yanamala, N., Kisin, E.R., Tkach, A.V., Shurin, G.V., Murray, A.R., et al., 2014. Graphene oxide attenuates Th2-type immune responses, but augments airway remodeling and hyperresponsiveness in a murine model of asthma. ACS Nano 8 (6), 5585–5599.

Shvedova, A.A., Tkach, A.V., Kisin, E.R., et al., 2013. Carbon nanotubes enhance metastatic growth of lung carcinoma via up-regulation of myeloid-derived suppressor cells. Small 9 (0), 1691–1695.

Staal, Y.C., van Triel, J.J., Maarschalkerweerd, T.V., Arts, J.H., Duistermaat, E., Muijser, H., van de Sandt, J.J., Kuper, C.F., 2014. Inhaled multiwalled carbon nanotubes modulate the immune response of trimellitic anhydride-induced chemical respiratory allergy in brown Norway rats. Toxicol. Pathol. 42, 1130–1142.

Teste, B., Malloggi, F., Siaugue, J.M., Varenne, A., Kanoufi, F., Descroix, S., 2011. Microchip integrating magnetic nanoparticles for allergy diagnosis. Lab. Chip. 11 (24), 4207–4213.

Thompson, E.A., Sayers, B.C., Glista-Baker, E.E., Shipkowski, K.A., Ihrie, M.D., Duke, K.S., et al., 2015. Role of signal transducer and activator of transcription 1 in murine allergen-induced airway remodeling and exacerbation by carbon nanotubes. Am. J. Respir. Cell Mol. Biol. 53 (5), 625–636.

Tkach, A.V., Shurin, G.V., Shurin, M.R., Kisin, E.R., Murray, A.R., Young, S.H., et al., 2011. Direct effects of carbon nanotubes on dendritic cells induce immune suppression upon pulmonary exposure. ACS Nano 5 (7), 5755–5762.

Zhang, L., Wang, M., Kang, X., et al., 2009. Oxidative stress and asthma: proteome analysis of chitinase-like proteins and FIZZ1 in lung tissue and bronchoalveolar lavage fluid. J. Proteome Res. 8 (4), 1631–1638.

CHAPTER

11

Cardiovascular System

*Jennifer B. Raftis**, *Nicholas L. Mills***, *Rodger Duffin**

**Centre for Inflammation Research, Edinburgh University, Edinburgh, United Kingdom;
**Centre for Cardiovascular Science, Edinburgh University, Edinburgh, United Kingdom

OUTLINE

Introduction 255

Diagnostic Applications in Cardiovascular Disease 256
- *Nanotechnology in Medical Imaging* 257
- *Superparamagnetic Iron Oxide Nanoparticles* 260
- *Lipid-Based Nanoparticles* 261
- *Gold Nanoparticles* 263

Particokinetics of Nanomaterials for Diagnostic Use 263

Combustion-Derived Nanoparticles in Cardiovascular Disease 264

Toxicity of Engineered Nanoparticles in Cardiovascular Disease 265
- *In Vitro Studies* 265
- *In Vivo Studies* 267
- *Ex Vivo Studies* 268

Conclusions 269

References 269

INTRODUCTION

Nanotechnology aims for the development of nanoparticles (NPs, particles with at least one dimension less than 100 nm) and other nanomaterials with novel properties, which cannot be obtained through use of the same material in bulk or molecular form. It is currently being applied in all manner of commercial industries to confer unique properties on products or improve existing ones (Medina et al., 2007). Some of the most significant applications are likely to occur in the field of medicine. Many engineered NPs and nanosurfaces have already been evaluated in the clinic (Linkov et al., 2008) and it is likely nanomaterials will have a major impact in areas, such as diagnostics, medical imaging and therapeutics. While the nanotechnologies develop novel engineered NPs, we are already exposed to numerous NPs in

Adverse Effects of Engineered Nanomaterials. http://dx.doi.org/10.1016/B978-0-12-809199-9.00011-2
Copyright © 2017 Elsevier Inc. All rights reserved.

the environment though inhalation of ambient particles (particles less than 10 μm in diameter; PM_{10}) from combustion, industrial and occupational sources and through the ingestion of food and water. Epidemiological and experimental studies have identified a range of adverse health effects related to $PM_{10,}$ and the key components and underlying mechanisms responsible are beginning to emerge (Barlow et al., 2005; Donaldson et al., 1997; Mills et al., 2008a,b; Renwick et al., 2004). Interestingly, the "ultrafine" or nanoparticulate component of $PM_{10,}$ which is considered to have the greatest potential for toxicity and harm. Extrapolations from environmental research and particle toxicology studies are used to guide our approach to the risk assessment of engineered nanoparticles. Combustion-derived nanoparticles (CDNPs) share some characteristics with engineered NPs, such as size, charge, and surface area that influence toxicity. Exposure to CDNPs has been shown to impair vascular function (Mills et al., 2005), promote myocardial ischemia (Mills et al., 2007a), and induce prothrombotic changes in the circulation in humans (Lucking et al., 2008) and may directly contribute to the formation of atherosclerotic plaque (Hoffmann et al., 2007) These observations highlight an important paradox between the harmful effects of CDNP in the environment and the potential for engineered NPs being used for diagnosis, imaging, and therapy causing harm in a clinical setting. In this chapter, we review the clinical applications of engineered NPs focusing on currently available NPs with diagnostic and therapeutic roles in cardiovascular medicine. The chemical and physical properties that are functionally important for each particle and applications are outlined, and strategies to optimize performance through modification of these properties identified. In addition, we highlight the potential for engineered NPs to adversely affect cellular function.

DIAGNOSTIC APPLICATIONS IN CARDIOVASCULAR DISEASE

Atherothrombosis remains the leading cause of death in developed countries. Successful therapies and strategies for prevention are dependent on identification of at risk individuals using clinical risk scores based on age, sex, blood pressure, serum cholesterol, and cigarette smoking (Viera and Sheridan, 2010; Wang, 2011). While these risk scores are used widely and identification of traditional risk factors is the basis of most primary prevention screening policies, these calculators are imperfect; the majority of persons presenting with an adverse cardiac event have no prior history or any established risk factors (Wang, 2011). The converse is also true, many patients with risk factors for cardiovascular disease do not go onto develop atherothrombosis or have an acute cardiovascular event.

An alternative and perhaps preferable strategy would be to identify individuals with early atherosclerotic disease in an attempt to modify the course of the disease and prevent the clinical consequences of plaque rupture, thrombosis, and infarction. Clinical and preclinical research has focused on the identification of novel biomarkers that could indicate the early development of atherothrombotic disease. Before a biomarker is introduced to the clinic as a diagnostic tool, it must satisfy the following criteria: it must be highly specific, easy to measure, provide novel information about the disease, and reflect disease burden accurately helping the clinician decide on an appropriate course of treatment. These basic criteria are not easily met for a heterogeneous condition like atherothrombosis.

Atherothrombosis is a complex inflammatory disease and is classified according to plaque composition (van Gils et al., 2009). Plaque rupture and subsequent thrombosis involve a number of inflammatory processes including endothelial dysfunction, leukocyte recruitment and migration, platelet activation, matrix metalloproteinase activity, and degradation of the fibrous cap resulting in plaque rupture and thrombosis (Slevin et al., 2008). Imaging strategies that target these pathological processes in atherosclerotic plaque development may provide more sensitive methods to monitor disease progression, permit early detection of disease, and have a role in the assessment of treatment efficacy. The ability to identify plaque structure and function/activity would allow the clinician to identify those individuals at highest risk of plaque rupture.

Nanotechnology in Medical Imaging

The application of nanotechnology to medical imaging is an exciting new frontier in medical science. To date, only a handful of nanoparticles are used routinely in the clinic (Rosner and Auerbach, 2011; Weidner et al., 2011), but many more are in development or under investigation in clinical trials (Kim et al., 2010). As a research tool, engineered nanomaterials are invaluable (Mulder et al., 2007). Unlike traditional imaging strategies, nanoparticle-based or molecular imaging modalities enable us to study the underlying mechanisms of disease in vivo to identify the biological, chemical, and genetic factors contributing to disease (McCarthy et al., 2010). On a simpler level, nanoparticles have been used to improve the effectiveness of current clinical diagnostic tools. For example, superparamagnetic iron oxide nanoparticles (SPION) are used as a contrast agent in patients undergoing magnetic resonance imaging (MRI) to improve spatial resolution.

Imaging as a diagnostic tool has gained importance over the years and is now indispensable in the clinic. Systems, such as positron-emission tomography (PET), single-photon-emission computed tomography (SPECT), fluorescence reflectance imaging, fluorescence-mediated tomography (FMT), fibre-optic microscopy, optical frequency-domain imaging, will provide the future platforms for noninvasive molecular cardiovascular imaging. Comprehensive reviews of the nanomaterials in development for cardiovascular imaging are available elsewhere (Jaffer et al., 2006a,b; Nahrendorf et al., 2008, 2009; Sosnovik et al., 2008).

Nanoparticles are unique in terms of their size and structure. Their small size allows them to interact with the body on a molecular level and confers upon them a high ratio of surface area to volume. They can be engineered to have different shapes, sizes, structures, surface functionalization, and coatings, allowing them to be specifically targeted to a biomarker of disease (Song et al., 2010). The ability to understand and manipulate the properties of nanomaterials will ultimately determine their usefulness in nanomedicine (Cormode et al., 2009). Table 11.1, gives examples of nanomaterials intended for clinical use.

Nanomaterial synthesis has progressed rapidly in recent years and has generated complex state-of-the-art nanoscale structures with diverse and complex functionalities (Cormode et al., 2009). The ability to construct such nanomaterials has led to the development of multimodal and multifunctional nanoparticles to facilitate MRI, CT, and PET imaging or for targeted drug delivery (Kim et al., 2011). The development of such nanoparticles has led to the terms “theragnostic” or “theranostic” to describe a nanoparticle that can be used both for diagnostic and therapeutic applications (Cormode et al., 2009; Schladt et al., 2011).

TABLE 11.1 Nanomaterials in Clinical Use and Development for Imaging, Diagnosis, and Therapy

Nanomaterials	Commercial name	Application	Target	Adverse effects	Manufacturer	Current status
Metallic						
Iron oxide	Feridex	MRI	Liver	Back pain, vasodilation	Bayer Schering	FDA approved
	Resovist	MRI	Liver	None	Bayer Schering	FDA approved
	Combidex	MRI	Lymph nodes	None	Advanced Magnetics	In phase 3 clinical trials
	Nano Therm	Cancer therapy	Various	Acute urinary retention	MagForce	In phase 3 clinical trials
	Feraheme	Iron replacement therapy	RES macrophages of the liver, spleen, and bone marrow	Diarrhea, nausea, dizziness, hypotension, constipation, peripheral edema anaphylactic-type reactions, cardiac arrest, Tachycardia, angioedema, ischemic myocardial events.	AMAG Pharmaceuticals	FDA approved
Gold	Verigene	In vitro diagnostics	Genetic	N/A	Nanosphere	FDA approved
	Aurimmune	Cancer therapy	Various	Fever	CytImmune Sciences	In phase 2 clinical trials
Nanoshells	Auroshell	Cancer therapy	Head and Neck	Under investigation	Nanospectra Biosciences	In phase 1 clinical trials

Semiconductor						
Quantum dot	Qdots, EviTags, semiconductor nanocrystals	Fluorescent contrast In vitro diagnostics	Tumors, cells, tissues, and molecular sensing structures	N/A	Life Technologies, eBioscience, Nanoco, CrystalPlex, Cytodiagnostics	Research use only
Organic						
Protein	Abraxane	Cancer therapy	Breast	Cytopenia	Abraxis Bioscience	FDA approved
Liposome	Doxil/Caelyx	Cancer therapy	Various	Hand-foot syndrome, stomatitis	Ortho Biotech	FDA approved
Polymer	Oncaspar	Cancer therapy	Acute lymphoblastic leukemia	Urticaria, rash	Rhône-Poulenc Rorer	FDA approved
	CALAA-01	Cancer therapy	Various	Mild renal toxicity	Calando	In phase 2 clinical trials
Dendrimer	VivaGel	Microbicide	Cervicovaginal	Abdominal pain, dysuria	Starpharma	In phase 2 clinica1 trials
Micelle	Genexol-PM	Cancer therapy	various	Peripheral sensory neuropathy, neutropenia	Samyang	For phase 4 clinical trials

Adapted from Kim et al. 2010 (Kim et al., 2010).

Superparamagnetic Iron Oxide Nanoparticles

Stark et al. (1985) used MRI to determine the quantitative relationship between relaxation rates and iron levels in patients with iron overload and it was quickly deduced that the increase in T2 relaxation rates in these patients could be artificially replicated and used to provide contrast in magnetic resonance imaging (Renshaw et al., 1986). MRI imaging is used routinely to assess the extent of damage after a myocardial infarct Fig. 11.1 or to monitor the extent of peripheral vascular disease (Uppal and Caravan, 2010). SPIONs were originally used to provide contrast when imaging liver (Fukukura et al., 2010), spleen (Beduneau et al., 2009) and lymph nodes (Weidner et al., 2011) as this is where these particles naturally accumulated. With improvements in nanoparticle production iron oxide nanoparticles were designed to be more biocompatible with increased circulation times to assist with MRI imaging (Krombach et al., 2002). Particle size and surface coating can be modified to target SPIONs to different sites. Larger particles, 50–150 nm in diameter, predominantly produce a signal decrease and are used as contrast media for imaging the liver and spleen. Smaller particles, less than 20 nm, are distributed more widely in vivo and can improve the identification of lymph nodes (Harisinghani et al., 1997, 2003) or assist in the characterization of vulnerable atherothrombotic plaque. SPIONs have been used to enhance plaque imaging in a number of experimental models of atherosclerosis (Briley-Saebo et al., 2008; Klug et al., 2008, 2009; Sigovan et al., 2009), however the clinical studies published to date are less consistent (Morishige et al., 2010; Richards et al., 2011; Tang et al., 2008; Yilmaz et al., 2011).

Macrophages are the most common target for molecular imaging agents as there is a large body of evidence implicating them in the disease and, as a naturally phagocytic cell they, tend to scavenge foreign material without much manipulation. Several experimental studies have demonstrated that SPIONs or ultrasmall paramagnetic iron oxide nanoparticles (USPIONs) can locate to atherosclerotic plaques and can be used to evaluate its metabolic state (Klug et al., 2009; Kooi et al., 2003; Richards et al., 2011; Schmitz et al., 2001; Trivedi et al., 2004). Targeted molecular imaging has been shown to result in localization of engineered nanoparticles within macrophage in atherosclerotic plaque (Kooi et al., 2003; Ruehm et al., 2001; Trivedi et al., 2003).

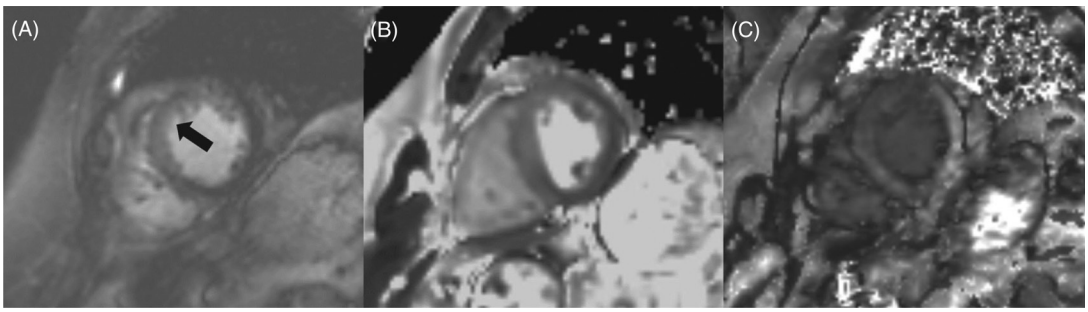


FIGURE 11.1 Patient imaged 3 days after an antero-septal myocardial infarction. Images show late gadolinium enhancement (LGE) in the region of the myocardial infarct (A). The middle image (B) shows myocardial edema (high T2 signal) on unenhanced T2 mapping in the region of the infarct. The image on the right (C) shows greater accumulation of USPIO, 24 h after infusion, in the region of the infarct compared to other areas of myocardium. *Source: Image courtesy of Dr. Colin Stirrat.*

Other applications of SPIONs include the assessment of myocardial perfusion (Jing et al., 2008; Krombach et al., 2002) and characterization of aortic aneurysm formation (Richards et al., 2011). SPIONs with a modified coat have also been used in molecular imaging, such as receptor-directed imaging (Eck et al., 2010), cell labelling for in vivo monitoring of stem cell migration (Thu et al., 2009), and in labelling gene constructs for localization in gene therapy studies (Zhao et al., 2010). A recent study by Florian et al. (2014) even suggested that intravenous iron oxide nanoparticles improved infarct healing in patients with acute ST-elevation myocardial infarction.

Ultimately, the usefulness of SPIONs in a clinical setting will require the combination of conventional imaging techniques with the design and synthesis of particles with specific properties. Whether designing a simple nanoparticle structure or a complex multilayered particle the choice of the core and monolayer material is critical as each layer dictates function and biopersistance (Lodhia et al., 2010). For molecular imaging purposes SPIONs need to be biocompatible, nontoxic, have a high magnetic moment, high colloidal stability, uniform particle size and the ability to bind various types of biological molecules (Lodhia et al., 2010).

The particle surface, functionalization or coating acts as the interface between the body and the core of the nanoparticle often acting as a barrier to stabilize the core thus ultimately determining the reactivity and solubility of the nanoparticle core (Mahmoudi et al., 2011). Often the monolayer is used to target the core molecule directing it to a specific area of interest. Drug delivery to a specific area of the body without the associated systemic side effects is one of the most attractive attributes of a nanoparticle-based drug delivery platform (Mahmoudi et al., 2011). Delivery of SPIONs to a specific area of interest or disease state is dictated by their surface functionalization (Uppal and Caravan, 2010; Uppal et al., 2011).

For the most part SPIONs are in development as MRI contrast agents, which relies on them having a high saturation magnetism value or electromagnetic units per gram (Lodhia et al., 2010). This value will determine the overall effectiveness of the contrast agent. Relaxation rates are a measure of the ability of a contrast agent to enhance the relaxation rate of water protons. SPIONs with high T2 values have faster relaxation with the surrounding water protons and therefore faster relaxation rates. Synthesis is critical in determining the magnetization value of SPIONs. Properties that effect the magnetization value of a SPION include size and nanoparticle aggregation with the highest values occurring in particles between 6 and 20 nm in diameter. When each particle is separate it will act independently, which increases the net magnetism per gram of particles. The more particles aggregate the lower their magnetization values with surface coatings are often used to prevent aggregation.

Research focusing on understanding the various interactions of SPIONs with the glycocalyx and biomolecules in the body system (i.e., protein corona, see Chapter 1) would lead to novel SPIONs with optimum surface properties that could overcome the problems of toxicity (see below) and permit more successful targeting (Mahmoudi et al., 2011; Shaw and Murthy, 2010). Examples of SPIONs in development or clinical use are given in Table 11.2.

Lipid-Based Nanoparticles

Although more commonly associated with drug delivery a number of studies have assessed the diagnostic potential of lipid-based nanoparticles (Briley-Saebo et al., 2011; Krombach et al., 2002; Liu et al., 2011). Liposomes contain amphiphilic molecules, which have both

TABLE 11.2 Iron-Oxide Based Nanomaterials in Clinical, Preclinical, and Development for Diagnosis and Therapy

Name	Status	Application	Administration	Relaxivities (m/mol/sec)	Coating	Core size (nm)
Lumirem and Gastromark	USA and Europe	GI	Oral	T2-72 T1-3.2	Silica	300
Abdoscan	Europe	GI	Oral	N/A	Polystyrene	300
Endorem/ Feriedex	USA	Liver/Spleen	IV	T2-98.3 T1-23.9	Dextran	5.6
Resovist	Withdrawn from some markets	Liver/Spleen	IV	T2-151.0 T1-25.4	Carbo-Dextran	4.2
Sinerem/ Combidex	Clinical trial	Lymph node Bone marrow	IV	T2-44.1 T1-21.6	Dextran	4–6
Clariscan	Discontinued	Perfusion/ angiography	IV	T2-35 T1-20	Carbohydrate-PEG	5–7
Supravist	Preclinical	Perfusion Lymph node Bone marrow	IV	T2-57 T1-7.3	Carbo-Dextran	3–5
MION46	Preclinical	Angiography Lymph node Tumor infarction	IV	N/A	Dextran	4–6
Feraheme	USA	Iron replacement therapy	IV	Polyglucose sorbitol carboxymethylether	N/A	2–10

Adapted from Lodhia et al (Lodhia et al., 2010).

hydrophobic and hydrophilic groups that self-assemble in water resulting in the formation of a hydrophilic core, whereas a micelle is a single layer of amphiphilic molecules with a hydrophobic core. These constructs can be 'loaded' with drugs, contrast agents, such as gadolinium, or gases for in vivo imaging (Liu et al., 2011; Mulder et al., 2007; Yang et al., 2008). Amirbekian et al. demonstrated that micelles containing gadolinium (Gd-micelles:100 nm) and targeted to the macrophage scavenging receptor CD204 enhanced MR imaging of atheroma compared to untargeted Gd-micelles, with increases in signal intensity directly related to the macrophage content of atheromatous plaque (Amirbekian et al., 2007). Briley-Saebo et al. used lipid-coated USPIONs and SPIONs functionalized to target oxidized phospholipids or malondialdehyde-lysine for plaque imaging and demonstrated signal loss on T2* weighted MR images of atherosclerotic lesions with preferential accumulation of the epitope-targeted PEG-nanoparticles within lipid-rich macrophage foam cells (Mulder et al., 2007). The authors suggest this was due to binding of particles to extracellular oxidized low density lipoproteins (LDL) at these sites, or binding to oxidized LDL on macrophage scavenger receptors and enhanced uptake. They conclude that these particles may provide a platform to target vulnerable atherosclerotic plaques (Briley-Saebo et al., 2011). A recent study demonstrated delivery of prednisolone to macrophages of iliofemoral atherosclerotic plaques (van der Valk et al., 2015). The study did not demonstrate any antiinflammatory effect, but it is a very important starting point for the

development of nanoparticles for use in a clinical setting. Combining nanoparticle technologies may yield more versatile particles with both diagnostic and therapeutic applications.

Gold Nanoparticles

Certain metal nanoparticles, such as gold, with the application of light, generate a strong surface plasmon field by oscillating electrons around the metal surface. The field strength may depend upon the metal type, particle size, and the wavelength of light. Gold is a metal of interest as it is considered chemically inert and can generate an intense surface plasmon field. At present, most colloidal synthesis protocols are based around the reduction of chloroauric acid $HauCl_4$ in the presence of citrate to produce monodisperse particles with controlled equivolume diameters of 10–60 nm (Khlebtsov and Dykman, 2011). Different reduction agents are used if other shaped nanoparticles are desired. Rod-shaped gold nanoparticles are useful for near-infrared imaging and in combination with photoacoustic imaging show promise in detection and diagnosis of cardiovascular disease (Pan et al., 2010). van Schooneveld et al. (2010) developed a trimodal gold nanoparticle for enhanced imaging using MRI, CT and FI of macrophage cells in vitro and liver in vivo with potential applications for imaging chronic inflammatory diseases and a recently reported that functionalized gold nanoparticles demonstrated increased uptake in the brain potentially allowing for greater delivery of drugs to the brain while reducing the systemic dose (Clark and Davis, 2015).

PARTICOKINETICS OF NANOMATERIALS FOR DIAGNOSTIC USE

Understanding the particokinetics of engineered nanomaterials is a critical consideration when designing them for biomedical uses. Engineered NPs accumulate differentially throughout the body depending on their physiochemical properties; characteristics of the NP, such as size, surface area, charge and surface chemistry as well as the method by which they are introduced (intravenous infusion, subdermal injection, inhalation, or ingestion) determine the ultimate fate of the NP once inside the body.

Surface characteristics are important in determining whether or not a particle will be cleared by the recticulo-endothelial system after intravenous administration (Al-Jamal et al., 2009). When hydrophobic nanoparticles are injected directly into the circulation, they rapidly become opsonized by blood serum proteins and are cleared by the reticulo-endothelial system. However, this opsonization and first pass clearance can be limited by alteration of the surface coatings of the nanoparticles. The most common method of prolonging circulation is conjugation of the nanoparticle with polyethylene glycol (PEG) polymer, providing the particle with steric hindrance to opsonization or phagocytosis (Li and Huang, 2008) and allowing the PEGylated form to remain in the circulation for much longer increasing interaction with blood components. A number of studies have demonstrated altered distribution patterns based on modification of the surface coating. Beduneau et al. (2009) covalently conjugated IgG molecules to the surface of SPIONs showing accelerated uptake by monocytes and enhanced retention in vivo without affecting monocyte viability. This technique could be very useful in the monitoring and tracking of disease progression. It is clear that surface modification has the ability to alter the distribution and clearance patterns of nanoparticles in the body, with

TABLE 11.3 Size-Based Biodistribution of Nanomaterials

Hydrodynamic diameter	Tissue uptake	Renal excretion
>30nm	Rapidly cleared by reticuloendothelial system (RES)	Not excreted by kidneys, excretion involves degradation of NP in RES tissue
10–30nm	Longer circulation time leads to increased uptake by non-RES tissues.	Little, <5%, excretion by renal system excretion involves degradation of NP in RES tissue
4–10nm	Longer circulation time leads to increased uptake by non-RES tissues.	Renal clearance increases as particle size decreases
<4nm	Uptake in most cell types for particles not excreted by kidneys	Rapid clearance of nanoparticles by kidneys with retention of between 5% and 10% of injected dose

small changes in surface moiety resulting in major differences in behavior of these particles in the body (Jayagopal et al., 2007; van Tilborg et al., 2008).

Hydrodynamic size also determines distribution (De Jong et al., 2008). Normally, in a healthy individual, the endothelium is very effective at preventing extravazation of nanoparticles due to the presence of tight gap junctions (<2 nm in diameter). However, when the endothelium is compromised, as is the case in atherosclerosis, these normally tight junctions become leaky and could potentially allow passage of nanoparticles to the underlying basement membrane. The final destination of particles in the body has been reported to be directly related to their size; larger particles, greater than 100 nm in diameter, deposit in the spleen due to its discontinuous endothelium with large fenestrations and smaller particles, less than 100 nm in diameter, deposit in the liver passing through the fenestrated endothelium and finally residing in the underlying parenchymal cells. Kinetic studies with gold nanoparticles shave shown their pattern of distribution to be size dependent (Hirn et al., 2011). De Jong et al. (2008) intravenously administered gold nanoparticles in rats and found that the biodistribution was dependent on particle size with the smallest particles (10 nm) having the most widespread organ distribution. This becomes very important when using nanoparticles to deliver drugs as the drug may reaches unintended parts of the body in higher concentrations than had been intended. Table 11.3 breaks down the size-dependent biodistribution of systemic nanoparticles.

COMBUSTION-DERIVED NANOPARTICLES IN CARDIOVASCULAR DISEASE

While nanotechnology aims to develop new engineered NPs with novel properties there are numerous sources of NPs in the environment from natural and anthropogenic sources. There have been many epidemiological, in vitro, in vivo and ex vivo studies demonstrating the adverse health effects of PM_{10} with the majority of investigators identifying the "ultrafine" or nanoparticulate component as the most toxic of airborne pollutants (Barlow et al., 2005; Brown et al., 2003; Donaldson et al., 1997; Geiser et al., 2005; Mills et al., 2008b;

Moller et al., 2005; Renwick et al., 2004). These studies have raised concerns about the potential for engineered NPs to exert similar adverse health effects. Exposure to ambient particles is associated with more hospitalizations with acute cardiovascular events and an increase in cardiovascular deaths (Brook et al., 2002; Delfino et al., 2005; Mills et al., 2007b, 2009). These effects are thought to be mediated by particle induced oxidative stress and inflammation leading to vascular dysfunction, impaired fibrinolysis, and changes in the autonomic regulation of heart rate and rhythm (Langrish et al., 2008; Mills et al., 2009). Combustion-derived nanoparticles (CDNPs) have been shown to be prothrombotic (Mills et al., 2007a) and may contribute to the formation of atherosclerotic plaques. While CDNPs differ greatly in chemical composition compared to engineered NPs they share similarities in other characteristics considered toxicologically significant, such as size and surface area. It is therefore plausible that for engineered nanoparticles will exert similar proinflammatory and prothrombotic effects; especially those manufactured for direct intravenous administration as a contrast agent in medical imaging (Donaldson, 2006; Donaldson and Seaton, 2007). Given the huge amount of published data implicating CDNPs in the pathogenesis of cardiovascular diseases (Donaldson et al., 2005) an understanding of the toxicology of nanoparticles intended for medical use is central to their future use.

TOXICITY OF ENGINEERED NANOPARTICLES IN CARDIOVASCULAR DISEASE

It is not surprising that a number of recent reviews focusing on the effects of occupational exposure to engineered nanomaterials have raised concerns that they could have similar health implications as CDNP (Castranova, 2011; Eisen et al., 2011; Moller et al., 2011). Resolution of these issues will require a collaborative approach between industry, government, academic researchers, and clinicians. In addition to occupational exposure to engineered nanomaterials the direct exposures to engineered nanoparticles through medical use in susceptible populations is of immediate concern. Engineered nanomaterials unlike novel pharmacological therapies are not routinely tested to determine their effect on the cardiovascular system prior to clinical use. Tables 11.1 and 11.2 highlight the many different nanoparticles that are being developed for clinical use. All of these nanoparticles, not just those intended for the diagnosis and treatment of cardiovascular disease, have the potential to interact with cells associated with the progression of cardiovascular disease. To date, there are no studies linking exposure to engineered nanomaterials and cardiovascular events, however a number of preclinical studies have reported that exposure to engineered nanomaterials, directly or indirectly, can cause endothelial dysfunction and platelet activation, and may plausibly contribute to ischemic heart disease (McGuinnes et al., 2011; Sun et al., 2011; Zhu et al., 2010, 2011). The schematic diagrams in Figs. 11.2 and 11.3 identify mechanisms and potential targets for engineered NPs in circulation.

In Vitro Studies

Cardiovascular disease is a complex and dynamic process with many contributing factors and therefore a single in vitro model to study the adverse effects of engineered nanomaterials

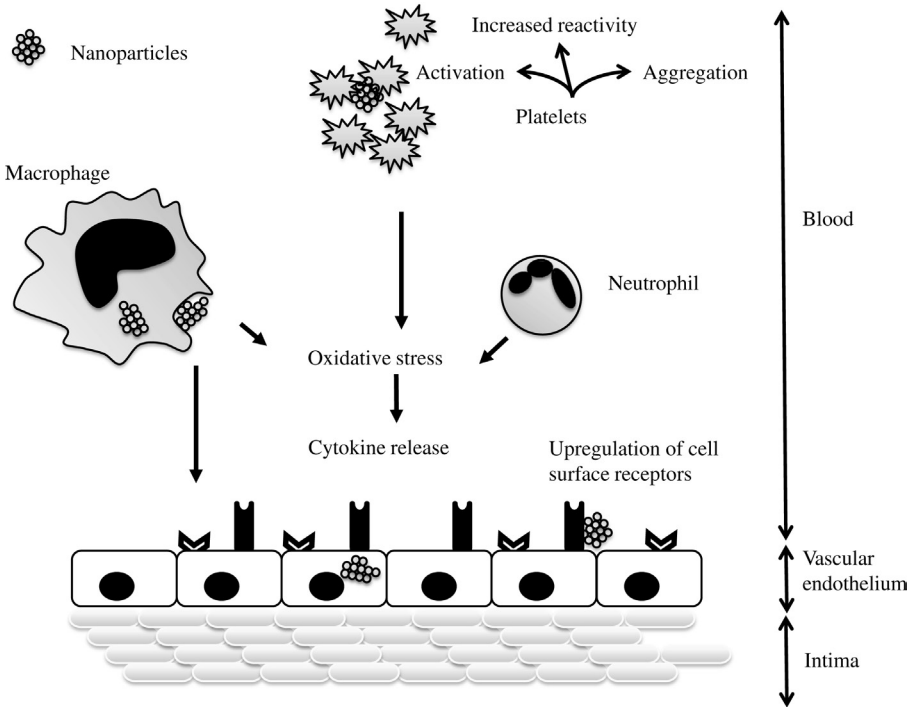


FIGURE 11.2 **Overview of interactions of blood-borne nanoparticles.**

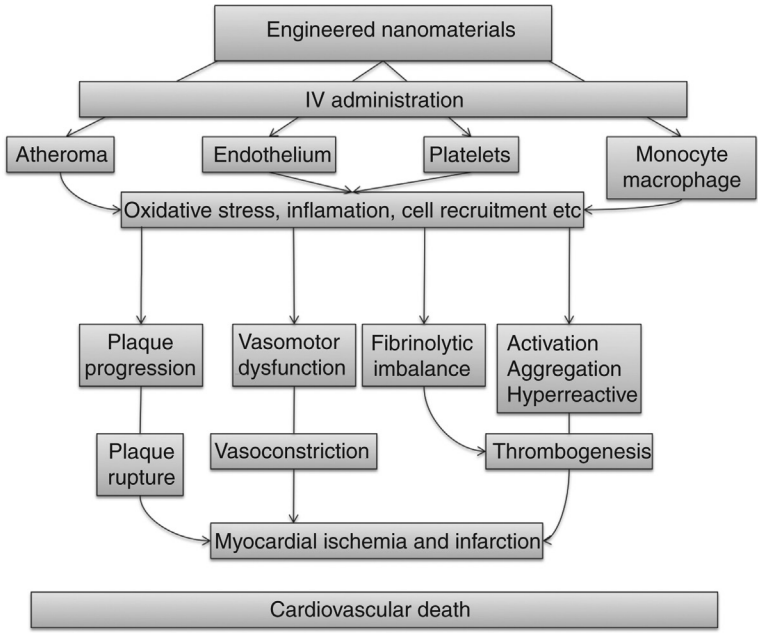


FIGURE 11.3 **Potential endpoints of systemic engineered nanoparticles.**

on cardiovascular system is unlikely to emerge. However, platelets, platelet-monocyte aggregation and the vascular endothelium have emerged as useful in vitro systems given their key role in the pathogenesis of coronary and ischemic heart disease (Bihari et al., 2010; McGuinnes et al., 2011; Neun and Dobrovolskaia, 2011).

Compatibility of nanoparticles with blood components is important and a number of studies have employed assays of hemolysis (Lu et al., 2009), platelet aggregation and activation (Radomski et al., 2005), and platelet-leukocyte aggregation (McGuinnes et al., 2011). Direct and indirect toxicity to the cardiovascular system can be determined using cell lines with assays of oxidative stress, inflammation, and cell death (Dick et al., 2003). Whether observations from these in vitro models apply in vivo or in the clinic is uncertain, and in the absence of any standards for the assessment of the cardiovascular toxicity of engineered nanomaterials the role of in vitro assessments remain controversial.

Recent studies have shown that engineered NPs can interact with platelets causing activation, aggregation (McGuinnes et al., 2011; Miller et al., 2009; Radomski et al., 2005) and hyperreactivity (unpublished observations). Surface charge is thought to be a critical determinant in platelet-nanoparticle activation (McGuinnes et al., 2011). When studying the impact of engineered nanomaterials on a complex multisystem disease it is important to take into consideration potential downstream, indirect effects as well as direct toxicity. Shaw et al (Shaw et al., 2011) demonstrate that CDNP induce moderate toxicity and inflammatory effects on endothelial cells following direct incubation, these effects were amplified when endothelial cells were exposed to conditioned supernatant from monocyte-derived macrophages exposed to CDNP due to the release of cytokines from CDNP laden macrophage. This approach utilizing coculture and other techniques that model the secondary effects of CDNP (Inoue and Takano, 2010; Ishii et al., 2005; Porter et al., 2007), could be very useful in studying the cardiovascular effects of engineered nanomaterials. Nanomaterials are increasingly being used in cell tracking to monitor disease progression (Jendelova et al., 2004; Jing et al., 2008; Voura et al., 2004). It is no longer sufficient to assess the potential toxicity of engineered nanoparticles on the cell carriers alone; it is essential that these cells do not become "time bombs" capable of releasing vast quantities of proinflammatory mediators in a vulnerable atherosclerotic plaque. This mechanism may be equally important in the occupational setting where inhalation of engineered nanomaterials has been shown to have systemic effects on the cardiovascular system (Adamkiewicz et al., 2004; Alfaro-Moreno et al., 2007; Antonini, 2003; Behndig et al., 2006; Brook et al., 2003).

In Vivo Studies

In vivo studies carried out in susceptible animals have contributed significantly to our understanding of cardiovascular disease. In the ApoE knock-out mouse CDNP instilled into the lungs increases atherogenesis (Jacobsen et al., 2009; Lucking et al., 2008; Tornqvist et al., 2007) and causes more complex and possibly more vulnerable plaques to form (Miller et al., 2013). Indeed engineered nanomaterials, such as carbon nanotubes and TiO_2 can accelerate plaque progression in susceptible animal models (Moller et al., 2011). Mills et al. exposed healthy human volunteers to dilute diesel exhaust, pure carbon nanoparticles, filtered diesel exhaust, or filtered air, in a randomized double blind cross-over study demonstrating that the adverse vascular effects of diesel exhaust could be attributed mainly to the

nanoparticulate component and not to the associated gaseous pollutants (Mills et al., 2011). Observations in vivo were replicated in vitro using wire-myography providing a rationale for testing the effects of engineered nanomaterials on vascular function before introduction to the clinic.

Ex Vivo Studies

Ex vivo studies have been very informative in modeling the effects of CDNP on cardiovascular disease and have potential use in determining the effects of engineered nanomaterials on cardiovascular disease. They allow measurements to be taken in tissues or artificial environments outside the organism but with as little as possible deviation from natural conditions with no risk to the patient. Ex vivo techniques have not been used widely to study the effects of engineered nanomaterials on the cardiovascular system due to the need for specialist equipment, training, and the high cost involved, however given the potential to generate meaningful data in the near patient setting these systems are worth consideration. Ex vivo techniques also side-step possible ethical issues associated with exposing healthy or disease populations to the unknown effects of engineered nanoparticles. Traditional ex vivo models have assessed the direct effects of CDNP in isolated vessels to assess vasomotor dysfunction. This chapter does not intend to review previous studies as they have largely focused on CDNP; however, this topic is very well covered by many comprehensive reviews, and the findings could easily be applied to the study of the adverse effects of engineered nanomaterial (Moller et al., 2011).

A recent ex vivo study used the Langendorff heart as a model system to study the cardiovascular effects of engineered nanoparticles (Stampfl et al., 2011). The isolated heart is a well-established method of modeling cardiac dysfunction and is used extensively to monitor electrophysiological parameters after exposure to investigational products. This model assesses the direct effects of engineered nanoparticles on the intact heart without the contribution from other systemic effects (Moller et al., 2011). The authors report that SiO_2, TiO_2 nanoparticles and engineered nanoparticles made of flame soot caused a significant dose-dependent increase in heart rate and promoted cardiac arrhythmia. They hypothesize that the nanoparticle-induced increase in heart rate might be caused by the release of catecholamines from the sympathetic nerve endings in the heart, which was supported by studies using β1-receptor antagonism postexcision and by depleting catecholamine stores in the neurons preexcision.

As many medical nanoparticles in development are for use in populations considered particularly vulnerable, it would be desirable to model this ex vivo. The Badimon chamber (Fig. 11.4) is an ex vivo model of deep arterial injury and thrombus formation used previously to study the effects of diesel exhaust inhalation on thrombosis in man (Lucking et al., 2008; Lundback et al., 2009). The chamber permits an assessment thrombus formation in whole native blood without the need for anticoagulants or exposure of the patient to the compounds being tested. While principally used to assess the impact of novel antithrombotic agents it can just as easily be used to assess the effects of engineered nanomaterials on thrombosis. A pump is used to draw blood from an antecubital vein through the chambers maintained at 37°C in a water bath. The rheological conditions in the first chamber simulate those of patent coronary arteries (low-shear rate), whereas those in the second and third chambers simulate those of

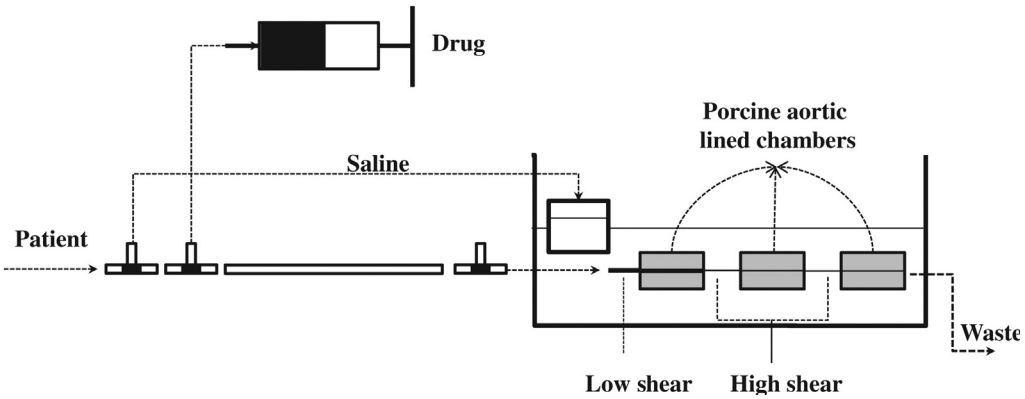


FIGURE 11.4 Schematic of Badimon chamber set-up. Consult text for details.

stenosed coronary artery (high-shear rate) (Lucking et al., 2008). The thrombus formed in the low shear chamber is macrophage rich whereas the thrombus formed in high shear conditions is platelet rich (Lucking et al., 2008). The chamber is therefore potentially able to assess nanoparticle interactions with specific cells and types of thrombus. Our preliminary investigations suggest that engineered nanomaterials may increase thrombus formation and that surface charge may be an important determinant.

CONCLUSIONS

Engineered nanomaterials are likely to have a big impact on diagnostic assays, medical imaging, and targeted therapeutics in the near future. Characteristics of these nanoparticles, such as size, surface area, charge, and surface chemistry as well as the method by which they are to be delivered will determine their distribution, efficacy and potential toxicity. Nanoparticles designed for medical use are usually derived from materials deemed biologically safe, but it remains unclear how we define a "biologically safe" material and in particular, what investigations should be mandatory in the development of these materials. Unfortunately, the behavior of nanoparticles cannot be determined from their bulk properties; when relatively inert or safe materials in their bulk form are presented in the nanoscale they may exert previously unseen harmful effects on cells and systems. If we are to maximize the potential of nanotechnologies in the field of medicine, for instance for diagnostic applications in cardiovascular disease, we must ensure that novel nanomaterials are rigorously tested before introduction to the clinic.

References

Adamkiewicz, G., Ebelt, S., Syring, M., Slater, J., Speizer, F.E., Schwartz, J., et al., 2004. Association between air pollution exposure and exhaled nitric oxide in an elderly population. Thorax 59 (3), 204–249.

Alfaro-Moreno, E., Nawrot, T.S., Nemmar, A., Nemery, B., 2007. Particulate matter in the environment: pulmonary and cardiovascular effects. Curr. Opin. Pulm. Med. 13 (2), 98–106.

Al-Jamal, W.T., Al-Jamal, K.T., Cakebread, A., Halket, J.M., Kostarelos, K., 2009. Blood circulation and tissue biodistribution of lipid-quantum dot (L-QD) hybrid vesicles intravenously administered in mice. Bioconjug. Chem. 20 (9), 1696–1702.
Amirbekian, V., Lipinski, M.J., Briley-Saebo, K.C., Amirbekian, S., Aguinaldo, J.G., Weinreb, D.B., et al., 2007. Detecting and assessing macrophages in vivo to evaluate atherosclerosis noninvasively using molecular MRI. Proc. Natl. Acad. Sci. USA 104 (3), 961–966.
Antonini, J.M., 2003. Health effects of welding. Crit. Rev. Toxicol. 33 (1), 61–103.
Barlow, P.G., Clouter-Baker, A., Donaldson, K., Maccallum, J., Stone, V., 2005. Carbon black nanoparticles induce type II epithelial cells to release chemotaxins for alveolar macrophages. Part. Fibre Toxicol. 2, 11.
Beduneau, A., Ma, Z., Grotepas, C.B., Kabanov, A., Rabinow, B.E., Gong, N., et al., 2009. Facilitated monocyte-macrophage uptake and tissue distribution of superparmagnetic iron-oxide nanoparticles. PLoS One 4 (2), e4343.
Behndig, A.F., Mudway, I.S., Brown, J.L., Stenfors, N., Helleday, R., Duggan, S.T., et al., 2006. Airway antioxidant and inflammatory responses to diesel exhaust exposure in healthy humans 11. Eur. Respir. J. 27 (2), 359–365.
Bihari, P., Holzer, M., Praetner, M., Fent, J., Lerchenberger, M., Reichel, C.A., et al., 2010. Single-walled carbon nanotubes activate platelets and accelerate thrombus formation in the microcirculation. Toxicology 269 (2–3), 148–154.
Briley-Saebo, K.C., Mani, V., Hyafil, F., Cornily, J.C., Fayad, Z.A., 2008. Fractionated Feridex and positive contrast: in vivo MR imaging of atherosclerosis. Magn. Reson. Med. 59 (4), 721–730.
Briley-Saebo, K.C., Cho, Y.S., Shaw, P.X., Ryu, S.K., Mani, V., Dickson, S., et al., 2011. Targeted iron oxide particles for in vivo magnetic resonance detection of atherosclerotic lesions with antibodies directed to oxidation-specific epitopes. J. Am. Coll. Cardiol. 57 (3), 337–347.
Brook, R.D., Brook, J.R., Urch, B., Vincent, R., Rajagopalan, S., Silverman, F., 2002. Inhalation of fine particulate air pollution and ozone causes acute arterial vasoconstriction in healthy adults. Circulation 105 (13), 1534–1536.
Brook, R.D., Brook, J.R., Rajagopalan, S., 2003. Air pollution: the "Heart" of the problem. Curr. Hypertens. Rep. 5 (1), 32–39.
Brown, L.M., Collings, N., Harrison, R.M., Maynard, A.D., Maynard, R.L., 2003. Ultrafine Particles in the Atmosphere. Imperial College Press, London.
Castranova, V., 2011. Overview of current toxicological knowledge of engineered nanoparticles. J. Occup. Environ. Med. 53 (6 Suppl.), S14–S17.
Clark, A.J., Davis, M.E., 2015. Increased brain uptake of targeted nanoparticles by adding an acid-cleavable linkage between transferrin and the nanoparticle core. Proc. Natl. Acad. Sci. USA 112 (40), 12486–12491.
Cormode, D.P., Skajaa, T., Fayad, Z.A., Mulder, W.J., 2009. Nanotechnology in medical imaging: probe design and applications. Arterioscler. Thromb. Vasc. Biol. 29 (7), 992–1000.
De Jong, W.H., Hagens, W.I., Krystek, P., Burger, M.C., Sips, A.J., Geertsma, R.E., 2008. Particle size-dependent organ distribution of gold nanoparticles after intravenous administration. Biomaterials 29 (12), 1912–1919.
Delfino, R.J., Sioutas, C., Malik, S., 2005. Potential role of ultrafine particles in associations between airborne particle mass and cardiovascular health. Environ. Health Perspect. 113 (8), 934–946.
Dick, C.A., Brown, D.M., Donaldson, K., Stone, V., 2003. The role of free radicals in the toxic and inflammatory effects of four different ultrafine particle types. Inhal. Toxicol. 15 (1), 39–52.
Donaldson, K., 2006. Resolving the nanoparticles paradox. Nanomedicine 1 (2), 229–234.
Donaldson, K., Seaton, A., 2007. The Janus faces of nanoparticles. J. Nanosci. Nanotechnol. 7 (12), 4607–4611.
Donaldson, K., Brown, D.M., Mitchell, C., Dineva, M., Beswick, P.H., Gilmour, P., et al., 1997. Free radical activity of PM10: iron-mediated generation of hydroxyl radicals. Environ. Health Perspect. 105, 1285–1289.
Donaldson, K., Tran, L., Jimenez, L., Duffin, R., Newby, D.E., Mills, N., et al., 2005. Combustion-derived nanoparticles: a review of their toxicology following inhalation exposure 1. Part. Fibre Toxicol. 2 (1), 10.
Eck, W., Nicholson, A.I., Zentgraf, H., Semmler, W., Bartling, S., 2010. Anti-CD4-targeted gold nanoparticles induce specific contrast enhancement of peripheral lymph nodes in X-ray computed tomography of live mice. Nano Lett. 10 (7), 2318–2322.
Eisen, E.A., Costello, S., Chevrier, J., Picciotto, S., 2011. Epidemiologic challenges for studies of occupational exposure to engineered nanoparticles; a commentary. J. Occup. Environ. Med. 53 (6 Suppl.), S57–S61.
Florian, A., Ludwig, A., Rosch, S., Yildiz, H., Klumpp, S., Sechtem, U., et al., 2014. Positive effect of intravenous iron-oxide administration on left ventricular remodelling in patients with acute ST-elevation myocardial infarction—a cardiovascular magnetic resonance (CMR) study. Int. J. Cardiol. 173 (2), 184–189.
Fukukura, Y., Kamiyama, T., Takumi, K., Shindo, T., Higashi, R., Nakajo, M., 2010. Comparison of ferucarbotran-enhanced fluid-attenuated inversion-recovery echo-planar, T2-weighted turbo spin-echo, T2*-weighted

gradient-echo, and diffusion-weighted echo-planar imaging for detection of malignant liver lesions. J. Magn. Reson. Imaging 31 (3), 607–616.
Geiser, M., Rothen-Rutishauser, B., Kapp, N., Schurch, S., Kreyling, W., Schulz, H., et al., 2005. Ultrafine particles cross cellular membranes by nonphagocytic mechanisms in lungs and in cultured cells. Environ. Health Perspect. 113 (11), 1555–1560.
Harisinghani, M.G., Saini, S., Slater, G.J., Schnall, M.D., Rifkin, M.D., 1997. MR imaging of pelvic lymph nodes in primary pelvic carcinoma with ultrasmall superparamagnetic iron oxide (Combidex): preliminary observations. J. Magn. Reson. Imaging 7 (1), 161–163.
Harisinghani, M.G., Barentsz, J., Hahn, P.F., Deserno, W.M., Tabatabaei, S., van de Kaa, C.H., et al., 2003. Noninvasive detection of clinically occult lymph-node metastases in prostate cancer. N. Engl. J. Med. 348 (25), 2491–2499.
Hirn, S., Semmler-Behnke, M., Schleh, C., Wenk, A., Lipka, J., Schaffler, M., et al., 2011. Particle size-dependent and surface charge-dependent biodistribution of gold nanoparticles after intravenous administration. Eur. J. Pharm. Biopharm. 77 (3), 407–416.
Hoffmann, B., Moebus, S., Mohlenkamp, S., Stang, A., Lehmann, N., Dragano, N., et al., 2007. Residential exposure to traffic is associated with coronary atherosclerosis. Circulation 116 (5), 489–496.
Inoue, K., Takano, H., 2010. Another systemic impact of inhaled diesel exhaust particles. Toxicol. Sci. 115 (2), 607–608.
Ishii, H., Hayashi, S., Hogg, J.C., Fujii, T., Goto, Y., Sakamoto, N., et al., 2005. Alveolar macrophage-epithelial cell interaction following exposure to atmospheric particles induces the release of mediators involved in monocyte mobilization and recruitment. Respir. Res. 6, 87.
Jacobsen, N.R., Moller, P., Jensen, K.A., Vogel, U., Ladefoged, O., Loft, S., et al., 2009. Lung inflammation and genotoxicity following pulmonary exposure to nanoparticles in ApoE-/- mice. Part. Fibre Toxicol. 6, 2.
Jaffer, F.A., Libby, P., Weissleder, R., 2006a. Molecular and cellular imaging of atherosclerosis: emerging applications. J. Am. Coll. Cardiol. 47 (7), 1328–1338.
Jaffer, F.A., Sosnovik, D.E., Nahrendorf, M., Weissleder, R., 2006b. Molecular imaging of myocardial infarction. J. Mol. Cell Cardiol. 41 (6), 921–933.
Jayagopal, A., Russ, P.K., Haselton, F.R., 2007. Surface engineering of quantum dots for in vivo vascular imaging. Bioconjug. Chem. 18 (5), 1424–1433.
Jendelova, P., Herynek, V., Urdzikova, L., Glogarova, K., Kroupova, J., Andersson, B., et al., 2004. Magnetic resonance tracking of transplanted bone marrow and embryonic stem cells labeled by iron oxide nanoparticles in rat brain and spinal cord 112. J. Neurosci. Res. 76 (2), 232–243.
Jing, X.H., Yang, L., Duan, X.J., Xie, B., Chen, W., Li, Z., et al., 2008. In vivo MR imaging tracking of magnetic iron oxide nanoparticle labeled, engineered, autologous bone marrow mesenchymal stem cells following intra-articular injection. Joint Bone Spine. 75 (4), 432–438.
Khlebtsov, N., Dykman, L., 2011. Biodistribution and toxicity of engineered gold nanoparticles: a review of in vitro and in vivo studies. Chem. Soc. Rev. 40 (3), 1647–1671.
Kim, B.Y., Rutka, J.T., Chan, W.C., 2010. Nanomedicine. N. Engl. J. Med. 363 (25), 2434–2443.
Kim, D., Yu, M.K., Lee, T.S., Park, J.J., Jeong, Y.Y., Jon, S., 2011. Amphiphilic polymer-coated hybrid nanoparticles as CT/MRI dual contrast agents. Nanotechnology 22 (15), 155101.
Klug, G., Bauer, L., Bauer, W.R., 2008. Patterns of USPIO deposition in murine atherosclerosis. Arterioscler. Thromb. Vasc. Biol. 28 (9), E157–E159.
Klug, K., Gert, G., Thomas, K., Christan, Z., Marco, P., Elisabeth, B., et al., 2009. Murine atherosclerotic plaque imaging with the USPIO Ferumoxtran-10. Front. Biosci. 14, 2546–2552.
Kooi, M.E., Cappendijk, V.C., Cleutjens, K.B., Kessels, A.G., Kitslaar, P.J., Borgers, M., et al., 2003. Accumulation of ultrasmall superparamagnetic particles of iron oxide in human atherosclerotic plaques can be detected by in vivo magnetic resonance imaging 2. Circulation 107 (19), 2453–2458.
Krombach, G.A., Wendland, M.F., Higgins, C.B., Saeed, M., 2002. MR imaging of spatial extent of microvascular injury in reperfused ischemically injured rat myocardium: value of blood pool ultrasmall superparamagnetic particles of iron oxide. Radiology 225 (2), 479–486.
Langrish, J.P., Mills, N.L., Newby, D.E., 2008. Air pollution: the new cardiovascular risk factor. Intern. Med. J. 38 (12), 875–878.
Li, S.D., Huang, L., 2008. Pharmacokinetics and biodistribution of nanoparticles. Mol. Pharm. 5 (4), 496–504.
Linkov, I., Satterstrom, F.K., Corey, L.M., 2008. Nanotoxicology and nanomedicine: making hard decisions. Nanomedicine 4 (2), 167–171.

Liu, Z., Lammers, T., Ehling, J., Fokong, S., Bornemann, J., Kiessling, F., et al., 2011. Iron oxide nanoparticle-containing microbubble composites as contrast agents for MR and ultrasound dual-modality imaging. Biomaterials 32 (26), 6155–6163.
Lodhia, J., Mandarano, G., Ferris, N., Eu, P., Cowell, S., 2010. Development and use of iron oxide nanoparticles (Part 1): synthesis of iron oxide nanoparticles for MRI. Biomed. Imaging Interv. J. 6 (2), e12.
Lu, S., Duffin, R., Poland, C., Daly, P., Murphy, F., Drost, E., et al., 2009. Efficacy of simple short-term in vitro assays for predicting the potential of metal oxide nanoparticles to cause pulmonary inflammation. Environ. Health Perspect. 117 (2), 241–247.
Lucking, A.J., Lundback, M., Mills, N.L., Faratian, D., Barath, S.L., Pourazar, J., et al., 2008. Diesel exhaust inhalation increases thrombus formation in man. EurHeart J. 29 (24), 3043–3051.
Lundback, M., Mills, N.L., Lucking, A., Barath, S., Donaldson, K., Newby, D.E., et al., 2009. Experimental exposure to diesel exhaust increases arterial stiffness in man. Part. Fibre Toxicol. 6, 7.
Mahmoudi, M., Sant, S., Wang, B., Laurent, S., Sen, T., 2011. Superparamagnetic iron oxide nanoparticles (SPIONs): development, surface modification and applications in chemotherapy. Adv. Drug Deliv. Rev. 63 (1–2), 24–46.
McCarthy, J.R., Korngold, E., Weissleder, R., Jaffer, F.A., 2010. A light-activated theranostic nanoagent for targeted macrophage ablation in inflammatory atherosclerosis. Small 6 (18), 2041–2049.
McGuinnes, C., Duffin, R., Brown, S., Mills, L., Megson, I.L., MacNee, W., et al., 2011. Surface derivatization state of polystyrene latex nanoparticles determines both their potency and their mechanism of causing human platelet aggregation in vitro. Toxicol. Sci. 119 (2), 359–368.
Medina, C., Santos-Martinez, M.J., Radomski, A., Corrigan, O.I., Radomski, M.W., 2007. Nanoparticles: pharmacological and toxicological significance. Br. J. Pharmacol. 150 (5), 552–558.
Miller, V.M., Hunter, L.W., Chu, K., Kaul, V., Squillace, P.D., Lieske, J.C., et al., 2009. Biologic nanoparticles and platelet reactivity. Nanomedicine (Lond). 4 (7), 725–733.
Miller, M.R., McLean, S.G., Duffin, R., Lawal, A.O., Araujo, J.A., Shaw, C.A., et al., 2013. Diesel exhaust particulate increases the size and complexity of lesions in atherosclerotic mice. Part. Fibre Toxicol. 10, 61.
Mills, N.L., Tornqvist, H., Robinson, S.D., Gonzalez, M., Darnley, K., MacNee, W., et al., 2005. Diesel exhaust inhalation causes vascular dysfunction and impaired endogenous fibrinolysis. Circulation 112 (25), 3930–3936.
Mills, N.L., Tornqvist, H., Gonzalez, M.C., Vink, E., Robinson, S.D., Soderberg, S., et al., 2007a. Ischemic and thrombotic effects of dilute diesel-exhaust inhalation in men with coronary heart disease. N. Engl. J. Med. 357 (11), 1075–1082.
Mills, N.L., Miller, J.J., Anand, A., Robinson, S.D., Frazer, G.A., Anderson, D., et al., 2007b. Increased arterial stiffness in patients with chronic obstructive pulmonary disease; a mechanism for increased cardiovascular risk. Thorax 63 (4), 306–311.
Mills, N.L., Donaldson, K., Hadoke, P.W., Boon, N.A., MacNee, W., Cassee, F.R., et al., 2008a. Adverse cardiovascular effects of air pollution. Nat. Clin. Pract. Cardiovasc. Med. 6 (1), 36–44.
Mills, N.L., Robinson, S.D., Fokkens, P.H., Leseman, D.L., Miller, M.R., Anderson, D., et al., 2008b. Exposure to concentrated ambient particles does not affect vascular function in patients with coronary heart disease. Environ. Health Perspect. 116 (6), 709–715.
Mills, N.L., Donaldson, K., Hadoke, P.W., Boon, N.A., MacNee, W., Cassee, F.R., et al., 2009. Adverse cardiovascular effects of air pollution. Nat. Clin. Pract. Cardiovasc. Med. 6 (1), 36–44.
Mills, N.L., Miller, M.R., Lucking, A.J., Beveridge, J., Flint, L., Boere, A.J., et al., 2011. Combustion-derived nanoparticulate induces the adverse vascular effects of diesel exhaust inhalation. Eur. Heart J. 32 (21), 2660–2671.
Moller, W., Brown, D.M., Kreyling, W.G., Stone, V., 2005. Ultrafine particles cause cytoskeletal dysfunctions in macrophages: role of intracellular calcium. Part. Fibre Toxicol. 2, 7.
Moller, P., Mikkelsen, L., Vesterdal, L.K., Folkmann, J.K., Forchhammer, L., Roursgaard, M., et al., 2011. Hazard identification of particulate matter on vasomotor dysfunction and progression of atherosclerosis. Crit. Rev. Toxicol. 41 (4), 339–368.
Morishige, K., Kacher, D.F., Libby, P., Josephson, L., Ganz, P., Weissleder, R., et al., 2010. High-resolution magnetic resonance imaging enhanced with superparamagnetic nanoparticles measures macrophage burden in atherosclerosis. Circulation 122 (17), 1707–1715.
Mulder, W.J., Strijkers, G.J., Briley-Saboe, K.C., Frias, J.C., Aguinaldo, J.G., Vucic, E., et al., 2007. Molecular imaging of macrophages in atherosclerotic plaques using bimodal PEG-micelles. Magn. Reson. Med. 58 (6), 1164–1170.
Nahrendorf, M., Zhang, H., Hembrador, S., Panizzi, P., Sosnovik, D.E., Aikawa, E., et al., 2008. Nanoparticle PET-CT imaging of macrophages in inflammatory atherosclerosis. Circulation 117 (3), 379–387.

Nahrendorf, M., Sosnovik, D.E., French, B.A., Swirski, F.K., Bengel, F., Sadeghi, M.M., et al., 2009. Multimodality cardiovascular molecular imaging. Part II. Circ. Cardiovasc. Imaging 2 (1), 56–70.
Neun, B.W., Dobrovolskaia, M.A., 2011. Method for in vitro analysis of nanoparticle thrombogenic properties. Methods Mol. Biol. 697, 225–235.
Pan, D., Pramanik, M., Senpan, A., Wickline, S.A., Wang, L.V., Lanza, G.M., 2010. A facile synthesis of novel self-assembled gold nanorods designed for near-infrared imaging. J. Nanosci. Nanotechnol. 10 (12), 8118–8123.
Porter, M., Karp, M., Killedar, S., Bauer, S.M., Guo, J., Williams, D., et al., 2007. Diesel-enriched particulate matter functionally activates human dendritic cells. Am. J. Respir. Cell Mol. Biol. 37 (6), 706–719.
Radomski, A., Jurasz, P., onso-Escolano, D., Drews, M., Morandi, M., Malinski, T., et al., 2005. Nanoparticle-induced platelet aggregation and vascular thrombosis 3. Br. J. Pharmacol. 146 (6), 882–893.
Renshaw, P.F., Owen, C.S., McLaughlin, A.C., Frey, T.G., Leigh, Jr., J.S., 1986. Ferromagnetic contrast agents: a new approach. Magn. Reson. Med. 3 (2), 217–225.
Renwick, L.C., Brown, D., Clouter, A., Donaldson, K., 2004. Increased inflammation and altered macrophage chemotactic responses caused by two ultrafine particle types. Occup. Environ. Med. 61 (5), 442–447.
Richards, J.M., Semple, S.I., Macgillivray, T.J., Gray, C., Langrish, J.P., Williams, M., et al., 2011. Abdominal aortic aneurysm growth predicted by uptake of ultrasmall superparamagnetic particles of iron oxide: a pilot study. Circ. Cardiovasc. Imaging 4 (3), 274–281.
Rosner, M.H., Auerbach, M., 2011. Ferumoxytol for the treatment of iron deficiency. Expert Rev. Hematol. 4 (4), 399–406.
Ruehm, S.G., Goyen, M., Barkhausen, J., Kroger, K., Bosk, S., Ladd, M.E., et al., 2001. Rapid magnetic resonance angiography for detection of atherosclerosis 82. Lancet 357 (9262), 1086–1091.
Schladt, T.D., Schneider, K., Schild, H., Tremel, W., 2011. Synthesis and bio-functionalization of magnetic nanoparticles for medical diagnosis and treatment. Dalton Trans. 40 (24), 6315–6343.
Schmitz, S.A., Taupitz, M., Wagner, S., Wolf, K.J., Beyersdorff, D., Hamm, B., 2001. Magnetic resonance imaging of atherosclerotic plaques using superparamagnetic iron oxide particles. J. Magn. Reson. Imaging 14 (4), 355–361.
Shaw, S., Murthy, P.V., 2010. Magnetic targeting in the impermeable microvessel with two-phase fluid model—non-Newtonian characteristics of blood. Microvasc. Res. 80 (2), 209–220.
Shaw, C.A., Robertson, S., Miller, M.R., Duffin, R., Tabor, C.M., Donaldson, K., et al., 2011. Diesel exhaust particulate-exposed macrophages cause marked endothelial cell activation. Am. J. Respir. Cell Mol. Biol. 44 (6), 840–851.
Sigovan, M., Boussel, L., Sulaiman, A., Sappey-Marinier, D., Alsaid, H., sbleds-Mansard, C., et al., 2009. Rapid-clearance iron nanoparticles for inflammation imaging of atherosclerotic plaque: initial experience in animal model. Radiology 252 (2), 401–409.
Slevin, M., Wang, Q., Font, M.A., Luque, A., Juan-Babot, O., Gaffney, J., et al., 2008. Atherothrombosis and plaque heterology: different location or a unique disease? Pathobiology 75 (4), 209–225.
Song, S., Qin, Y., He, Y., Huang, Q., Fan, C., Chen, H.Y., 2010. Functional nanoprobes for ultrasensitive detection of biomolecules. Chem. Soc. Rev. 39 (11), 4234–4243.
Sosnovik, D.E., Nahrendorf, M., Weissleder, R., 2008. Magnetic nanoparticles for MR imaging: agents, techniques and cardiovascular applications. Basic Res. Cardiol. 103 (2), 122–130.
Stampfl, A., Maier, M., Radykewicz, R., Reitmeir, P., Gottlicher, M., Niessner, R., 2011. Langendorff heart: a model system to study cardiovascular effects of engineered nanoparticles. ACS Nano. 5 (7), 5345–5353.
Stark, D.D., Moseley, M.E., Bacon, B.R., Moss, A.A., Goldberg, H.I., Bass, N.M., et al., 1985. Magnetic resonance imaging and spectroscopy of hepatic iron overload. Radiology 154 (1), 137–142.
Sun, J., Wang, S., Zhao, D., Hun, F.H., Weng, L., Liu, H., 2011. Cytotoxicity, permeability, and inflammation of metal oxide nanoparticles in human cardiac microvascular endothelial cells: cytotoxicity, permeability, and inflammation of metal oxide nanoparticles. Cell Biol. Toxicol. 27 (5), 333–342.
Tang, T.Y., Howarth, S.P., Miller, S.R., Graves, M.J., King-Im, J.M., Li, Z.Y., et al., 2008. Correlation of carotid atheromatous plaque inflammation using USPIO-enhanced MR imaging with degree of luminal stenosis. Stroke 39 (7), 2144–2147.
Thu, M.S., Najbauer, J., Kendall, S.E., Harutyunyan, I., Sangalang, N., Gutova, M., et al., 2009. Iron labeling and preclinical MRI visualization of therapeutic human neural stem cells in a murine glioma model. PLoS One 4 (9), e7218.
Tornqvist, H., Mills, N.L., Gonzalez, M., Miller, M.R., Robinson, S.D., Megson, I.L., et al., 2007. Persistent endothelial dysfunction in humans after diesel exhaust inhalation. Am. J. Respir. Crit. Care Med. 176 (4), 395–400.
Trivedi, R., King-Im, J., Gillard, J., 2003. Accumulation of ultrasmall superparamagnetic particles of iron oxide in human atherosclerotic plaque. Circulation 108 (19), e140.

Trivedi, R.A., King-Im, J.M., Graves, M.J., Cross, J.J., Horsley, J., Goddard, M.J., et al., 2004. In vivo detection of macrophages in human carotid atheroma: temporal dependence of ultrasmall superparamagnetic particles of iron oxide-enhanced MRI. Stroke 35 (7), 1631–1635.

Uppal, R., Caravan, P., 2010. Targeted probes for cardiovascular MRI. Future Med. Chem. 2 (3), 451–470.

Uppal, R., Catana, C., Ay, I., Benner, T., Sorensen, A.G., Caravan, P., 2011. Bimodal thrombus imaging: simultaneous PET/MR imaging with a fibrin-targeted dual PET/MR probe--feasibility study in rat model. Radiology 258 (3), 812–820.

van der Valk, F.M., van Wijk, D.F., Lobatto, M.E., Verberne, H.J., Storm, G., Willems, M.C., et al., 2015. Prednisolone-containing liposomes accumulate in human atherosclerotic macrophages upon intravenous administration. Nanomedicine 11 (5), 1039–1046.

van Gils, J.M., Zwaginga, J.J., Hordijk, P.L., 2009. Molecular and functional interactions among monocytes, platelets, and endothelial cells and their relevance for cardiovascular diseases. J. Leukoc. Biol. 85 (2), 195–204.

van Schooneveld, M.M., Cormode, D.P., Koole, R., van Wijngaarden, J.T., Calcagno, C., Skajaa, T., et al., 2010. A fluorescent, paramagnetic and PEGylated gold/silica nanoparticle for MRI, CT and fluorescence imaging. Contrast Media Mol Imaging 5 (4), 231–236.

van Tilborg, G.A., Strijkers, G.J., Pouget, E.M., Reutelingsperger, C.P., Sommerdijk, N.A., Nicolay, K., et al., 2008. Kinetics of avidin-induced clearance of biotinylated bimodal liposomes for improved MR molecular imaging. Magn. Reson. Med. 60 (6), 1444–1456.

Viera, A.J., Sheridan, S.L., 2010. Global risk of coronary heart disease: assessment and application. Am. Fam. Physician 82 (3), 265–274.

Voura, E.B., Jaiswal, J.K., Mattoussi, H., Simon, S.M., 2004. Tracking metastatic tumor cell extravasation with quantum dot nanocrystals and fluorescence emission-scanning microscopy. Nat. Med. 10 (9), 993–998.

Wang, T.J., 2011. Assessing the role of circulating, genetic, and imaging biomarkers in cardiovascular risk prediction. Circulation 123 (5), 551–565.

Weidner, A.M., van Lin, E.N., Dinter, D.J., Rozema, T., Schoenberg, S.O., Wenz, F., et al., 2011. Ferumoxtran-10 MR lymphography for target definition and follow-up in a patient undergoing image-guided, dose-escalated radiotherapy of lymph nodes upon PSA relapse. Strahlenther Onkol. 187 (3), 206–212.

Yang, F., Li, L., Li, Y., Chen, Z., Wu, J., Gu, N., 2008. Superparamagnetic nanoparticle-inclusion microbubbles for ultrasound contrast agents. Phys. Med. Biol. 53 (21), 6129–6141.

Yilmaz, A., Rosch, S., Klingel, K., Kandolf, R., Helluy, X., Hiller, K.H., et al., 2011. Magnetic resonance imaging (MRI) of inflamed myocardium using iron oxide nanoparticles in patients with acute myocardial infarction—preliminary results. Int. J. Cardiol. 163 (2), 175–182.

Zhao, Y., Zhao, L., Zhou, L., Zhi, Y., Xu, J., Wei, Z., et al., 2010. Quantum dot conjugates for targeted silencing of bcr/abl gene by RNA interference in human myelogenous leukemia K562 cells. J. Nanosci. Nanotechnol. 10 (8), 5137–5143.

Zhu, M.T., Wang, Y., Feng, W.Y., Wang, B., Wang, M., Ouyang, H., et al., 2010. Oxidative stress and apoptosis induced by iron oxide nanoparticles in cultured human umbilical endothelial cells. J. Nanosci. Nanotechnol. 10 (12), 8584–8590.

Zhu, M.T., Wang, B., Wang, Y., Yuan, L., Wang, H.J., Wang, M., et al., 2011. Endothelial dysfunction and inflammation induced by iron oxide nanoparticle exposure: risk factors for early atherosclerosis. Toxicol. Lett. 203 (2), 162–171.

CHAPTER

12

Neurological System

*Myrtill Simkó**, *Mats-Olof Mattsson**, *Robert A. Yokel*****

***AIT Austrian Institute of Technology GmbH, Tulln, Austria;**

****College of Pharmacy, University of Kentucky, Lexington, KY, United States**

OUTLINE

Introduction 275

ENM Translocation to and Within the Nervous System 277

ENM Effects on Blood–Brain Barrier Permeability and Function 278

Transport Across Cell Membranes and Intercellular Transport of ENMs 279

ENM Interactions With Biological Structures Including the Nervous System 281

Biotransformation and Persistence of ENMs in the Nervous System 284

Adverse Effects of ENMs Related to Nervous System Functions and Disease 285

Nervous System Effects of In Vivo ENM Exposure 286

In Vitro Studies of ENM Effects 290

In Silico Studies of ENM Effects 297

Human Studies of ENM Effects 299

Risk Assessment of Nanomaterials 301

Take-Home Messages 302

References 302

INTRODUCTION

The number of people affected by neurodegenerative diseases is rapidly growing as the worldwide population's age is increasing. Thus, there is an emergent need to develop new methodologies to target and deliver drugs into the central nervous system (CNS) for treating pathological conditions. To circumvent or cross the blood–brain barrier (BBB) and transport the medication directly to the desired tissue, various strategies have been developed, such as chemical modifications to disrupt the BBB by changing membrane permeability, the

Adverse Effects of Engineered Nanomaterials. http://dx.doi.org/10.1016/B978-0-12-809199-9.00012-4

Copyright © 2017 Elsevier Inc. All rights reserved.

use of receptor-mediated drug delivery, but also intraventricular, intracerebral, and intraspinal applications of drugs (for a review see Tosi et al., 2014). However, all these applications are of limited effectiveness. Therefore, medical and pharmacological research focuses on the application of engineered nano(sized)-materials (ENM), for the targeted delivery of drugs. Such drug delivery systems (or nanocarriers) should and may overcome physiological barriers, such as the BBB, and to be useful for targeted delivery. They may minimize induced side effects by the spatial and temporal distribution of pharmaceutical drugs in the brain. Nanocarrier systems coated with drugs are already in clinical use (see review by Simkó and Mattsson, 2014). Unfortunately, side effects associated with their use are generally not taken into consideration. In fact, the knowledge about potential toxicity of ENMs is still far from comprehensive.

The BBB is formed by brain capillary endothelial cells linked together by tight junctions. Together with adjacent processes from astrocytes, pericytes, and a basement membrane, it restricts the transfer of most substances from the bloodstream into the brain. Therefore, substances may gain access to the CNS, for example, by lipid-mediated free diffusion or potentially by receptor-mediated endocytosis or by transcytosis. Since in the BBB tight junctions have a gap of only 4–6 nm, nanoparticles pass through the endothelial cell membrane rather than via interendothelial junctions. It has been shown that nanoparticles from the blood circulation may influence endothelial cell membrane integrity and/or disrupt the BBB, and may induce certain mechanisms of intracellular transport (vesicular transport) to gain access into the CNS. Nanocarriers require surface or other forms of functional modifications for receptor-mediated transport through the brain capillary endothelium to deliver drugs to the CNS. Therefore, surface modifications of nanoparticles are presently intensely studied for nanomedical applications like diagnosis and therapy aiming to influence the target-oriented pharmacokinetic behavior of nanocarriers. Different approaches to obtain suitable modifications are discussed and under development (Calvo et al., 2001; Kreuter, 2004; Kreuter et al., 2002; Olivier, 2005; Tiwari and Amiji, 2006; Zensi et al., 2009; Zheng et al., 2005).

There has been much research reported on methods to enhance brain delivery, but very much less on the adverse effects. ENMs can induce significant unintended (toxic) effects due to their unique physicochemical properties (for review see De Jong and Borm, 2008). Besides the chemical properties, this can be due to their electric, optical, and magnetic properties that are related to physical dimensions, but also the surface of the material can be involved in catalytic and oxidative reactions which can induce cytotoxicity. This toxicity can be greater than that of a similar bulk material because the surface area-to-volume ratio for nanomaterials is much greater.

How can ENMs reach the brain? Beside the intended medical application of ENMs (e.g., systemic application by injection) there are also other routes by which ENM can enter the human body: (1) the main route is via inhalation, followed by (2) dermal exposure. The third route which has not been very well understood so far is the gastrointestinal pathway. The uptake of ENMs goes via intra- and intercellular transport and the blood circulation to other organs. In certain cases, ENMs can reach secondary organs, such as the brain via, for example, axonal transport along the olfactory and trigeminal nerves.

In the present chapter, we give an overview of how ENMs can translocate from the point of entry of the body, especially the respiratory tract, to the circulation and possibly pass the BBB with subsequent effects on the brain. We differentiate between intended and unintended

exposure to ENMs. We discuss several different classes of ENMs, but do not consider soluble nanomaterials, such as liposomes or micelles. Furthermore, our focus is on presenting illustrative data rather than the accumulation of all available studies.

ENM TRANSLOCATION TO AND WITHIN THE NERVOUS SYSTEM

The phenomenon of ENM transport from point of entry to other parts of the body, secondary organs, has been extensively discussed in many previous reviews (Simkó and Mattsson, 2010, 2014). It is generally considered that the principal entry pathways include inhalation, ingestion, dermal uptake, and systemic administration. To reach the nervous system, the taken up ENM needs to be transported via the circulation to the target organ, and there be able to penetrate the local barrier systems that protect the nervous system from various damaging agents. These barriers include the blood–brain (discussed in more detail in subsequent sections), the blood–cerebrospinal fluid, and the blood–nerve barriers.

A further barrier that has been recently studied is the blood–retina barrier. Thus, Henrich-Noack et al. (2012) showed that fluorescently labeled polybutyl cyanoacrylate particles (mean diameter 69 nm) can translocate to the retina after intraperitoneal injection to male Lister hooded rats. The fate of the material was followed in vivo by means of a confocal neuroimaging approach developed by the authors. The fluorescent particles were visible within seconds, and reached the retina within minutes, where they ended up in cellular structures. The animals were investigated up to several days after ENM administration, and the injected particles were still present in the retina 9 days after administration.

Besides the major entry points mentioned previously, a further uptake route has been investigated in several animal studies. This route relies on retrograde axonal transport from primarily the olfactory epithelium in nasal structures via the olfactory tract to the olfactory bulb in the brain. Possibly, further translocation from the olfactory bulb to other regions of the brain can take place. This transport mechanism was discussed in some detail in previous overviews (Simkó and Mattsson, 2010, 2014), but there are some recent findings that merit mentioning in the present context.

Hopkins et al. (2014) recently showed that quantum dots (QDs; CdSE/ZnS nanocrystals; 15–20 nm diameter) can translocate from the olfactory tract to the brain after inhalation. The study describes how the QDs were visualized in the olfactory bulb of adult C57BL/6 mice 3 h after a 1-h nasal inhalation exposure. The presence of the nanoparticles was seen by means of fluorescence and transmission electron microscopy. Furthermore, the uptake of the nanoparticles was accompanied by microglial activation as seen by microscopy and immunostaining.

Garcia et al. (2015) performed a study with the aim to estimate the dose of inhaled nanoparticles that deposit in the human olfactory epithelium during nasal breathing at rest. This deposited material would then be available for further translocation by means of axonal transport. This is a modeling study based on computational fluid dynamics and simulated in nasal anatomies of two healthy adults. The authors also used previously published modeling data of the olfactory dose of inhaled nanoparticles for 18-week-old male F344 rats. The depositions of particles ranging from 1 to 100 nm was studied and the results showed that the deposition in the human nose is substantially higher for the smallest particles (about 65% of the 1-nm particles) than for the larger ones (e.g., 1% for 13 nm, 0.3% for 100 nm) at a resting

breathing rate (15 L/min). Of the deposited material, only a fraction was found in the olfactory epithelium of the nasal cavity (c. 1% for 1–2 nm particles, 0.01% for 100-nm particles). When comparing the data with those from rats, the authors stated that rats have higher olfactory deposition. However, due to higher respiratory rates in humans, the olfactory dose per surface area unit is actually higher in humans for the smallest particles. For larger sizes (i.e., >7 nm), the doses in the two species are comparable.

ENM Effects on Blood–Brain Barrier Permeability and Function

To assess the effect of metal ENMs on BBB permeability and function, primary cultured rat brain microvessel endothelial cells (rBMECs) were exposed to ~28-, 48-, or 102-nm silver; 40- or 60-nm copper; or 2.9-, 5.3-, 9-, 13-, 26-, or 59-nm gold (Trickler et al., 2014). Silver accumulation in rBMECs; increased permeability through an rBMEC monolayer; and release of PGE_2, TNF-α, & IL-1β from rBMECs were inversely related to ENM size. The larger copper ENMs produced greater permeability than the silver ENMs, at 50% of the silver ENM concentration, and increased TNF-α & IL-1β, but not PGE_2, release, which was not size dependent. Gold accumulation in rBMECs also decreased as size increased. The 3-nm gold was more toxic than larger gold sizes. The 3- and 5-nm gold had less effect on permeability and PGE_2, TNF-α, and IL-1β release than silver ENMs (Trickler et al., 2014). Given that ENM uptake and effects inversely related to size, these studies indicate that copper and silver were more toxic than gold. Similar studies with these ENMs and porcine brain microvessel endothelial cells (pBMECs) showed that copper ENMs increased permeability more than silver, which was more effective than the gold ENMs. Silver increased PGE_2 & TNF-α release (inversely related to size) more than Cu (which was size independent); they similarly increased IL-1β release; whereas gold ENMs did not (Trickler et al., 2014). A series of studies compared the effects of 20–30, 50–60, and 130–150 nm aluminum, copper, and silver given by the intravenous, intraperitoneal, intracarotid, and intracerebrovascular routes to mice and rats. The extent of BBB opening inversely related to ENM size, was more pronounced with silver than copper than aluminum ENMs, was greater in 9–10 and 30–35 week old than 18–20 week old rats, and greater in the presence of heat-induced stress, sleep disruption, and a model of diabetes (Sharma et al., 2013, 2015). Daily intraperitoneal injections of a 50–60 nm silica for a week increased the BBB permeability produced by hypertension and partial restraint stress in rats (Sharma et al., 2013). Studies with human BMECs, mouse BMECs, a triple coculture, and rats showed decreased tight junction proteins after exposure to 8–12 nm aluminum oxide, 25-nm titania, 7-nm silver, and 40-nm gold (Brun et al., 2012; Chen et al., 2008; Li et al., 2015; Xu et al., 2015a), which would contribute to increased BBB permeability. Activation of the kallikrein–kinin contact system that is involved in vascular permeability by a negatively charged silver nanoparticles caused bradykinin release, decrease of an adherens junction protein, and elevated retinal vascular permeability (Long et al., 2015). ENM-induced release of bradykinin might mediate BBB opening, but no reports were found showing ENM-induced release of bradykinin. Intravenous injection of reduced graphene oxide produced a transient opening of the rat BBB accompanied by transient reduction of proteins that maintain BBB integrity (Mendonça et al., 2015). One hundred forty nanometer uncoated and polysorbate 80-coated poly(*n*-butylcyanoacrylate) (PBCA) ENMs concentration-dependently, up to 1000 μg/mL, increased metabolic activity of a porcine BBB model after 1-h incubation. Over time this

response reversed, to near total suppression, and increased lactic dehydrogenase release, at 24 h. One to 25 ENM concentration-dependently decreased resistance across the model, to a total loss with the highest concentration. The authors compare these tested concentrations to ~490-μg/mL PBCA in rat blood after an intravenous injection that increased brain drug levels (Kolter et al., 2015).

Transport Across Cell Membranes and Intercellular Transport of ENMs

The BBB is probably the best protective network of different cells and vessels in the body. However, once ENMs have entered this barrier they will be taken up by cells actively or passively. Subsequently they can translocate further to cells of the brain parenchyma and there trigger various effects.

There are various pathways by which nanoparticles can be taken up by cells. The ENM size can determine the pathway of cell entry (Jana, 2011; Rejman et al., 2004). EMNs are not restricted to one but can activate multiple pathways to enter the cell. These uptake routes include phagocytosis (mainly used by professional phagocytic cells, such as monocytes, neutrophils, or macrophages) (Sahay et al., 2010); clathrin-dependent endocytosis which is reported as the main pathway for ENM uptake and has been documented in, for example, muscle cells, adipocytes, and endothelial cells (Zhang et al., 2013); clathrin- and caveolin-independent endocytosis [less frequently employed by ENM (Zhang et al., 2013], but plays a role in signal transduction and transcytosis); and macropinocytosis which is considered as a nonspecific entry-point detected in various cell types (Sahay et al., 2010). Iversen et al. (2011) summarized that since the size of caveolae are around 50–80 nm, the uptake and accumulation of larger ENMs is very unlikely. Mao et al. (2013) demonstrated that clathrin-mediated endocytosis is "limited" by around 120 nm, caveolin-mediated by c. 60 nm, and clathrin/caveolin independent endocytosis by around 90 nm, whereas phagocytic uptake can process objects in the micrometer range. Similar findings have been shown by Jana (2011) as well. Shang et al. (2014) summarized the influence of size-dependent ENM uptake: "(i) There is an optimal size for efficient endocytosis of ENMs independent of the particle composition. (ii) This critical size can vary with cell type and surface properties of the ENM. (iii) Small ENMs have a higher probability to be internalized by passive uptake than large ones. (iv) Under otherwise identical conditions, small ENMs are more likely to cause toxic cellular responses…" The ENM size–related influence on cells is usually related to the primary size of the particle which depends on the ENM synthesis method (Kato et al., 2012). Which pathway or entry-point is activated by the ENM uptake is furthermore influenced by the particle's agglomeration, aggregation, and/or stability. Inside the cell, particles can be active in different ways: easily soluble particles can release ions mediating toxicity, while particles with a more persistent surface oxide or poor solubility can be stable for a longer time, accumulating and exerting toxicity through reactions on the particle surface. Particles often end up in lysosomes with a lower pH (around pH 4.5–5) that can enhance the rate of dissolution of some particles. In a recent publication Guarnieri et al. (2014) demonstrated that the cytotoxicity of ENMs is strictly reliant on the pathway of cellular internalization. The authors showed that gold, silver, and iron oxide ENMs are nontoxic if they were forced through the cell membrane (bypassing any form of active mechanism, such as endocytosis). The EMNs exhibited rather free random Brownian motion within the cytosol and did not accumulate in lysosomes. These

findings suggest that intracellular accumulation of metallic nanoparticles into endolysosomal compartments is the leading cause of nanotoxicity, due to continuous nanoparticle degradation and in situ release of metal ions.

Limbach et al. (2007) reported that an increased level of intracellular reactive oxygen species (ROS) was induced from cobalt oxide nanoparticles compared to cobalt salt ($CoCl_2$). The authors showed that a so-called "Trojan horse" type uptake of cobalt oxide nanoparticles leads to a higher release of cobalt ions from inside the cell and suggested the possibility of different pathways passing through the cell membrane.

Studies have shown that autophagy is the degradation of mitochondria and other intracellular structures within lysosomes (Kobzik, 1995). A portion of cytoplasmic constituents, such as long-lived proteins and damaged organelles, are enclosed in double-membrane vesicles named autophagosomes, which subsequently fuse with lysosomes to form autolysosomes. This process leads to the degradation of the engulfed contents by lysosomal enzymes. Lin et al. (2014) showed that a silver ENM induced autophagy in cancer cells by activating the PtdIns3K signaling pathway. This was characterized by an enhanced autophagosome formation, normal cargo degradation, and no disruption of lysosomal function. Yu et al. (2014) demonstrated that nanoscale SiO_2 (silica)-induced autophagy and autophagic cell death is triggered by ROS generation in HepG2 cells. Exposure to nanoalumina, but not to nanocarbon, induced a dose-dependent mitochondrial potential collapse, increased autophagy of brain endothelial cells, and decreased expression of tight junction proteins, occludin and claudin-5 as shown by (Chen et al., 2013).

The electrical surface charge (zeta potential) plays an important role in activating certain receptors, especially in the uptake of titania-, iron oxide- and quartz-containing nanoparticles (Kobzik, 1995). Uncharged particles, such as carbon-containing nanoparticles or diesel emission particles activate the same receptors as bacteria, viruses, or fungi (Inoue et al., 2006). Particles that are enclosed in phagosomes fuse with the lysosomes, and release enzymes and free radicals to digest the invading pathogen (e.g., bacterium) (Park, 2003). Depending on the activated receptor, the corresponding cell signaling cascades are triggered, for example, to activate the immune system (refer to Chapter 13 for a detailed discussion). If particles cannot be digested, they can remain within cells for up to 700 days (Oberdorster et al., 2005) and thus cause cell damage. This can lead to cell death, which means the particles are retained in the respective organ (Brown et al., 2004). The cycle begins again: the particles are ingested which trigger the continuous formation of free radicals leading to oxidative stress. This, in turn, can lead to chronic reactions which have often been associated with various diseases, such as cancer, neurodegenerative and cardiovascular diseases.

Another relevant parameter is the shape of the ENM which might play an important role in cellular uptake and toxicity, as demonstrated by Gratton et al. (2008). The authors detected differences in the internalization of cubic and cylindrical ENMs with various aspect ratios. Furthermore, the authors described that rod-like particles were favored by the investigated cells. Oh and Park (2014) concluded that macrophages are more efficient in rod-shaped ENM uptake, while cancer and lung epithelial cells internalize spherical ENM more efficiently. The fibrous forms of nanotubes can lead to frustrated phagocytosis and increased toxicity if they are enclosed for a longer time by the cell (Murphy et al., 2012) (and see Chapter 9).

The highly sensitive nanotubular structures that mediate membrane continuity between mammalian cells are not yet well recognized in the nanotoxicological area. The so-called

tunneling nanotubes (TNTs) that were discovered in 2004 (Rustom et al., 2004) are F-actin-based membrane tubes that form between cells in culture and in tissues leading to the generation of complex cellular networks. They have been shown to facilitate the intercellular transfer of organelles as well as, on a limited scale, of membrane components and cytoplasmic molecules (Wang and Gerdes, 2015). It has been proposed that TNTs represent a novel and general biological principle of cell-to-cell communication and it becomes increasingly apparent that they fulfill important functions in the physiological processes of multicellular organisms (Gerdes et al., 2007). Schoelermann et al. (2015) showed that nanotitania was transferred between cells in direct contact with TNTs, along with endosomes and lysosomes. The authors detected that the small GTPase Arf6 facilitates the intercellular transfer of smaller ENMs and agglomerates and the nanoparticles modified the cellular ability to communicate with surrounding cells. Spherical, anatase nano-TiO_2 (5 and 40 nm) did not affect the cellular viability. A recent study by Tosi et al. (2014) investigated the mechanisms of cell-to-cell transport of glycopeptide (g7-ENMs) modified poly-lactide-*co*-glycolide (PLGA) ENMs, known to cross the BBB after in vivo administration in rodents. The authors showed intra- and intercellular transport of the ENMs within vesicles after vesicular internalization and the cell-to-cell transport by TNTs in cell lines and most interestingly in glial and neuronal cells in vitro and in vivo. The intra- and intercellular transport of novel ENMs represents a novel strategy for CNS drug delivery.

ENM INTERACTIONS WITH BIOLOGICAL STRUCTURES INCLUDING THE NERVOUS SYSTEM

The impact of the physicochemical nature of ENMs, including the biological corona, on their biological activity related to the nervous system has been reviewed (Albanese et al., 2012; Duan and Li, 2013; Fadeel et al., 2013; Jo et al., 2015). An important distinction is between the properties of the synthetic (pristine) identity (the ENM as made) and its bioidentity (biological identity, altered from the synthetic identity by protein coating, age, and other factors that affect surface properties). The relevance is that ENM-biological interaction is greatly affected by the surface physicochemical nature of the ENM, for example, what the cell sees (Walczyk et al., 2010). ENMs become protein coated (opsonized) in contact with blood, making them more recognizable by macrophages for their clearance from blood. One might assume this would decrease the ability of ENMs to enter the brain. Much work has focused on using specific blood proteins to target the brain for ENM delivery, and additional proteins that enhance ENM entry into brain or between endothelial cells have been identified (Kim et al., 2015; Mahmoudi et al., 2015), but the ability of blood proteins, and specific proteins, to protect the brain has not been assessed. Many ENM physicochemical properties might impact on its biological interaction, including chemical composition, size, shape, surface charge, and surface coating. A complication to determining whether an ENM property influences biological interaction is the ability to vary that one property, but not others (Fadeel et al., 2013).

ENM interaction with the nervous system can be described as pharmacokinetic [biological effects on the ENM, such as absorption, distribution (translocation) and persistence, metabolism (biotransformation), and elimination] or pharmacodynamic (ENM effects on the biological substrate). It would be expected that ENM chemical composition would affect its

biological interaction. This was supported by greater primary rat and porcine brain microvascular endothelial cell (BMEC) response to copper and silver than gold, adverse response inversely related to ENM size, and greater adverse effects in the rat and mouse produced by copper and silver than aluminum ENMs, as discussed previously. Similarly, 20-nm silver was also more toxic to rat primary neurons and a mixed culture of neurons, astrocytes, and some oligodendrocytes than 20-nm gold (Haase et al., 2012). Another study found 20-nm titania produced greater cytotoxicity to rat primary microglia than a similarly sized silica, or a 45-nm Fe_3O_4 (magnetite) (Xue et al., 2012). Many other studies reported metal/metal oxide ENM chemical composition effects their biological interaction, whereas studies with organic composition ENMs were not found that address this.

Many studies have shown that ENM size inversely relates to nervous system distribution and effects. Studies on endosomal localization and cytotoxicity of 25–75 nm silica with C17.2 neural progenitor cells (Soenen et al., 2013), permeation of 30- and 100-nm silica across human BMECs (Hanada et al., 2014), and cytotoxicity of 20- and 40-nm peptide-coated silver to rat primary neurons (Haase et al., 2012) illustrate this. An exception was better uptake of 40-nm than 20- or 100-nm carboxylated polystyrene by 1321N1 tumor cells, suggesting tumor cells might respond differently than healthy cells (Varela et al., 2012). Similarly, many in vivo studies have shown an inverse relationship between ENM size and brain association. Examples include studies with 1.4–200 nm monosulfonated triphenylphosphine gold and 25–500 nm polystyrene in rat brain (Hirn et al., 2011; Kulkarni and Feng, 2013). The transport rate of 40–200 nm carboxylated and PEG-coated polystyrene through brain slices was also inversely size related (Nance et al., 2014).

No studies rigorously comparing ENMs of different shapes, while holding other variables constant, were found; therefore, no conclusions can be drawn regarding the influence of ENM shape on interactions with the nervous system.

Attributing an ENM effect due to its surface charge versus coating is difficult due to the difficulty of isolating individual ENM physicochemical characteristics (Fadeel et al., 2013). Much work has focused on developing surface coatings to enhance ENM brain uptake. Given the negative charge of the endothelial cells comprising the BBB that are the first line of the brain's defense against substance entry (Johansson, 2001), it would be expected to repel ENMs with a negative surface potential. Opsonization by proteins with a net-negative surface charge, such as albumin (~ -24 mV) should provide similar protection. This has not been demonstrated. Studies showed greater permeation of neutral than cationic particles, which was greater than anionic-coated 60-nm maltodextrin (polysaccharide) through a bovine BMEC/rat astrocyte coculture (Fenart et al., 1999). Permeability through a primary rat BMEC/pericyte coculture was greater for 50-nm QDs with a surface charge of -7.3 mV than one with a surface charge of -32 mV (Hanada et al., 2014). Addition of C6- and di-C6-quaternary ammonium increased gold-induced stem cell cytotoxicity, suggesting positive zeta potential and/or lipophilicity increased cytotoxicity (Manshian et al., 2014). The cationic agent CTAB (trimethylammonium bromide) was more cytotoxic than anionic PEG coating (Hutter et al., 2010). When introduced into dissected human or rat cortex less anionic polystyrene ENMs penetrated further than more anionic ones (Nance et al., 2012, 2014). These studies show reduced BBB and brain permeation as ENM surface charge becomes more negative. It appears that BBB and brain permeation and BBB toxicity can be reduced by avoiding ENMs with a positive surface charge. Results of several in vivo studies showed greater toxicity associated with more cationic

surface charge. For example, intracerebroventricular injection of micelles (32 mV) produced toxicity whereas anionic (−7 mV) micelles did not (Knudsen et al., 2014).

Many studies have shown increased uptake by cells, penetration through the BBB, and brain effects with selected surface coatings. These findings are primarily the result of investigations aimed at enhancing brain delivery. To reduce unintended brain entry and effects these surface coatings should be avoided. Agents that increased cell uptake, BBB permeation, brain uptake, or tumor cell toxicity include glucose compared to glutathione (Gromnicova et al., 2013), polysorbate 80 (Reimold et al., 2008), cationic albumin (Lu et al., 2007), apolipoprotein A1 (Zensi et al., 2010), apolipoproteins B and E (Kreuter et al., 2002), transferrin (Cui et al., 2013; Yan et al., 2013), lactoferrin (Qiao et al., 2012), and insulin (Shilo et al., 2014). More relevant to adverse effects of ENMs on the brain, reduction of single-walled carbon nanotube (SWCNT) toxicity to PC12 cells (derived from rat pheochromocytoma, phenotypically resembling dopaminergic neurons) was achieved by PEG coating, which creates a "stealth" effect making the ENMs less recognizable by macrophages (Johansson, 2001) and to multiwalled carbon nanotube (MWCNT)-induced toxicity to dorsal root ganglion (DRG) cells by carboxylate coating (Lai et al., 2013), consistent with the previously cited studies showing greater toxicity of more cationic ENMs.

The present knowledge regarding different dose concepts relevant for nanomaterials is very limited, with possible exception of data from a few in vitro studies. Relevant data, such as dose relation studies especially for neurological in vivo situations is scanty. Even in in vitro studies dose estimation is not trivial because ENM diffuse, settle, and agglomerate in cell culture media depending on different factors like media density and viscosity, particle size, shape, charge, and density. Teeguarden and coworkers (Hinderliter et al., 2010; Teeguarden et al., 2006) developed a particokinetic model to estimate cellular dose in vitro considering different factors like the dynamic precipitation rate in cell culture media which depends on particle size and follows more Brownian motion than gravitation. In a recent critical overview it has been suggested that the adoption of already developed dosimetric methodologies will facilitate the determination of effective dose delivered to cells in vitro, thereby improving the accuracy of the data (Cohen et al., 2015).

Dose–response relationship studies need to deal with the physicocemical properties and not only with the mass of the ENM. There are mathematical tools available, such as QSAR models, with the potential to predict potential ENM toxicity (Benfenati et al., 2013; Winkler et al., 2013) (and see Chapter 4). Other approaches aim to apply new testing by the use of the so-called "grouping of substances" (or category) approach even though it is not clear which properties of the ENM and which biological endpoint(s) are relevant for computational approaches (Arts et al., 2014) (and see Chapter 8 for a discussion on grouping of ENM in the context of risk assessment). Simkó et al. (2014) presented a dose model which takes the total deposited ENM surface area with the potential to induce biological effects, as the deposited dose, into account. This can be weighted by factors quantifying the effects of several other physicochemical properties of the ENM, leading to the equivalent dose and considering the biokinetics of the ENM. The retained particle surface area of the ENM is favored as a dose metric or paradigm by others as well. Maynard and Kuempel (2005) concluded that especially for poorly soluble, low toxicity particles, regardless of the particle physicochemical compositions, the surface area dose–response relationship appears to be comparable.

BIOTRANSFORMATION AND PERSISTENCE OF ENMs IN THE NERVOUS SYSTEM

The biopersistence of ENMs can be viewed as those that readily degrade, such as polymers versus those that are quite insoluble in biological fluids, such as carbon-based and some of the metal and metal oxide ENMs. Polymer-based ENMs are being extensively studied as diagnostic, theranostic, and therapeutic delivery systems, often releasing their payload as they dissolve in vivo. The concern for adverse effects related to biopersistent ENMs focuses on potential untoward outcomes from their prolonged residence.

A challenge determining the biopersistence of carbon-based ENMs is their quantification, given carbon's ubiquitous presence. Approaches to this challenge have included quantification of cobalt as a component of carbon nanotubes (CNTs), Raman spectroscopy, and the ENM's electron diffraction pattern. Inhalation of MWCNTs resulted in estimated half-lives in the lung that increased with exposure increases from ~50 to 375 days, the latter perhaps indicating overload of the lung's ability to clear the ENM (Oyabu et al., 2011; Pauluhn, 2010). These inhalation studies and a few studies where SWCNTs were given intravenously or subcutaneously either did not see the ENM in the brain or did not look at the brain. Seven days after injection of PEG-functionalized SWCNTs into rat hippocampus some of the ENM was detected by Raman spectroscopy, providing the best available information about carbon-based ENM brain persistence (Dal et al., 2015). Subcutaneously implanted 18 × 825 nm tangled, oxidized MWCNTs persisted for 2 years, undergoing some biodegradation in macrophage lysosomes (Sato et al., 2013). When administered into mouse brain motor cortex, 20–30 × 500–2000 nm amino-functionalized MWCNTs were internalized into vesicular structures, presumably phagolysosomal vesicles, and within 2 days began to unravel from their concentric conformation and become decomposed (Nunes et al., 2012). Recent studies have show peroxidase-mediated biodegradation of oxidized CNTs (Andón et al., 2013).

Much research has been conducted with iron oxide–containing ENMs targeting the brain. In the only study found that informs about the biopersistence of iron in the brain from ENMs, no decrease of ^{59}Fe was seen in rat brain for 50 days after intratracheal administration of 22-nm $^{59}Fe_2O_3$. It is not clear if the ^{59}Fe was from nanoiron or solubilized iron (Zhu et al., 2009). Nanotitania retention half-lives of ~30–400 days were reported in the lung after inhalation. The longer half-lives were attributed to particle overload (Bermudez et al., 2004). No information of brain nanotitania retention was found. Brain aluminum did not decrease over 113 days after inhalation of ~10- and 40-nm primary particle–sized commercial calcined γ-AlO(OH) boehmite (Pauluhn, 2009). Differences of Cd and Te determined by inductively coupled plasma mass spectrometry and chemical vapor generation following intravenous administration CdTe core and ZnS shell and CdSeTe core ZnS shell QDs to mice and rats revealed ENM dissolution up to 16 weeks after administration, including release of Te and Cd in the brain, although much lower concentrations than in peripheral organs (Liu et al., 2013a; Su et al., 2015). QD release of cadmium, a carcinogen that has possible adverse effects on the nervous system, and tellurium, that has demonstrated adverse nervous system effects, is of concern. However, several studies did not find cadmium or QDs in the brain after QD administration.

After a single 85-mg/kg intravenous injection of 30-nm ceria, brain and cerebrospinal fluid cerium concentration increased 90 days later compared to earlier times, perhaps the result of partial dissolution of the nanoceria in organs containing most of this ENM (liver and spleen) (Graham et al., 2014; Yokel et al., 2012). Similarly, an increase of brain cerium was seen 4 and 5 months after a single 20-mg/kg intravenous injection of 2.9-nm ceria (Heckman et al., 2013). Some decrease is brain cerium concentration was seen from 30 to 90 days after a 6-mg intravenous dose of 30-nm ceria (Yokel et al., 2014), suggesting higher doses may have overwhelmed the rat's ability to eliminate cerium. Injection of nanoceria into each eye of rats resulted in estimated half-lives of 525 days in the eye and 414 days in the retina (Wong et al., 2013). The cerium ion is retained for a long time in mammals, shown by a half-life of ~285 days in the liver and skeleton after a single inhalation of $^{144}CeCl_3$ (Hahn et al., 1997).

No information was found on the persistence of nanocopper, which is soluble in artificial lysosomal fluid. Following a subcutaneous injection of 50–100 nm silver to rats, brain silver concentration increased at 12 weeks and remained fairly constant for another 12 weeks (Tang et al., 2008c). Four weeks after a 12-week course of intranasal polyvinylpyrrolidone (PVP)-coated nanosilver administration brain silver had not decreased compared to the end of the exposure (Wen et al., 2016). Brain silver was higher after an intravenous injection of 20- than 200-nm silver, and increased over 28 days as liver silver decreased (Dziendzikowska et al., 2012). After 28 days of oral intake of 100 or 500 mg/kg/day of 10- or 25-nm silver, brain silver concentration slightly increased in the first and second month, and then decreased after 4 months. The half-lives were 60–260 days, shorter for the larger ENM and dose, but generally longer than liver, spleen, and kidneys (Lee et al., 2013). Brain silver did not appreciably decrease over 8 weeks after 28 days oral gavage of a polymer-coated 18-nm silver ENM (van der Zande et al., 2012). With the exception of visualization of particles believed to be silver in the brain (Dziendzikowska et al., 2012), the previous studies did not show if the silver in the brain was nanoscale or ionic. Given the solubility of nanosilver it was probably ionic, consistent with the longer half-life of silver in the CNS than other organs (Rungby and Danscher, 1983). The contribution of metal ion from a metal or metal oxide ENM may account for much of the previously reported metals in the brain.

The general lack of much information on ENM biopersistence in the nervous system is probably a product of the lack of much distribution of ENMs into the nervous system. Even when increased brain ENM concentrations are claimed, they are usually not supported by evidence of increased ENM in brain parenchyma. Most of the ENM may reside in the vascular system or associated with the BBB.

ADVERSE EFFECTS OF ENMs RELATED TO NERVOUS SYSTEM FUNCTIONS AND DISEASE

This section highlights some of the more robust and credible nervous system adverse effects following in vivo ENM administration, followed by a discussion on in vitro studies and in silico studies and, finally, human studies. We will discuss each class of ENM separately.

Nervous System Effects of In Vivo ENM Exposure

Nanotitania

Nanoscale titanium dioxide (nanotitania) is the most studied ENM for its adverse effects on the nervous system. Oral administration of 5–50 mg/kg, compared to the typical human intake of 0.1 mg/kg, altered brain electrolytes; increased acetylcholine, glutamate, and nitric oxide but decreased norepinephrine, dopamine, and serotonin and their metabolites; increased oxidative stress and reduced antioxidant defense mechanisms; increased apoptosis; and impaired Y-maze spatial recognition memory (Hu et al., 2010). Nanotitania has many commercial applications that might produce human nasal passage exposure, providing a rationale to study its uptake and effects from that route. Intranasal application of 500 μg of 80- and 155-nm titania in the mouse (~20 mg/kg) led to increased titanium, pathological changes, oxidative stress, and immune response in the hippocampus (Wang et al., 2008). The hippocampus seems to be a target for nanotitania not only after intranasal administration, as it is intimately connected to the olfactory tract originating in the nasal cavity, but following intraperitoneal administration as well (Ma et al., 2010). Studies with 5–6 nm titania introduced into the nasal cavity as 2.5–10 mg/kg daily for 90 days produced dose-dependent oxidative stress, pyramidal cell edema, hemorrhage, glial cell proliferation, and alteration of genes regulating cellular processes, signal transduction, protein metabolism, brain development, oxidative stress, and other neurological functions (Ze et al., 2014 and prior reports from this group). A dose of 2.5 mg/kg is 35 times a 24-h exposure at the U.S. NIOSH recommended airborne exposure limit for nanoscale titania of 0.3 mg/m^3, based on inhalation of 10 L/min (~15 m^3/day) and 100% deposition of aerosolized nanotitania in the nasal passages. Increased brain titanium was only seen after dermal application of 21-nm Degussa P25, but not 10-nm anatase or 25- or 60-nm rutile titania, consistent with most studies that did not find increased brain titanium. No histopathology was seen in the brain (Wu et al., 2009). Intraperitoneal injection of 5–150 mg/kg 5-nm titania produced elevated brain titanium (in the absence of reports of nanotitania in the brain) and oxidative stress, reduced antioxidant defense, microglial activation, neurotransmitter disruption (Ma et al., 2010). When 0.1 mg was given subcutaneously 4 times to mice (total ~4 mg/kg) during gestation, nanotitania was seen in the olfactory bulb and cerebral cortex of offspring at 6 weeks of age that had more olfactory bulb caspase-3 positive cells than controls (Takeda et al., 2009). Gestational exposure also resulted in alteration of genes related to cell death, apoptosis, oxidative stress, inflammation, and neurotransmitters (Shimizu et al., 2009). Synaptic plasticity and long-term potentiation changes that were more pronounced after maternal lactation than gestation exposure (Gao et al., 2011) and reduced cell proliferation and learning and memory disruption (Mohammadipour et al., 2014) were also reported, but these followed daily oral nanotitania doses of 100 mg/kg, 1000-fold greater than the typical human intake of titanium.

Nanosilver

Interpretation of adverse effects of nanosilver is confounded by the role of released silver ions due to its solubility. Intragastric nanosilver to rats daily for 14 days produced damage to synaptic components and decreased pre- and postsynaptic proteins at a dose (0.2 mg/kg) in the range of the typical daily intake of silver by humans (0.1 μg/kg). The effects were more pronounced in the hippocampus than cortex and seen with silver ions (Skalska et al., 2015).

Higher exposure produced neuron and astrocyte damage attributed to inflammation (Xu et al., 2015b). Intranasal nanosilver (0.1–1 mg/kg daily for 14 weeks) increased glial fibrillary acidic protein and caspase-3, indicating reactive gliosis to neurotoxicity and apoptosis as well as cerebellar degeneration and motor performance deficits (Yin et al., 2015a,b). Higher exposure (50 mg/kg for 7 days) increased the oxidative stress-responsive gene Hmox1 in the hippocampus but not cortex, impairment in Morris water maze performance, and nanosilver sequestration in the choroid plexus, illustrating this mechanism to protect the brain from bloodborne metals (Davenport et al., 2015). Nanosilver injections during gestation resulted in impaired performance by 35- to 49-day-old offspring in the Morris water maze and a decrease in GAP-43 (a neuron-specific phosphoprotein that plays a major role in neural development and remodeling) (Ghaderi et al., 2015; Wu et al., 2015). Given the biopersistence of silver (see preceding section) these findings are of some concern, but they have been obtained with exposures several orders of magnitude greater than typical human silver intake.

Nanoalumina

Nanoalumina creates a potential health concern because excessive aluminum exposure in renal-impaired humans has been shown to produce a fatal encephalopathy (dialysis encephalopathy), a fracturing osteodystrophy, and anemia, and adverse effects on mental development and the skeletal system in neonates receiving aluminum-contaminated parenteral nutrition solutions. It has been suggested to contribute to Alzheimer's disease (AD) (Willhite et al., 2014). Aluminum welding releases 10–75 nm fume particles that can result in subtle neurocognitive deficits in chronically exposed welders (Willhite et al., 2014). Characterization of adverse effects of nanoscale alumina has been investigated in mice and rats via nose-only inhalation, which did not reveal any distribution into or effects on the nervous system (Pauluhn, 2009), and oral, intraperitoneal, and intranasal exposure. Oral administration of 50–2000 mg/kg, compared to typical oral aluminum intake by humans of 0.1 mg/kg, showed increased ROS and disruption of antioxidant defenses, effects not seen with bulk alumina (Prabhakar et al., 2012; Shrivastava et al., 2014). Behavioral deficits in the Morris water maze, increased apoptosis, disruption of synaptic proteins, and changes in processing of amyloid precursor protein to amyloid β peptides, as seen in AD, were demonstrated after intraperitoneal exposure to 300 mg/kg over 3 weeks (Shah et al., 2015). Microglial activation was seen in the olfactory bulb after intranasal administration of 1 mg/kg 40-nm aluminum oxide every other day for 60 days (Li et al., 2013). If these effects in animals would be seen with human-relevant exposures has not been reported.

Nanocadmium and Quantum Dots

Most of the studies of nanoscale cadmium have been conducted with cadmium-containing QDs. Injection of maltodextrin-coated CdS QDs into the air sac of chicken eggs resulted in anomalies, when the 1 mL dose was 9.84 nM or greater, that included the CNS and neural tube, suggesting embryotoxic potential (Rodriguez-Fragoso et al., 2012). Intracortical and intrahippocampal injection of PEG CdTe QD 705 or Cd–Se QDs produced an increase of glial fibrillary acidic protein, indicating neurotoxicity (Maysinger et al., 2007), and decreased field excitatory postsynaptic potential slopes, population spike amplitude, and long-term potentiation indicating impaired synaptic transmission, plasticity, and learning and memory; and increased hippocampal SOD activity, glutathione, and malondialdehyde, indicating greater

oxidative stress (Tang et al., 2009). The section on biopersistence in this chapter cites some studies suggesting in vivo QD dissolution that result in small concentrations of free cadmium and tellurium in the brain, although other studies did not find either intact QDs or their dissolution products. In one of the few studies that related experimental ENM exposure in animals to human exposure, intratracheal instillation of cadmium oxide 5 days weekly for 3 or 6 weeks shifted the spectrum of spontaneous cortical electrical activity to higher frequencies and increased the latency of somatosensory-, visual-, and auditory-evoked potentials (also seen with nanolead and nanomanganese) (Papp et al., 2012). Oxidant and proinflammatory responses were seen after intravenous administration of CdSeS QDs (reduction of MnSOD and increased malondialdehyde) (Yang et al., 2013) and intranasal inhalation of PEG-phosphatidyl ethanolamine–coated CdSe/ZnS QDs (Hopkins et al., 2014).

Nanocarbon

Intravenous injection of 3.125, 6.25, or 12.5 mg/kg SWCNTs daily for 9 days to mice resulted in pyramidal cell damage, evidenced by Nissl staining, increased brain caspase-3 activity and oxidative stress, and impaired performance in the Morris water maze and open field tests after the highest two doses (Liu et al., 2014). MWCNT-induced impaired performance was associated with reduced expression of a glutaminergic (NMDA) receptor subunit and the synaptic protein synaptophysin, reduced field excitatory postsynaptic potential slope, consistent with impaired cognition, and increased autophagy (Gao et al., 2015). Increased oxidative stress was seen in mouse brain after motor cortex injection of MWCNTs that was more pronounced with shorter, 200–300 nm (oxidized), than 500–1000 nm ENM (Bardi et al., 2013). When MWCNTs were intravenously injected into mice the day of mating and 3 days later, increased anxiety was demonstrated in offspring in the elevated plus maze on postnatal day 22 and despair in the forced swimming test on postnatal day 23, which was not dose (1 or 10 mg/kg) related. Nonsignificant effects were seen in several other neurobehavioral tests, which were also generally not dose related (Ivani et al., 2012). Intravenous injection of SWCNTs and three diameters of MWCNTs (<8-, 20–30, and ~50-nm, all 500–2000 nm long) to mice on gestation days 10.5, 12.5, and 15.5 resulted in brain tissue malformations in 15% of fetuses on day 17.5 in those exposed to the largest MWCNT, but not in any of the smaller ENMs (Huang et al., 2014). The effect was seen in $p53^{-/-}$, but not $p53^{+/+}$ or $p53^{+/-}$ fetuses, suggesting a protective role for p53. MWCNT inhalation, equivalent to 108 work day exposure to the NIOSH recommended exposure level, increased heart rate variability during exposure, which did not persist 1 and 7 days later, indicating a transient adverse effect on the autonomic NS (Zheng et al., 2016). Intranasal administration of G4 PAMAM-NH_2 dendrimer altered mRNA gene expression in three brain regions; most consistently affecting pluripotent network and TGF-beta receptor signaling (Win-Shwe et al., 2014). A review of dendrimers and dendriplexes concluded that they are nontoxic in vivo (Shcharbin et al., 2014). Oral administration of 60 mg/kg/day for 5 days of chemically reduced graphene oxide to mice produced no significant effects on Morris water maze, open field or rotorod behavior, other than initial reduced rotorod performance (neuromuscular coordination) and locomotor activity in the open field. No significant oxidative stress or change in acetylcholine synthesis or catabolism enzymes was seen (Zhang et al., 2015). In contrast, intraperitoneal injection of 4 mg/kg of SWCNT produced a greater reduction of glutathione and increased ROS than graphene oxide or graphene (Shang et al., 2015), consistent with very low oral absorption of ENMs.

Nanocopper

Repeated intraperitoneal injection of 0.5 mg/kg of 10–70 nm copper oxide for 14 days to rats produced increased oxidative stress, apoptosis, and cytotoxicity in the hippocampus accompanied by impairment in Morris water maze performance (An et al., 2012). Studies with repeated, higher doses of intranasally administered 23.5-nm copper resulted in some changes in neurotransmitters and their metabolites, particularly in the olfactory bulb, that were not dose related (Zhang et al., 2012). Intranasally administered 23.5-nm copper and intraperitonealy injected 20-nm copper produced neuronal damage (Bai et al., 2014; Privalova et al., 2014). In one of the few reports comparing pristine to aged ENMs, sulfidation of 4-nm spherical copper resulted in various shaped ENMs that produced less decrease of brain acetylcholinesterase activity in zebrafish exposed to 0.01 ppm for 48 h (Dharsana et al., 2015).

Nanogold

Intraperitoneal injection of 0.5–14.6 mg/kg 17- or 37-nm gold to mice produced an increase of brain dopamine, but only the smaller ENM reduced serotonin, and after 3.7 mg/kg only the smaller ENM impaired learning in a passive avoidance test (Chen et al., 2010). This is consistent with the trend of greater effect on the nervous system of smaller ENMs, discussed in section "ENM Interactions With Biological Structures Including the Nervous System," of this chapter. In contrast, a single intraperitoneal injection of a 30-nm gold ENM produced more DNA strand breaks than a 10-nm one. After daily injection for 28 days there was no significant difference in the increase produced by the two sizes (Cardoso et al., 2014).

Nanoiron

Although there has been much research conducted to assess the application of iron oxide containing ENMs as brain imaging agents, little has been reported on their adverse effects potential in the nervous system. Bilateral intranasal instillation of 130 μg of α- and γ-Fe_2O_3 every other day for 40 days decreased neuron number in the hippocampus, apoptosis in the striatum and cerebral cortex, and microglial activation in these brain regions and the olfactory bulb (Wang et al., 2011b). Oral doses up to 1000 mg/kg daily for 28 days decreased brain acetylcholinesterase, total ATPase, Na-K-ATPase, Mg-ATPase, and Ca-ATPase. Increased lipid peroxidation, catalase activity, glutathione reductase, and glutathione S transferase; and decreased glutathione were seen only after the higher doses (Kumari et al., 2012; Reddy et al., 2015). After sciatic nerve injection of 150 nmoles of 3–15 nm DMSA-Fe_2O_3, DMSA-Fe_3O_4, PEG-Fe_3O_4, or PEG-Au-Fe_3O_4 in the rat subperineurial edema and cell accumulation suggestive of disrupted blood–nerve barrier, macrophage/monocyte and lymphocyte infiltration, neural inflammation/stress, and apoptosis were seen (Kim et al., 2013).

Nanolead

Repeated intratracheal nanoscale lead introduction produced effects in the rat characteristic of lead neurotoxicity. Ambulation and local activity increased and rearing decreased in the open field; somatosensory evoked potential latency increased; and tail nerve conduction velocity decreased, latency increased, and amplitude decreased (Oszlanczi et al., 2011). Lead sulfide increased intracellular free calcium and Ca-ATPase and L-type calcium channel expression, that presumably allowed calcium influx; hippocampal cell structural damage (Cao et al., 2013).

Nanomanganese

Manganese oxide ENM administration by the intratracheal, intranasal, and oral routes increased brain manganese, but none of the studies determined the presence of the ENM in brain parenchyma. Given the known dissolution of manganese oxide ENM, particularly at pH 4.5, the results may be due to the manganese ion. In some studies the manganese oxide ENM produced a decrease in immobility and an increase in horizontal activity in the open field, and an increase in fast bands and decrease in slow bands of the cortical electrical activity, effects that were also seen with the manganese ion and nanolead (Mate et al., 2015; Oszlanczi et al., 2010a,b). The intratracheal doses given daily for 6 weeks in one study would correspond to approximately 100 and 200-fold the US EPA reference concentration for 8-h daily chronic manganese inhalation exposure (Mate et al., 2015). Decreased brain acetylcholinesterase, total ATPase, Na-K-ATPase, Mg-ATPase, and Ca-ATPase were also seen, as was seen with nanoiron (Singh et al., 2013).

Nanosilica

Intranasal administration of 150 μg daily of 10- and 80-nm silica for 15, 30, or 90 days produced changes in three brain regions characteristic of oxidative stress, and after 90 days apoptosis and deficits in rotarod and forelimb grip strength performance and reduced locomotor activity (Parveen et al., 2014, 2015). The intraperitoneal injection of 50 mg/kg nanosilica daily for a week exacerbated rotarod and inclined plane performance deficits and neuronal damage produced by induced hypertension, partial immobilization stress, and elevated temperature, and produced brain edema in the hypertensive, stressed rats in a 33°C environment (Sharma et al., 2013).

Nanoceria

A single intravenous nanoceria administration initially increased hippocampal oxidative stress in rats, temporally followed by an inflammatory response and autophagy, and 90 days later, a restoration of redox balance (Hardas et al., 2014). These changes were seen in the absence of appreciable amounts of nanoceria in the brain, but associated with nanoceria biotransformation in the liver that might have released cerium ion or circulating factors that influenced the brain response (Graham et al., 2014). Brain glutathione was decreased after repeated oral nanoceria (Kumari et al., 2014), consistent with an increase in the hippocampal oxidized/reduced glutathione ratio (Hardas et al., 2014).

Nanoscale Zinc Oxide

Animal studies with nanoscale zinc oxide have not shown profound adverse effects. Although some decrement in Morris water maze acquisition and reacquisition behavior occurred in rats injected with nanoscale zinc oxide from 4 to 12 weeks of age, long-term potentiation was enhanced (Han et al., 2011). When compared to nanoalumina and nanotitania, nanoscale zinc oxide had the greatest effect on superoxide dismutase antioxidation and neurotransmission (increased norepinephrine and dopamine), but the least effect on the glutathione antioxidant defense system and catalase (Shrivastava et al., 2014).

In Vitro Studies of ENM Effects

In vitro models of the BBB are useful for mechanistic studies and can also to some extent complement and replace animal and human studies (see Lippmann et al., 2011, 2012, 2013;

Naik and Cucullo, 2012 for recent reviews). Cells can be isolated from whole brain tissue or from biopsied tissue samples. From these sources primary cultures of different cell types (endothelial cells, astrocytes, pericytes, and neurons) can be attained. Cells can be cultivated in monocultures or in coculture(s) in different combinations of glia cells, astrocytes, pericytes, and neurons. Newer studies show that even stem cells, such as neural progenitor cells, the in vitro precursors to neurons, astrocytes, and oligodendrocytes, can be used to study BBB induction and thus toxic effects (Lippmann et al., 2013). Other developments include the creation of dynamic 3D-models of the BBB in cell culture.

Nanotitania

Titania is one of the best investigated EMNs in vitro and in vivo for nervous system adverse effects. It has been proposed that once an ENM enters the cell it can cause different toxic effects. In a recent study it has been shown that nanotitania (anatase isoform), induced toxicity on human glial (D384) and neuronal (SH-SY5Y) cell lines, by altering mitochondrial function, membrane integrity, cell morphology and proliferation (Coccini et al., 2015). Other studies demonstrated that nanotitania was toxic to rat neuroglia cells, inhibiting the cell survival rate concentration dependently (Liu et al., 2013b), inducing oxidative stress and mitochondrial damage in human and rat glial cells (Huerta-García et al., 2014). The group detected that the uptake process was accompanied by the formation of pseudopodia and intracellular vesicles, indicating that this process was mediated by endocytosis, without any specific localization of nanotitania in organelles but into large vesicles in the cytoplasm. By using specific inhibitor the authors detected that macropinocytosis is the main process of internalization in a human glioblastoma cell line (U373 cells) leading to nanotitania accumulation and thus toxic effects (Huerta-Garcia et al., 2015). Erriquez et al. (2015) used DRG sensory neurons and satellite glial cells and detected the internalization of TiO_2ENMs in DRG cells accompanied by the induction of apoptosis in a dose-dependent manner, ROS production, and changes in expression of the proinflammatory cytokine IL-1β in both cell types. Axonal retrograde transport was altered in the neurons.

An interesting study showed that the process of uptake of TiO_2ENM involved both endocytosis and exocytosis in neural stem cells (NSC). This process was observed irrespective of the type and dose of TiO_2ENMs. In the first 24 h after removing TiO_2ENMs from the culture medium, about 35–40%, of the ENMs were released (exocytosed) (Wang et al., 2013).

Wu et al. (2010) investigated the effects of titania macro and nanoparticles at various doses on several endpoints in differentiated PC12 cells that were exposed for 24 h. For all investigated endpoints (cell survival and death, cell cycle regulation, and oxidative stress), the anatase form of the particles exerted much stronger toxicological effects on the cells than rutile particles and the nanosized particles were much more potent than the microsized particles of the same substance, and the anatase particles caused significant toxic responses in PC12 cells in a concentration-dependent manner. The authors suggested that exposure caused increased oxidative stress that in turn triggered induction of apoptotic pathways via the mitochondria, also arresting the cells in the G2/M phase of the cell cycle.

A different approach investigated electrophysiological processes on primary cultures from the frontal cortex of embryonic mice. Cocultures containing glia cells and neurons growing on multielectrode arrays (MEA) were treated with nanotitania (91 nm), carbon black, and hematite (50 nm, Fe_2O_3) ENMs showing that all three substances affected the spike and

burst rate, burst duration, number of spikes within a burst, etc. in a dose-dependent manner (Gramowski et al., 2010). The strongest effects were exerted by nanotitania and ROS production was upregulated (titania at 5 and 10 μg/cm^2, 24-h exposure). A more recent study employing MEAs was published by Strickland et al. (2016). Cortical neuronal cell cultures were derived from newborn Long–Evans rat pups and treated (1, 24, 48 h) with nanotitania or nanoceria of different sizes and at different concentrations. The major endpoints measured were total number of spikes per well and number of active electrodes per well. Overall, there were several exposure conditions that affected the physiological activities, although in all cases effects were subtle and significantly lower than responses to chemicals with known action mechanisms and effects. The effects were concentration dependent where present, but not consistently increasing or decreasing the recorded activities. No specific cytotoxicity was recorded from any of the ENMs irrespective of size or concentrations. Several in vitro studies showed that the morphological (mitochondria, lysosome, and cytoskeleton) and/or functional impairment of neuronal or glial cells after exposure to nanotitania leading to apoptosis induction, ROS production, and the activation of proinflammatory mediators occur in a dose-dependent manner (Sheng et al., 2015). There is also increasing evidence that small ENMs (~5 nm) are able to cross BBB, regardless of the exposure route or administration pathway (Catalan-Figueroa et al., 2016). It has also been demonstrated that small ENMs can persist in brain tissue, even after 4 months of oral exposure (Lee et al., 2013) and activate microglia cells which is related to neurodegenerative processes (Block et al., 2007).

Nanosilver

There is a discussion whether the toxicity of nanosilver is induced by the intracellular, relatively high ion concentration after internalization and the subsequent ion release from the ENMs or if the ENM itself is toxic (i.e., a particle effect). In primary cerebellar granule cells of rats, after nanosilver incubation excitotoxicity was detected which is a specific neuronal death process. This was accompanied by calcium imbalance, mitochondrial dysfunction and ROS production (Zieminska et al., 2014). Yin et al. (2013) showed decreased viability of rat cerebellum granule cells after nanosilver exposure caused by apoptosis that was coupled to oxidative stress. Nanosilver and silver ions induced neurotoxicities in rat cerebral astrocytes through distinct pathways by causing apoptosis via intracellular ROS generation coupled with JNK phosphorylation, whereas silver ions predominantly led to necrosis via compromising the cell membrane integrity and binding with cellular thiol groups directly (Sun et al., 2016). The authors investigated neuroinflammation as well and found effects at the noncytotoxic level of nanosilver via the regulation of cytokine secretion in astrocytes. This effect was not detected from silver ion release, suggesting particle-specific effects in nanosilver-mediated neurotoxicitiy. A recent study by Xu et al. (2015a) showed on a triple coculture BBB model of rat brain (microvascular endothelial cells, pericytes, and astrocytes) that after nanosilver the BBB permeability was significantly increased, also observed between microvascular endothelial cells, and the expression of the tight junction protein ZO-1 was decreased. TEM investigation showed severe mitochondrial shrinkage, vacuolations, and endoplasmic reticulum expansion in the astrocytes. Nanosilver induced also inflammation and apoptosis through the modulation of the MAPK pathway, and according to the author's interpretation, this could also be induced by B-cell lymphoma-2 expression or mTOR activity in astrocytes. Huang et al. (2015) detected the induction of proinflammatory cytokine secretion,

oxidative stress, and Aβ degradation in mouse neural cells suggesting a nanosilver-induced neuroinflammatory response.

Mixed cortical primary cultures from rats were used for the studies on nanosilver by Haase et al. (2012) and Xu et al. (2013) on effects of silver ENM on primarily neurons and astrocytes. Nanosilver uptake was documented only in astrocytes, not in neurons. Both cell types exhibited increased rates of cell death (LDH-leakage) when exposed to the ENMs, more so for astrocytes than for neurons. In addition to the cytotoxicity, acute Ca^{2+}-response (within minutes) that was seen in both cell types was detected followed by the induction of oxidative stress. The response was stronger to 20- than 40-nm particles, and even stronger than the response to Ag^{+} ions ($AgNO_3$). Thus, it was demonstrated that astrocytes are more vulnerable to nanosilver than neurons.

A review from Hohnholt et al. (2013) interprets the results more cautiously. The authors point out that cultured mammalian astrocytes are not acutely damaged by the ENMs, even at high concentrations and long exposure times. Astrocytes can accumulate significant amounts of ENMs, which are stored preferentially in lysosomes in an acidic environment causing the release of ions to the cytosol. This leads to the synthesis of metallothioneins in astrocytes, posing the potential protective effects in the brain, by taking up and safely storing ENMs that can release ions, and thus be toxic. Hadrup et al. (2012) investigated whether there was a difference in cell death mechanisms following exposure to 14-nm PVP-coated nanosilver, ionic silver (Ag–Ac) or a sub-nanofiltered fraction of the nanosilver preparation in neuronal-like PC12 cells. Nanosilver did not induce necrosis, but, apoptosis was increased in cells exposed to both, nanosilver and the subfiltered fraction, with Ag–Ac showing a greater potency. These findings suggest that ionic silver and a 14-nm nanosilver preparation have similar neurotoxic effects; the authors suggest that this could be the release and action of ionic silver from the surface of nanosilver.

Nanoiron

The use of magnetic nanoparticles or iron oxide ENMs is an area of increasing interest in both research and medicine. Since iron oxide ENMs have unique physical properties regarding their reaction to a magnetic force, they are seen as potential candidates for diagnostic and therapy and especially the so-called theranostics which includes both. Thus, they are either already in use as contrast agents for magnetic resonance imaging (MRI) and/or being investigated for cancer therapy in combination with hyperthermia. However, their potential neurotoxicity has been poorly investigated.

Astrocytes play an important role in the CNS because they regulate the metal homeostasis in the brain (Hohnholt et al., 2013). These authors have overviewed the literature concerning astrocytes and uptake and effects of magnetic iron oxide ENMs and concluded that astrocytes accumulate significant amounts primarily by endocytosis, and increasing the intracellular metal concentration by a factor of 100–1000. The main storage of the ingested ENMs occurs in the lysosomes, where the acid conditions may cause release of Fe^{2+}/Fe^{3+} ions, which in turn when reaching the cytosol can be sequestered by the protein ferritin. In this way, the authors argue that at least short-term detrimental effects of ENM uptake can be avoided.

Using an in vitro model of the BBB consisting of murine brain endothelial cells, Hoff et al. (2013) investigated the permeability of 11 different iron oxide ENMs, both as ferrofluid formulations and as powders. The authors concluded that the ferrofluid samples were more

prone to pass the BBB than the powders, and that the particle size showed the highest correlation to permeability, with smaller particles more permeable than large ones.

Pinkernelle et al. (2012) found significant FeO uptake by glial populations, most notably microglia. The cells were primary cultures from neonatal rats and contained cells from the cerebellum and also organotypic spinal cord cultures together with peripheral nerve grafts incubated with magnetic iron oxide ENMs (24 h) with a shell consisting of a green fluorescent dye and a polysaccharide mixture of glucuronic acid. Although all cell types internalized the particles, the microglia cells were by far the most active in that respect. In addition, incubation with the ENMs also stimulated microglia to proliferate, which suggests that the ENMs activated the cells. When comparing the uptake in primary cells with the PC12 cell line, it was shown that PC12 cells exhibited a much stronger uptake. This cell line was also the tool for a study by Kim et al. (2011) who employed 11-nm Fe_3O_4ENMs with a PEG-phospholipid shell. After 24-h incubation with various concentrations of ENMs, substantial uptake was observed into vesicular structures, presumably lysosomes. Interestingly, the presence of ENMs potentiated the effect of nerve growth factor (NGF) on differentiation of the PC12 cells. Furthermore, the cell adhesion molecule integrin β1 and phosphorylation of the signal transduction enzyme ERK1/2 were increased in ENM-exposed cells. No cytotoxic effects were seen at any concentration after 1 day of exposure, whereas a modest cytotoxic response was noted at the highest (40 μg/mL) concentration at days 3 and 5.

Nanocarbon

The use of CNTs in engineering and medicine raises great hopes for innovative applications and, consequently, considerable economic potential. It must be underlined that CNTs exist in great diversity, and their biological impact depends on their shape. Long (20 μm) fibers like CNT behave like asbestos fibers and cause chronic inflammation by frustrated phagocytosis. However, there is little or no such effect caused by short needle-shaped CNT (Donaldson et al., 2013; Poland et al., 2008). Some of the functionalized forms of CNT are water dispersible (Ntim et al., 2012) thus there is a growing interest in possible biomedical application/use. It is well established that nanotubes improve electrical connectivity between neurons owing to their electrical conductivity (Keefer et al., 2008). According to Nunes et al. (2012) the presence of metals, jointly with their aggregation, has been identified as responsible for the CNT toxicity. Purification may reduce their cytotoxicity. At the same time it has been reported that neuronal cells are not affected by carboxyl group-functionalized CNTs (Ni et al., 2005).

Phillips et al. (2013) investigated the effect of oxidized MWCNTs (ranging from 50 to 200 μg/mL) in PC12 neuronal cells in vitro and detected a slight cytotoxicity after a short time period while after longer time, no significant toxicity was observed. Xu et al. reported that carboxyl-terminated MWCNTs act as antagonists of three types of potassium channels in PC12 cells. The authors showed that carboxyl-terminated MWCNTs suppress the current densities of I(to), I(K), and I(K1) in a time-dependent and irreversible manner but did not induce any ROS or intracellular free calcium and also did not alter the mitochondrial membrane potential (Xu et al., 2009).

Another aspect of CNT was investigated by Wu et al. (2012), a study where crush lesions were induced in DRG sensory neuron axons of 8-week-old CD-1 mice. Subsequently, the DRG neurons were cultured in vitro and there subjected to treatment with MWCNTs (25-nm diameter; 10–20 μm length). Axonal regeneration (length of axonal elongation after 24 in vitro

treatments with 0.1–10 μg/mL MWCNT) was evaluated. It was reported that the DRG neurons exhibited stunted neurite outgrowth (69 vs. 330 μm in the controls), and fewer processes and branches than the vehicle-treated control cell cultures. No effects on apoptosis were seen. Lower concentrations caused inhibition of axonal regeneration, albeit at lower levels. No effects were seen only in cultures treated with the lowest concentration.

Nanoceria

Naked nanoceria (CeO_2) can act as an antioxidant to protect cells against oxidative damage. However, the genome-wide transcription effects of nanoceria and associated biological processes remain elusive. Thus studies on naked CeO ENM were performed by Lee et al. (2012) who compared the effects of 6-nm CeO particles with 100-nm ceria particles and Al_2O_3 particles (300 nm) on murine HT22 hippocampal cells in culture (8, 16, or 24 h) for gene expression analysis. Each treatment gave rise to a distinct expression pattern, where the 6-nm ceria particles displayed the highest number of unique gene responses (230 genes that were not affected in any of the other conditions). The genes belonged to a wide range of disease groups including "neurological disease" (13 genes). Remarkably, these genes included Htt (huntingtin) that was strongly downregulated in the array analysis, also confirmed by separate studies on the RNA (quantitative RT-PCR) and protein (Western blot) levels. Ciofani et al. (2013) exposed NGF-treated PC12 cells for 24 or 72 h to ceria (5–80 nm) at concentrations up to 100 μg/mL, and no effect on cell viability was seen. However, beneficial effects on cell differentiation and dopamine production were detected, which was suggested to be due to the decreased ROS levels. Kumari et al. (2014) investigated cytotoxic, genotoxic, and oxidative stress responses after exposure to different doses of nanoceria and its microsized ceria for 24 h in a human neuroblastoma cell line (IMR32). The authors used a huge battery of tests and summarized that nanoceria was more toxic than the microsized (MP) particles. However, it has to be pointed out that the significant differences between the nano- and microsized ceria were seen above 50- or 100-μg/mL CeO_2 (MTT, LDH, micronucleus, and comet assays).

Nanosilica

Nanosilica (SiO_2) ENMs are considered as good candidates for neuromedical applications. However, their neurotoxic behavior is not very well studied. Gilardino et al. (2015) studied calcium signaling in two neuronal cell lines (GT1-7 and GN11 cells) at the nontoxic level of 20 μg/mL, 50-nm silica. The authors detected the induction of long-lasting but reversible calcium signals that in most cases show a complex oscillatory behavior and suggested that this effect is not depending on ENMs internalization. Using fluorescent ENMs, they found a connection to calcium influx and a dependency on ENM size and surface charge. Furthermore, the involvement of voltage-dependent and transient receptor potential-vanilloid 4 (TRPV4) channels was suggested for this effect. Xue et al. (2012) performed a comparative study where primary cultures of microglia cells from postnatal Sprague-Dawley rats were exposed to several ENMs: silica (20 nm), hydroxyapatite (HAP; 60 and 12 nm respectively for the long and short diameter) and Fe_3O_4 (45 nm) at different concentrations. The study also included a positive control, the bacterial lipopolysaccharide LPS (1 μg/mL; 24 h). Treatment of the microglia with nanotitania and HAP caused NO production via iNOS activation and also chemokine (MCP-1, MIP-1) production. All four investigated ENMs caused cytokine (TNF-α, IL-1β, IL-6) production (at a level lower than LPS treatment). For all investigated endpoints,

the nanotitania treatment gave the strongest responses in the microglia cultures. The authors furthermore took cell-free culture supernatant from cells treated with the ENMs and exposed PC12 cells to these supernatants. The supernatants from nanotitania and HAP-treated cells caused decreased levels of tyrosine hydroxylase and also modest induction of cell death in the PC12 cultures.

To study the cytotoxicity of nanosilica, cell survival and other endpoints were investigated in two human cell lines with origin in the nervous system (Lai et al., 2010; Wang et al., 2011a). NGF-treated PC12 cells by Wang et al. were subjected to 20- and 50-nm silica for 12–48 h causing a concentration and time-dependent decrease in cell survival mediated by increased oxidative stress. In addition, the treatment had negative effects on NGF-induced differentiation where number of neurites/cell, neurite length, and intercellular contacts/cell all decreased as a function of dose. Similar findings were observed by Lai et al. (2010). The authors used U87 astrocytoma cells and exposed them for 48 h to 12-nm silica. A dose-dependent decrease in cell survival was seen at ≥25 μg/mL, accompanied by an increased activity of two mitochondrial enzymes and decreased levels of certain mitochondrial proteins and decreased ERK signaling.

Nanomanganese

Metal ENMs seem to be particularly neurotoxic. This was seen after occupational exposure of nonparticulate manganese (Mn), aluminum, and iron (Cannon and Greenamyre, 2011). Manganism is a condition that develops after chronic exposure to excessive manganese levels that can lead to a variety of psychiatric and motor disturbances. Gendelman et al. detected internalized ENMs in N27 dopaminergic neuronal cells that induced a time-dependent upregulation of the transporter protein transferrin. The exposure to nanomanganese (25–400 μg/mL) induced apoptosis in a time- and dose-dependent manner, a significantly increased ROS, a caspase-mediated proteolytic cleavage of proapoptotic protein kinase Cδ (PKCδ), the activation of phosphorylation, and the induction of autophagy. Thus nanomanganese activates mitochondrial-dependent apoptotic signaling and autophagy in dopaminergic neuronal cells. The cytotoxic effects of nanosized Mn were shown by Afeseh Ngwa et al. (2011) in a study where they employed rat mesencephalic dopaminergic cells (N27 cell line). According to the authors, the ENMs were either single ENMs (20 nm) or agglomerates (100–900 nm) during the experimental conditions. The particles were shown to be cytotoxic in a dose- and time-dependent manner and induced oxidative stress, transferrin upregulation, autophagy, and apoptosis whereby cell death induction was independent of oxidative stress. Also in murine primary mesencephalic nerve cell cultures, nanomanganese induced cell death and decreased neurite length. The study shows that the detrimental effects of Mn on dopaminergic neurons, seen in chemical toxicology studies, are also caused by Mn in the form of ENM.

Quantum Dots

QDs are already widely applied for diagnostics and cellular imaging. Chen et al. (2013) investigated the role of autophagy in the synaptic plasticity caused by CdSe/ZnS QDs, and demonstrated that QDs elicit autophagy in HeLa cells and also in cultured hippocampal neurons. The authors used autophagy inhibitors (wortmannin, 3-MA, and chloroquine) that suppressed QD-induced autophagic flux, partly blocked LTP (long-term potentiation)

impairment, and downregulated synapsin-I. Tang et al. (2008a) used unmodified CdSe QDs to investigate the cytoplasmic calcium levels ($[Ca^{2+}]_i$) in primary cultures of hippocampal neurons, in which both extracellular Ca^{2+} influx and internal Ca^{2+} release was involved. The authors showed, by using different specific inhibitors and chemicals that QDs elevated $[Ca^{2+}]_i$ which is involved in both extracellular and intracellular Ca^{2+}. Extracellular Ca^{2+} influx can be mainly through sodium channels, and partially through N-type calcium channels. The intracellular source was more complex: extracellular Na^+ influx induced Ca^{2+} release from mitochondria through the Na^+/H^+ exchanger (mNCX), this further induced much more Ca^{2+} release from ER through ryanodine receptors. In another study Tang et al. (2008b) used confocal laser scanning and standard whole-cell patch clamp techniques and detected that QDs induce neuron death dose dependently (1, 10, 20 nM). Furthermore, it was shown that QD treatment enhanced the activation and inactivation of I(Na), and reduced the fraction of available VGSCs. In a study (Xu et al., 2008) bioconjugated QDs (lysine-coated CdSe/CdS/ZnS) quantum rods (QRs) were used to cross an in vitro BBB model that consisted of primary human BMEC and normal human astrocyte (NHA) cells in two-compartment cell culture wells. QRs were conjugated with transferrin (Tf) or anti-claudin4 antibody (aC4). The authors showed that Tf-QRs were transported within human BMEC and NHA cells through the transferrin-receptor-mediated endocytosis pathway. The receptor-mediated uptake of Tf-QR into the cells was confirmed by presaturating the cells with free Tf, to block the available Tf receptors on the cell surface.

In summary, a large number of in vitro studies using either primary cultures from the mammalian nervous system (including experiments on both neurons and glial cells) or established cell lines with nervous system origin have been performed and many types of ENMs and endpoints have been studied. It is clear that biological effects to cells can be caused by ENMs since after nanoparticles enter the cell in an active or inactive manner they interact subsequently with the cytoplasm and subcellular organelles. Depending on the cell type and also on the ENMs, they can accumulate for a long period of time and thus induce specific cellular effects. The intracellular localization of the ENMs define if there is, for instance, a release of ions in case of soluble ENMs in lysosomes, thus nanoparticles can induce oxidative stress, apoptosis, and also cell cycle disturbances (Fig. 12.1). However, it has to be pointed out that the observed effects are dose dependent. Under in vivo conditions such high concentrations of unintended ENMs localized in the CNS are not very likely. Furthermore, these effects are not unique to nanosized particles since they can appear as consequences of exposure to the bulk material as well.

In Silico Studies of ENM Effects

The diversity of materials that have dimensions at the nanoscale is simply forbiddingly large, and is expected to grow to even larger numbers. If current approaches to assess the risks of these materials take place on a case-by-case basis, the assessment procedures will be too cumbersome to allow that even a small fraction of the materials are appropriately assessed. Thus, approaches that streamline the work are highly sought after, and will likely be the cornerstones of future assessments. However, until now, these ambitions have not taken off. A recent paper from Arts et al. (2014) summarizes current grouping protocols that are developed for use with ENMs. Their conclusion is that the suggested protocols are promising.

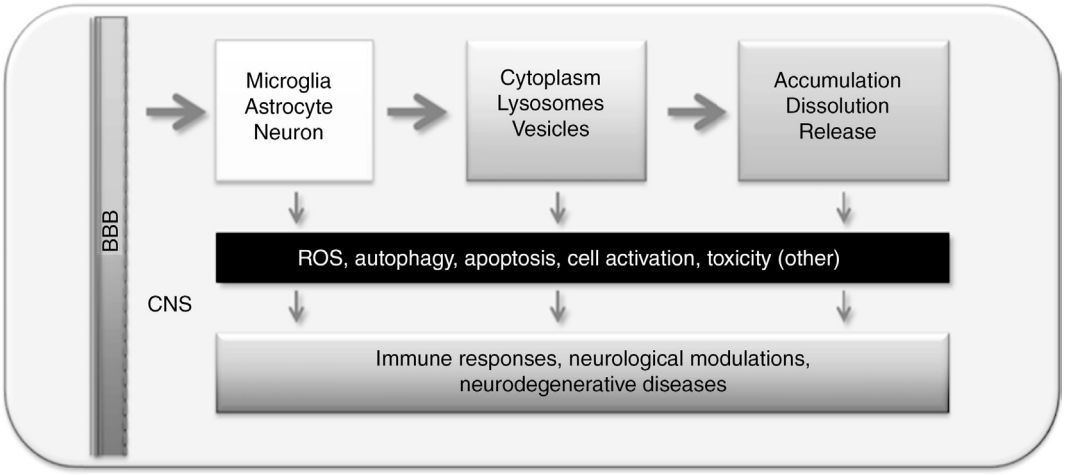


FIGURE 12.1 Pathway of engineered nanomaterials (ENM) after passing through the blood–brain barrier *(BBB).* Depending on the cell type (microglia, astrocyte, neuron) ENMs can accumulate for different periods of time in different parts of the cell (cytoplasm, lysosomes, vesicles). Depending on the intracellular localization, EMNs can accumulate, dissolve, or be released, inducing specific cellular effects, such as reactive oxygen species *(ROS)* production, autophagy, apoptosis, cell activation, and other effects. These effects can then lead to different systemic or neurodegenerative modulations.

They go beyond mere structure activity relationships (SARs), and should consider inclusion of the material's life cycle. This is also discussed in further detail in Chapter 8 on risk assessment strategies.

Several studies have promoted specific quantitative structure activity relationships (QSARs) that model ENM exposure to specific biological responses (Sayes et al., 2013; Winkler et al., 2013). Of special interest for nanotoxicology is the potential to use QSARs for grouping of materials (Burello and Worth, 2011), which is needed in a setting where novel nanomaterials are constantly developed. Also molecular modeling, where computational studies involving interactions of ENM with biological systems useful for constructing algorithms of assessing the likelihood of toxicity in a variety of natural environments, has been introduced as an appropriate tool (Gajewicz et al., 2012).

These computational approaches can be complemented by high-throughput screening (HTS) approaches, which also provide useful information regarding hazard identification and characterization. HTS assays can be based on biological macromolecules (i.e., genomics, transcriptomics, proteomics, metabolomics, and so on), and also on specific cellular or organismal endpoints, such as survival, cell proliferation, genotoxicity, and free radical homeostasis. The biological systems can involve in vitro (as described in Nel et al., 2013; Pisani et al., 2015), as well as in vivo models (George et al., 2011; Jung et al., 2015; Liu et al., 2012). There are only a few studies of this kind that are focused on neural system specific effects and disorders. Chorley et al. (2014) have published a study where the question was if nanosized silver particles of different sizes and with different coatings (PVP or citrate) have differentially toxic effects on oxidative stress sensitive neurons (rat N27 dopaminergic cells). The cells were exposed to nanosilver (10 or 75 nm; 8-h exposure) with either coating. Besides global

and specific gene expression, endpoints related to apoptosis and oxidative stress were investigated. The PVP-coating had more pronounced effects than citrate on the cell responses, and there were also differences in response to the two particle sizes. About 20% of the affected genes were responding to both 10- and 75-nm particles. Many of these genes were associated with oxidative stress pathways. Exposure to the 75-nm silver influenced mitochondrial dysfunction pathways, whereas 10-nm particles preferentially affected neuronal protective pathways. Development of a high-throughput neuromimetic screening system employing a coculture model (both neuronal and nonneuronal cell types) was presented by Jenkins et al. (2015). The initial set of studies using this screening platform focused on particle uptake, but can obviously be used for other endpoints. Although various computational and HTS approaches are available and possibly very useful for determining safety aspects of ENM, they are so far only sparingly used in the context of ENM effects on the nervous system and neuronal system disorders. An increased use of these methods would have the potential to significantly accelerate build-up of knowledge relevant for both hazard identification and understanding modes of action of ENMs.

Human Studies of ENM Effects

Nervous system disorders include a wide range of conditions, including vascular disorders (e.g., stroke), structural disorders (such as brain or spinal cord injury, tumors, and so on), functional (headaches, epilepsy, neuralgia, etc.), and degenerative disorders [Parkinson's disease (PD), multiple sclerosis, ALS, Huntington chorea, AD]. Such conditions comprise a significant part of the global burden of disease. Thus, World Economic Forum (2011) presented an estimate that these and related conditions represented costs in the trillion US$ range in 2010, and are projected to increase significantly in the coming decades.

The involvement of various types of environmental factors in the etiology and progression of most of these conditions is recognized. It is thus expected that any role for ENM in these disorders is or will be investigated. However, there are few human studies (experimental or epidemiological) that are addressing this issue.

Human unintended exposures to nanoparticles include both the ENMs that are the main topic of this chapter, but also to combustion-derived nanoparticles [primarily particulate matter (PM), diesel exhaust particles, and welding fumes; (Win-Shwe and Fujimaki, 2011)]. Human data are very limited in both cases, but we provide an overview here.

To the best of our knowledge, there are no relevant epidemiological studies that have assessed the possible influence of nonoccupational ENM exposure on neurological disorders. However, there are on-going and also finalized occupational studies. Liou et al. (2015) recently published an overview where they included data from 15 studies. Six were published, whereas the data from the other investigations were obtained from conference presentations or by other means. A number of biological markers were the independent variables. The material in the studies covered CNTs, nanosilver, nanotitania, iron oxides, and Ca_2CO_3. Exposure was assessed by mass concentration or particle count. According to the authors, some studies had inadequate exposure assessment. In general, the detected exposure levels were low compared to the ones employed in inhalation chamber studies. Furthermore, the studies often suffered from small sample sizes, inconsistent (or inadequate) exposure assessment, low exposure levels, and short intervals between exposure and a possible effect.

In summary, eleven of the studies (all cross sectional) showed a positive relationship between various biomarkers and ENM exposure. Three out of four longitudinal studies did not find any relationship between exposure and effect, whereas one study did. Of particular relevance for the present text, the findings did not include any nervous system–related outcome, with one unpublished exception.

A number of epidemiological studies have investigated nervous system disorders in relation to air pollution. It is recognized that air pollution is a chronic source of neuroinflammation, ROS production and neuropathology instigating CNS disease (Block and Calderon-Garciduenas, 2009). Thus, stroke as well as PD and AD are all linked to air pollution, which caused a number of systemic effects that can impinge on nervous system health. Air pollution includes PM, gases, and organic compounds and metals. Of special interest for the present text, the PM includes ultrafine particles (UFP, <100 nm). The UFP mostly emanate from tailpipe emissions from various mobile sources (motor vehicles, aircrafts, marine vessels; Muhlfeld et al., 2008).

Recent epidemiological studies have indeed investigated air pollution and neurodegenerative diseases. In a study by Palacios et al. (2014), the aim was to assess if exposure to PM air pollution is related to PD risk in the Nurse's Health Study, which is a prospective cohort study with female participants. Cumulative average exposure to different size fractions (including UFP) of PM up to 2 years prior to PD onset was taken into account. The study did not find any statistically significant associations between exposure and PD risk. Different results were obtained in another study (Zanobetti et al., 2014), where a multisite crossover analysis of all-cause and certain hospitalizations among Medicare enrolees during 1999–2010 in 121 US communities was done. The authors found that short-term exposure to $PM_{2.5}$ (particles <2.5 μm, and thus including UFP) up to 2 years prior to PD onset were significantly associated with hospitalization for PD and also for AD in subjects aged 65–75 at diagnosis.

Several studies investigating the effects of air pollution on nervous system disorders in Mexico City and other Mexican cities have been published by Calderon-Garciduenas and coworkers. Among these studies, one article published in 2004 (Calderon-Garciduenas et al., 2004) investigated whether residency in cities with high levels of air pollution is associated with human brain inflammation. This would have bearing on subsequent disease development. In this particular study, cyclooxygensase-2 (COX-2) and amyloidβ_{42} ($A\beta_{42}$ which makes up the plaques in AD) were measured in autopsy brain tissues of cognitively and neurologically intact lifelong residents of cities with either high or low levels of air pollution. In material from the high pollution cities, elevated levels of COX-2 were found in frontal cortex and hippocampus, whereas $A\beta_{42}$ was accumulated among both neurons and astrocytes in the same regions. Amyloid plaques were also noted in the olfactory bulb.

A subsequent study from the same group (Calderon-Garciduenas et al., 2008) investigated brain tissue samples from children and young adults that had died suddenly. Three inflammation markers were analyzed (COX-2, IL-1β, and CD14) in a number of brain regions. Corpses from individuals residing in highly polluted cities had higher levels of markers in the olfactory bulb, frontal cortex, substantia nigra, and vagus nerve. Microscopy also revealed disruptions in the BBB, endothelial activation, oxidative stress, and inflammatory cell trafficking in the specimens associated with a high degree of air pollution. Immunoreactivity for $A\beta_{42}$ was seen in 59% of APOE3/3 subjects, and in all carriers of the APOE4 allele that were investigated. Notably, all these subjects were younger than 25 years at the time of death. A marker for PD, α-synuclein, was detected in 24% of the corpses younger than 25 years from

the polluted cities. Finally, this study also documented the presence of UFP (as detected by transmission electron microscopy) in the olfactory bulb and in erythrocytes in CNS capillaries. Taken together, these two studies suggest a correlation between the presence of PM in air pollution and signs of brain damage and neurodegenerative diseases. Any UFP in the PM could originate from combustion processes, which may be most likely. However, the present knowledge does not allow the exclusion of ENM as another source of PM.

Still another article possibly connecting air pollution and CNS effects was published by Ranft et al. (2009). The study included 399 female subjects (68–79 years of age) that had lived more than 20 years at the same residential address. The study found consistent effects of traffic-related air pollution exposures on mild cognitive impairments. Distance to traffic was the main risk factor. This provides indirect evidence for a role of PM, if the closeness to traffic implies more PM pollution.

In conclusion, there are still very few epidemiological studies that have investigated the specific role of ENM for nervous system disorders. No studies have been found that address nonoccupational exposures. There is some evidence from epidemiological studies that air pollution, and by inference PM, is correlated to several nervous system disorders, including neurodegenerative diseases. However, at present it is not possibly to say if, and to what extent, the nanoparticle-sized UFP play any role in this scenario.

RISK ASSESSMENT OF NANOMATERIALS

A number of challenges have been identified that are specific for risk assessment of ENM (Hristozov et al., 2012, 2014) (and see Chapter 8). These challenges include the characterization of physical–chemical properties of ENM during various stages of the life cycle. Other challenges regard the actual exposure to man or the environment, the hazard potential for different forms of ENM, and their effects at relevant exposure levels. These considerations are also relevant regarding assessment of possible adverse effects of ENM on the nervous system. The scientific literature contains a significant number of studies that have relevance for risk assessment of ENM, particularly in the form of hazard identification studies. However, there are very few articles that actually perform a proper and complete risk assessment for a given ENM, taking exposure, hazard identification, and dose–response relationship studies as well as uncertainty analysis into consideration. As outlined in this chapter, ENM can during certain conditions enter and also pass through the BBB and other barriers that are protecting nervous system. It seems also to be possible that intranasal deposition of ENM can lead to entrance to the brain via retrograde axonal transport. Furthermore, both in vivo and in vitro studies provide evidence for effects of ENM on several aspects of nervous system function, including detrimental consequences. These findings thus support the notion that ENM possess properties that are hazardous to the nervous system. However, whether these findings are sufficient to claim that "real-life" exposure conditions can cause adverse health effects on the nervous system is still an open question. The risk assessment of ENPs to the brain has also to consider both acute and chronic exposure. As we stated previously (Simkó and Mattsson, 2010), any risk due to acute exposure of ENPs is expected to be low based on current knowledge. The lack of epidemiological studies that focus on long-term low-level exposure to ENM precludes any conclusions regarding chronic effects at present.

TAKE-HOME MESSAGES

- ENMs can, under certain conditions, enter the nervous system either by passing through blood–nervous system barriers, such as the BBB or via retrograde axonal transport from the nasal cavity.
- After entering the nervous system, a multitude of effects including adverse ones have been reported in both in vivo and in vitro studies.
- The relevance for adverse health consequences of these effects is still unclear; a substantial number of available in vitro and in vivo studies have used very high doses.
- Epidemiological studies are too few and often not designed to distinguish between ENM and other particle exposures.
- Important knowledge gaps include long-term, low-dose effects of ENMs; in addition, there is a need for HTS studies of ENMs.

References

Afeseh Ngwa, H., Kanthasamy, A., Gu, Y., Fang, N., Anantharam, V., Kanthasamy, A.G., 2011. Manganese nanoparticle activates mitochondrial dependent apoptotic signaling and autophagy in dopaminergic neuronal cells. Toxicol. Appl. Pharmacol. 256 (3), 227–240.

Albanese, A., Tang, P.S., Chan, W.C.W., 2012. The effect of nanoparticle size, shape, and surface chemistry on biological systems. Annu. Rev. Biomed. Eng. 14, 1–16.

An, L., Liu, S., Yang, Z., Zhang, T., 2012. Cognitive impairment in rats induced by nano-CuO and its possible mechanisms. Toxicol. Lett. 213 (2), 220–227.

Andón, F.T., Kapralov, A.A., Yanamala, N., Feng, W., Baygan, A., Chambers, B.J., Kagan, V.E., 2013. Biodegradation of single-walled carbon nanotubes by eosinophil peroxidase. Small 9 (16), 2721–2729.

Arts, J.H.E., Hadi, M., Keene, A.M., Kreiling, R., Lyon, D., Maier, M., Landsiedel, R., 2014. A critical appraisal of existing concepts for the grouping of nanomaterials. Regul. Toxicol. Pharmacol. 70 (2), 492–506.

Bai, R., Zhang, L., Liu, Y., Li, B., Wang, L., Wang, P., Chen, C., 2014. Integrated analytical techniques with high sensitivity for studying brain translocation and potential impairment induced by intranasally instilled copper nanoparticles. Toxicol. Lett. 226 (1), 70–80.

Bardi, G., Nunes, A., Gherardini, L., Bates, K., Al-Jamal, K.T., Gaillard, C., Kostarelos, K., 2013. Functionalized carbon nanotubes in the brain: cellular internalization and neuroinflammatory responses. PLoS One 8 (11), e80964/80961–e80964/80910, 80910 p..

Benfenati, E., Pardoe, S., Martin, T., Gonella Diaza, R., Lombardo, A., Manganaro, A., Gissi, A., 2013. Using toxicological evidence from QSAR models in practice. ALTEX 30 (1), 19–40.

Bermudez, E., Mangum, J.B., Wong, B.A., Asgharian, B., Hext, P.M., Warheit, D.B., Everitt, J.I., 2004. Pulmonary responses of mice, rats, and hamsters to subchronic inhalation of ultrafine titanium dioxide particles. Toxicol. Sci. 77 (2), 347–357.

Block, M.L., Calderon-Garciduenas, L., 2009. Air pollution: mechanisms of neuroinflammation and CNS disease. Trends Neurosci. 32 (9), 506–516.

Block, M.L., Zecca, L., Hong, J.S., 2007. Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. Nat. Rev. Neurosci. 8 (1), 57–69.

Brown, D.M., Donaldson, K., Stone, V., 2004. Effects of PM_{10} in human peripheral blood monocytes and J774 macrophages. Respir. Res. 5, 29, Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC545043/.

Brun, E., Carriere, M., Mabondzo, A., 2012. In vitro evidence of dysregulation of blood–brain barrier function after acute and repeated/long-term exposure to TiO_2 nanoparticles. Biomaterials 33 (3), 886–896.

Burello, E., Worth, A., 2011. Computational nanotoxicology: predicting toxicity of nanoparticles. Nat. Nanotechnol. 6 (3), 138–139.

Calderon-Garciduenas, L., Reed, W., Maronpot, R.R., Henriquez-Roldan, C., Delgado-Chavez, R., Calderon-Garciduenas, A., Swenberg, J.A., 2004. Brain inflammation and Alzheimer's-like pathology in individuals exposed to severe air pollution. Toxicol. Pathol. 32 (6), 650–658.

Calderon-Garciduenas, L., Solt, A.C., Henriquez-Roldan, C., Torres-Jardon, R., Nuse, B., Herritt, L., Reed, W., 2008. Long-term air pollution exposure is associated with neuroinflammation, an altered innate immune response, disruption of the blood–brain barrier, ultrafine particulate deposition, and accumulation of amyloid beta-42 and alpha-synuclein in children and young adults. Toxicol. Pathol. 36 (2), 289–310.

Calvo, P., Gouritin, B., Chacun, H., Desmaele, D., D'Angelo, J., Noel, J.P., Couvreur, P., 2001. Long-circulating PEGylated polycyanoacrylate nanoparticles as new drug carrier for brain delivery. Pharm. Res. 18 (8), 1157–1166.

Cannon, J.R., Greenamyre, J.T., 2011. The role of environmental exposures in neurodegeneration and neurodegenerative diseases. Toxicol. Sci. 124 (2), 225–250.

Cao, Y., Liu, H., Li, Q., Wang, Q., Zhang, W., Chen, Y., Cai, Y., 2013. Effect of lead sulfide nanoparticles exposure on calcium homeostasis in rat hippocampus neurons. J. Inorg. Biochem. 126, 70–75.

Cardoso, E., Rezin, G.T., Zanoni, E.T., Notoya, F.d.S., Leffa, D.D., Damiani, A.P., et al., 2014. Acute and chronic administration of gold nanoparticles cause DNA damage in the cerebral cortex of adult rats. Mutat. Res., Fundam. Mol. Mech. Mutagen. 766-767, 25–30.

Catalan-Figueroa, J., Palma-Florez, S., Alvarez, G., Fritz, H.F., Jara, M.O., Morales, J.O., 2016. Nanomedicine and nanotoxicology: the pros and cons for neurodegeneration and brain cancer. Nanomedicine 11 (2), 171–187.

Chen, Y.-S., Hung, Y.-C., Lin, L.-W., Liau, I., Hong, M.-Y., Huang, G.S., 2010. Size-dependent impairment of cognition in mice caused by the injection of gold nanoparticles. Nanotechnology 21, 485102/485101–485102/485109.

Chen, L., Miao, Y., Chen, L., Jin, P., Zha, Y., Chai, Y., Wang, M., 2013. The role of elevated autophagy on the synaptic plasticity impairment caused by CdSe/ZnS quantum dots. Biomaterials 34 (38), 10172–10181.

Chen, L., Yokel, R.A., Hennig, B., Toborek, M., 2008. Manufactured aluminum oxide nanoparticles decrease expression of tight junction proteins in brain vasculature. J. Neuroimmune Pharmacol. 3 (4), 286–295.

Chorley, B., Ward, W., Simmons, S.O., Vallanat, B., Veronesi, B., 2014. The cellular and genomic response of rat dopaminergic neurons (N27) to coated nanosilver. Neurotoxicology 45, 12–21.

Ciofani, G., Genchi, G.G., Liakos, I., Cappello, V., Gemmi, M., Athanassiou, A., Mattoli, V., 2013. Effects of cerium oxide nanoparticles on PC12 neuronal-like cells: proliferation, differentiation, and dopamine secretion. Pharm. Res. 30 (8), 2133–2145.

Coccini, T., Grandi, S., Lonati, D., Locatelli, C., De Simone, U., 2015. Comparative cellular toxicity of titanium dioxide nanoparticles on human astrocyte and neuronal cells after acute and prolonged exposure. Neurotoxicology 48, 77–89.

Cohen, J.M., DeLoid, G.M., Demokritou, P., 2015. A critical review of in vitro dosimetry for engineered nanomaterials. Nanomedicine 10 (19), 3015–3032.

Cui, Y., Xu, Q., Chow, P.K.-H., Wang, D., Wang, C.-H., 2013. Transferrin-conjugated magnetic silica PLGA nanoparticles loaded with doxorubicin and paclitaxel for brain glioma treatment. Biomaterials 34 (33), 8511–8520.

Dal, B.L., Weber, G.E., Parfitt, G.M., Monserrat, J.M., Barros, D.M., Cordeiro, A.P., Santos, A.P., 2015. Biopersistence of PEGylated carbon nanotubes promotes a delayed antioxidant response after infusion into the rat hippocampus. PLoS One 10 (6), e0129156.

Davenport, L.L., Hsieh, H., Eppert, B.L., Carreira, V.S., Krishan, M., Ingle, T., Genter, M.B., 2015. Systemic and behavioral effects of intranasal administration of silver nanoparticles. Neurotoxicol. Teratol. 51, 68–76.

De Jong, W.H., Borm, P.J., 2008. Drug delivery and nanoparticles: applications and hazards. Int. J. Nanomed. 3 (2), 133–149.

Dharsana, U.S., Sai Varsha, M.K.N., Khan Behlol, A.A., Veerappan, A., Thiagarajan, R., 2015. Sulfidation modulates the toxicity of biogenic copper nanoparticles. RSC Adv. 5, 30238.

Donaldson, K., Poland, C.A., Murphy, F.A., MacFarlane, M., Chernova, T., Schinwald, A., 2013. Pulmonary toxicity of carbon nanotubes and asbestos—similarities and differences. Adv. Drug Deliv. Rev. 65 (15), 2078–2086.

Duan, X., Li, Y., 2013. Physicochemical characteristics of nanoparticles affect circulation, biodistribution, cellular internalization, and trafficking. Small 9 (9–10), 1521–1532.

Dziendzikowska, K., Gromadzka-Ostrowska, J., Lankoff, A., Oczkowski, M., Krawczynska, A., Chwastowska, J., et al., 2012. Time-dependent biodistribution and excretion of silver nanoparticles in male Wistar rats. J. Appl. Toxicol. 32 (11), 920–928.

Erriquez, J., Bolis, V., Morel, S., Fenoglio, I., Fubini, B., Quagliotto, P., Distasi, C., 2015. Nanosized TiO_2 is internalized by dorsal root ganglion cells and causes damage via apoptosis. Nanomedicine 11 (6), 1309–1319.

Fadeel, B., Feliu, N., Vogt, C., Abdelmonem, A.M., Parak, W.J., 2013. Bridge over troubled waters: understanding the synthetic and biological identities of engineered nanomaterials. Wiley Interdiscip. Rev. 5 (2), 111–129.

Fenart, L., Casanova, A., Dehouck, B., Duhem, C., Slupek, S., Cecchelli, R., Betbeder, D., 1999. Evaluation of effect of charge and lipid coating on ability of 60-nm nanoparticles to cross an in vitro model of the blood–brain barrier. J. Pharmacol. Expl. Ther. 291 (3), 1017–1022.

Gajewicz, A., Rasulev, B., Dinadayalane, T.C., Urbaszek, P., Puzyn, T., Leszczynska, D., Leszczynski, J., 2012. Advancing risk assessment of engineered nanomaterials: application of computational approaches. Adv. Drug Deliv. Rev. 64, 1663–1693.

Gao, X., Yin, S., Tang, M., Chen, J., Yang, Z., Zhang, W., et al., 2011. Effects of developmental exposure to TiO2 nanoparticles on synaptic plasticity in hippocampal dentate gyrus area: an in vivo study in anesthetized rats. Biol. Trace Elem. Res. 143 (3), 1616–1628.

Gao, J., Zhang, X., Yu, M., Ren, G., Yang, Z., 2015. Cognitive deficits induced by multi-walled carbon nanotubes via the autophagic pathway. Toxicology 337, 21–29.

Garcia, G.J., Schroeter, J.D., Kimbell, J.S., 2015. Olfactory deposition of inhaled nanoparticles in humans. Inhal. Toxicol. 27 (8), 394–403.

George, S., Xia, T., Rallo, R., Zhao, Y., Ji, Z., Lin, S., et al., 2011. Use of a high-throughput screening approach coupled with in vivo zebrafish embryo screening to develop hazard ranking for engineered nanomaterials. ACS Nano 5 (3), 1805–1817.

Gerdes, H.H., Bukoreshtliev, N.V., Barroso, J.F., 2007. Tunneling nanotubes: a new route for the exchange of components between animal cells. FEBS Lett. 581 (11), 2194–2201.

Ghaderi, S., Tabatabaei, S.R.F., Varzi, H.N., Rashno, M., 2015. Induced adverse effects of prenatal exposure to silver nanoparticles on neurobehavioral development of offspring of mice. J. Toxicol. Sci. 40 (2), 263–275.

Gilardino, A., Catalano, F., Ruffinatti, F.A., Alberto, G., Nilius, B., Antoniotti, S., Lovisolo, D., 2015. Interaction of SiO_2 nanoparticles with neuronal cells: ionic mechanisms involved in the perturbation of calcium homeostasis. Int. J. Biochem. Cell Biol. 66, 101–111.

Graham, U.M., Tseng, M.T., Jasinski, J.B., Yokel, R.A., Unrine, J.M., Davis, B.H., Butterfield, D.A., 2014. In vivo processing of ceria nanoparticles inside liver: impact on free radical scavenging activity and oxidative stress. ChemPlusChem 79 (8), 1083–1088.

Gramowski, A., Flossdorf, J., Bhattacharya, K., Jonas, L., Lantow, M., Rahman, Q., Dopp, E., 2010. Nanoparticles induce changes of the electrical activity of neuronal networks on microelectrode array neurochips. Environ. Health Perspect. 118 (10), 1363–1369.

Gratton, S.E., Ropp, P.A., Pohlhaus, P.D., Luft, J.C., Madden, V.J., Napier, M.E., DeSimone, J.M., 2008. The effect of particle design on cellular internalization pathways. Proc. Natl. Acad. Sci. USA 105 (33), 11613–11618.

Gromnicova, R., Davies, H.A., Sreekanthreddy, P., Romero, I.A., Lund, T., Roitt, I.M., et al., 2013. Glucose-coated gold nanoparticles transfer across human brain endothelium and enter astrocytes in vitro. PLoS One 8 (12), e81043/81041-e81043/81010, 81010 p.

Guarnieri, D., Sabella, S., Muscetti, O., Belli, V., Malvindi, M.A., Fusco, S., Netti, P.A., 2014. Transport across the cell-membrane dictates nanoparticle fate and toxicity: a new paradigm in nanotoxicology. Nanoscale 6 (17), 10264–10273.

Haase, A., Rott, S., Mantion, A., Graf, P., Plendl, J., Thunemann, A.F., Reiser, G., 2012. Effects of silver nanoparticles on primary mixed neural cell cultures: uptake, oxidative stress and acute calcium responses. Toxicol. Sci. 126 (2), 457–468.

Hadrup, N., Loeschner, K., Mortensen, A., Sharma, A.K., Qvortrup, K., Larsen, E.H., Lam, H.R., 2012. The similar neurotoxic effects of nanoparticulate and ionic silver in vivo and in vitro. Neurotoxicology 33 (3), 416–423.

Hahn, F.F., Boecker, B.B., Griffith, W.C., Muggenburg, B.A., 1997. Biological effects of inhaled 144CeCl3 in beagle dogs. Radiat. Res. 147 (1), 92–108.

Han, D., Tian, Y., Zhang, T., Ren, G., Yang, Z., 2011. Nano-zinc oxide damages spatial cognition capability via over-enhanced long-term potentiation in hippocampus of Wistar rats. Int. J. Nanomed. 6, 1453–1461.

Hanada, S., Fujioka, K., Inoue, Y., Kanaya, F., Manome, Y., Yamamoto, K., 2014. Cell-based in vitro blood–brain barrier model can rapidly evaluate nanoparticles' brain permeability in association with particle size and surface modification. Int. J. Mol. Sci. 15 (2), 1812–1825.

Hardas, S.S., Sultana, R., Warrier, G., Dan, M., Wu, P., Grulke, E.A., Butterfield, D.A., 2014. Rat hippocampal responses up to 90 days after a single dose of nanoceria extends a hierarchical oxidative stress model for nanoparticle toxicity. Nanotoxicology 8, 155–166.

Heckman, K.L., DeCoteau, W., Estevez, A., Reed, K.J., Costanzo, W., Sanford, D., Erlichman, J.S., 2013. Custom cerium oxide nanoparticles protect against a free radical mediated autoimmune degenerative disease in the brain. ACS Nano 7 (12), 10582–10596.

Henrich-Noack, P., Prilloff, S., Voigt, N., Jin, J., Hintz, W., Tomas, J., Sabel, B.A., 2012. In vivo visualisation of nanoparticle entry into central nervous system tissue. Arch. Toxicol. 86 (7), 1099–1105.
Hinderliter, P.M., Minard, K.R., Orr, G., Chrisler, W.B., Thrall, B.D., Pounds, J.G., Teeguarden, J.G., 2010. ISDD: a computational model of particle sedimentation, diffusion and target cell dosimetry for in vitro toxicity studies. Part. Fibre Toxicol. 7 (1), 36.
Hirn, S., Semmler-Behnke, M., Schleh, C., Wenk, A., Lipka, J., Schaffler, M., Kreyling, W.G., 2011. Particle size-dependent and surface charge-dependent biodistribution of gold nanoparticles after intravenous administration. Eur. J. Pharm. Biopharm. 77 (3), 407–416.
Hoff, D., Sheikh, L., Bhattacharya, S., Nayar, S., Webster, T.J., 2013. Comparison study of ferrofluid and powder iron oxide nanoparticle permeability across the blood–brain barrier. Int. J. Nanomed. 8, 703–710.
Hohnholt, M.C., Geppert, M., Luther, E.M., Petters, C., Bulcke, F., Dringen, R., 2013. Handling of iron oxide and silver nanoparticles by astrocytes. Neurochem. Res. 38 (2), 227–239.
Hopkins, L.E., Patchin, E.S., Chiu, P.-L., Brandenberger, C., Smiley-Jewell, S., Pinkerton, K.E., 2014. Nose-to-brain transport of aerosolised quantum dots following acute exposure. Nanotoxicology 8 (8), 885–893.
Hristozov, D.R., Gottardo, S., Critto, A., Marcomini, A., 2012. Risk assessment of engineered nanomaterials: a review of available data and approaches from a regulatory perspective. Nanotoxicology 6, 880–898.
Hristozov, D.R., Zabeo, A., Foran, C., Isigonis, P., Critto, A., Marcomini, A., Linkov, I., 2014. A weight of evidence approach for hazard screening of engineered nanomaterials. Nanotoxicology 8 (1), 72–87.
Hu, R., Gong, X., Duan, Y., Li, N., Che, Y., Cui, Y., Hong, F., 2010. Neurotoxicological effects and the impairment of spatial recognition memory in mice caused by exposure to TiO_2 nanoparticles. Biomaterials 31 (31), 8043–8050.
Huang, C.-L., Hsiao, I.L., Lin, H.-C., Wang, C.-F., Huang, Y.-J., Chuang, C.-Y., 2015. Silver nanoparticles affect on gene expression of inflammatory and neurodegenerative responses in mouse brain neural cells. Environ. Res. 136, 253–263.
Huang, X., Zhang, F., Sun, X., Choi, K.-Y., Niu, G., Zhang, G., Chen, X., 2014. The genotype-dependent influence of functionalized multiwalled carbon nanotubes on fetal development. Biomaterials 35 (2), 856–865.
Huerta-Garcia, E., Marquez-Ramirez, S.G., Ramos-Godinez Mdel, P., Lopez-Saavedra, A., Herrera, L.A., Parra, A., Lopez-Marure, R., 2015. Internalization of titanium dioxide nanoparticles by glial cells is given at short times and is mainly mediated by actin reorganization-dependent endocytosis. Neurotoxicology 51, 27–37.
Huerta-García, E., Pérez-Arizti, J.A., Márquez-Ramírez, S.G., Delgado-Buenrostro, N.L., Chirino, Y.I., Iglesias, G.G., López-Marure, R., 2014. Titanium dioxide nanoparticles induce strong oxidative stress and mitochondrial damage in glial cells. Free Rad. Biol. Med. 73, 84–94.
Hutter, E., Boridy, S., Labrecque, S., Lalancette-Hebert, M., Kriz, J., Winnik, F.M., Maysinger, D., 2010. Microglial response to gold nanoparticles. ACS Nano 4 (5), 2595–2606.
Inoue, K.-i., Takano, H., Yanagisawa, R., Hirano, S., Ichinose, T., Shimada, A., Yoshikawa, T., 2006. The role of toll-like receptor 4 in airway inflammation induced by diesel exhaust particles. Arch. Toxicol. 80 (5), 275–279.
Ivani, S., Karimi, I., Tabatabaei, S.R.F., 2012. Biosafety of multiwalled carbon nanotube in mice: a behavioral toxicological approach. J. Toxicol. Sci. 37 (6), 1191–1205.
Iversen, T.-G., Skotland, T., Sandvig, K., 2011. Endocytosis and intracellular transport of nanoparticles: present knowledge and need for future studies. Nano Today 6 (2), 176–185.
Jana, N.R., 2011. Design and development of quantum dots and other nanoparticles based cellular imaging probe. Phys. Chem. Chem. Phys. 13 (2), 385–396.
Jenkins, S.I., Roach, P., Chari, D.M., 2015. Development of a nanomaterial bio-screening platform for neurological applications. Nanomedicine 11 (1), 77–87.
Jo, D.H., Kim, J.H., Lee, T.G., Kim, J.H., 2015. Size, surface charge, and shape determine therapeutic effects of nanoparticles on brain and retinal diseases. Nanomedicine 11 (7), 1603–1611.
Johansson, B.B., 2001. Blood–brain barrier: role of brain endothelial surface charge and glycocalyx. Fukuuchi, Y., Tomita, M., Koto, A. (Eds.), Ischemic Blood Flow in the Brain, Vol. Part 2, Springer, Japan, pp. 33–38.
Jung, S.-K., Qu, X., Aleman-Meza, B., Wang, T., Riepe, C., Liu, Z., Zhong, W., 2015. A multi-endpoint, high-throughput study of nanomaterial toxicity in *Caenorhabditis elegans*. Environ. Sci. Technol. 49 (4), 2477–2485.
Kato, H., Nakamura, A., Takahashi, K., Kinugasa, S., 2012. Accurate size and size-distribution determination of polystyrene latex nanoparticles in aqueous medium using dynamic light scattering and asymmetrical flow field flow fractionation with multi-angle light scattering. Nanomaterials 2 (4), 15–30.
Keefer, E.W., Botterman, B.R., Romero, M.I., Rossi, A.F., Gross, G.W., 2008. Carbon nanotube coating improves neuronal recordings. Nat. Nanotechnol. 3 (7), 434–439.

Kim, S.Y., Choi, E.-S., Lee, H.-J., Moon, C., Kim, E., 2015. Transthyretin as a new transporter of nanoparticles for receptor-mediated transcytosis in rat brain microvessels. Colloids Surf. B 136, 989–996.
Kim, J.A., Lee, N., Kim, B.H., Rhee, W.J., Yoon, S., Hyeon, T., Park, T.H., 2011. Enhancement of neurite outgrowth in PC12 cells by iron oxide nanoparticles. Biomaterials 32 (11), 2871–2877.
Kim, Y., Kong, S.D., Chen, L.-H., Pisanic, T.R., Jin, S., Shubayev, V.I., 2013. In vivo nanoneurotoxicity screening using oxidative stress and neuroinflammation paradigms. Nanomedicine 9 (7), 1057–1066.
Knudsen, K.B., Northeved, H., Ek, P.K., Permin, A., Andresen, T.L., Larsen, S., Lykkesfeldt, J., 2014. Differential toxicological response to positively and negatively charged nanoparticles in the rat brain. Nanotoxicology 8 (7), 764–774.
Kobzik, L., 1995. Lung macrophage uptake of unopsonized environmental particulates. Role of scavenger-type receptors. J. Immunol. 155 (1), 367–376.
Kolter, M., Ott, M., Hauer, C., Reimold, I., Fricker, G., 2015. Nanotoxicity of poly(*n*-butylcyano-acrylate) nanoparticles at the blood–brain barrier, in human whole blood and in vivo. J. Control. Release 197, 165–179.
Kreuter, J., 2004. Influence of the surface properties on nanoparticle-mediated transport of drugs to the brain. J. Nanosci. Nanotechnol. 4 (5), 484–488.
Kreuter, J., Shamenkov, D., Petrov, V., Ramge, P., Cychutek, K., Koch-Brandt, C., Alyautdin, R., 2002. Apolipoprotein-mediated transport of nanoparticle-bound drugs across the blood–brain barrier. J. Drug Target. 10 (4), 317–325.
Kulkarni, S.A., Feng, S.-S., 2013. Effects of particle size and surface modification on cellular uptake and biodistribution of polymeric nanoparticles for drug delivery. Pharm. Res. 30 (10), 2512–2522.
Kumari, M., Kumari, S.I., Grover, P., 2014. Genotoxicity analysis of cerium oxide micro and nanoparticles in Wistar rats after 28 days of repeated oral administration. Mutagenesis 29 (6), 467–479.
Kumari, M., Rajak, S., Singh, S.P., Kumari, S.I., Kumar, P.U., Murty, U.S.N., Rahman, M.F., 2012. Repeated oral dose toxicity of iron oxide nanoparticles: biochemical and histopathological alterations in different tissues of rats. J. Nanosci. Nanotechnol. 12 (3), 2149–2159.
Lai, J.C., Ananthakrishnan, G., Jandhyam, S., Dukhande, V.V., Bhushan, A., Gokhale, M., Leung, S.W., 2010. Treatment of human astrocytoma U87 cells with silicon dioxide nanoparticles lowers their survival and alters their expression of mitochondrial and cell signaling proteins. Int. J. Nanomed. 5, 715–723.
Lai, J.C.K., Gao, W., Bhushan, A., Leung, S.W., 2013. Entry of short multi-wall carbon nanotubes into dorsal root ganglion (DRG) neurons induces cell death. In: Paper presented at the Nanotech Conference & Expo 2013 An Interdisciplinary Integrative Forum on Nanotechnology, Microtechnology, Biotechnology and Cleantechnology, Washington, DC, United States.
Lee, J.H., Kim, Y.S., Song, K.S., Ryu, H.R., Sung, J.H., Park, J.D., et al., 2013. Biopersistence of silver nanoparticles in tissues from Sprague-Dawley rats. Part. Fibre Toxicol. 10, 36.
Lee, T.L., Raitano, J.M., Rennert, O.M., Chan, S.W., Chan, W.Y., 2012. Accessing the genomic effects of naked nanoceria in murine neuronal cells. Nanomedicine 8 (5), 599–608.
Li, X.-B., Liu, R., Liang, G.-Y., Yin, L.-H., Zheng, H., 2013. Aluminum oxide nanoparticles upregulate ED1 expression in rat olfactory bulbs by repeated intranasal instillation. Adv. Mater. Res. 716 (3–9), 8.
Li, C.-H., Shyu, M.-K., Jhan, C., Cheng, Y.-W., Tsai, C.-H., Liu, C.-W., Kang, J.-J., 2015. Gold nanoparticles increase endothelial paracellular permeability by altering components of endothelial tight junctions, and increase blood–brain barrier permeability in mice. Toxicol. Sci. 148 (1), 192–203.
Limbach, L.K., Wick, P., Manser, P., Grass, R.N., Bruinink, A., Stark, W.J., 2007. Exposure of engineered nanoparticles to human lung epithelial cells: influence of chemical composition and catalytic activity on oxidative stress. Environ. Sci. Technol. 41 (11), 4158–4163.
Lin, J., Huang, Z., Wu, H., Zhou, W., Jin, P., Wei, P., Wen, L., 2014. Inhibition of autophagy enhances the anticancer activity of silver nanoparticles. Autophagy 10 (11), 2006–2020.
Liou, S.H., Tsai, C.S., Pelclova, D., Schubauer-Berigan, M.K., Schulte, P.A., 2015. Assessing the first wave of epidemiological studies of nanomaterial workers. J. Nanopart. Res. 17, 413.
Lippmann, E.S., Al-Ahmad, A., Palecek, S.P., Shusta, E.V., 2013. Modeling the blood–brain barrier using stem cell sources. Fluids and Barriers of the CNS 10 (1), 1–14.
Lippmann, E.S., Azarin, S.M., Kay, J.E., Nessler, R.A., Wilson, H.K., Al-Ahmad, A., et al., 2012. Derivation of blood–brain barrier endothelial cells from human pluripotent stem cells. Nat. Biotechnol. 30, 783–791.
Lippmann, E.S., Weidenfeller, C., Svendsen, C.N., Shusta, E.V., 2011. Blood–brain barrier modeling with co-cultured neural progenitor cell-derived astrocytes and neurons. J. Neurochem. 119.

Liu, R., Lin, S., Rallo, R., Zhao, Y., Damoiseaux, R., Xia, T., Cohen, Y., 2012. Automated phenotype recognition for zebrafish embryo based in vivo high throughput toxicity screening of engineered nano-materials. PLoS One 7 (4), e35014.

Liu, N., Mu, Y., Chen, Y., Sun, H., Han, S., Wang, M., Sun, Z., 2013a. Degradation of aqueous synthesized CdTe/ZnS quantum dots in mice: differential blood kinetics and biodistribution of cadmium and tellurium. Part. Fibre Toxicol. 10, 37.

Liu, Y., Xu, Z., Li, X., 2013b. Cytotoxicity of titanium dioxide nanoparticles in rat neuroglia cells. Brain Injury 27 (7–8), 934–939.

Liu, X., Zhang, Y., Li, J., Wang, D., Wu, Y., Li, R., Yu, S.C.T., 2014. Cognitive deficits and decreased locomotor activity induced by single-walled carbon nanotubes and neuroprotective effects of ascorbic acid. Int. J. Nanomed. 9, 823–839.

Long, Y.-M., Zhao, X.-C., Zhou, Q.-F., Liu, Q., Jiang, G.-B., Long, Y.-M., Yan, B., 2015. Negatively charged silver nanoparticles cause retinal vascular permeability by activating plasma contact system and disrupting adherens junction. Nanotoxicology 10, 501–511.

Lu, W., Wan, J., She, Z., Jiang, X., 2007. Brain delivery property and accelerated blood clearance of cationic albumin conjugated pegylated nanoparticle. J. Control. Release 118 (1), 38–53.

Ma, L., Liu, J., Li, N., Wang, J., Duan, Y., Yan, J., Hong, F., 2010. Oxidative stress in the brain of mice caused by translocated nanoparticulate TiO_2 delivered to the abdominal cavity. Biomaterials 31 (1), 99–105, Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19783296.

Mahmoudi, M., Sheibani, S., Milani, A.S., Rezaee, F., Gauberti, M., Dinarvand, R., Vali, H., 2015. Crucial role of the protein corona for the specific targeting of nanoparticles. Nanomedicine 10 (2), 215–226.

Manshian, B.B., Moyano, D.F., Corthout, N., Munck, S., Himmelreich, U., Rotello, V.M., Soenen, S.J., 2014. High-content imaging and gene expression analysis to study cell-nanomaterial interactions: The effect of surface hydrophobicity. Biomaterials 35 (37), 9941–9950.

Mao, Z., Zhou, X., Gao, C., 2013. Influence of structure and properties of colloidal biomaterials on cellular uptake and cell functions. Biomater. Sci. 1 (9), 896–911.

Mate, Z., Horvath, E., Kozma, G., Simon, T., Konya, Z., Paulik, E., et al., 2015. Size-dependent toxicity differences of intratracheally instilled manganese oxide nanoparticles: conclusions of a subacute animal experiment. Biol. Trace Elem. Res. 171, 156–166.

Maynard, A.D., Kuempel, E.D., 2005. Airborne nanostructured particles and occupational health. J. Nanopart. Res. 7 (6), 587–614.

Maysinger, D., Behrendt, M., Lalancette-Hebert, M., Kriz, J., 2007. Real-time imaging of astrocyte response to quantum dots: in vivo screening model system for biocompatibility of nanoparticles. Nano Lett. 7 (8), 2513–2520, Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17638392.

Mendonça, M.C.P., da, C.-H.M.A., Mendonca, M.C.P., Soares, E.S., de, J.M.B., da, C.-H.M.A., et al., 2015. Reduced graphene oxide induces transient blood–brain barrier opening: an in vivo study. J. Nanobiotechnol. 13, 78.

Mohammadipour, A., Fazel, A., Haghir, H., Motejaded, F., Rafatpanah, H., Zabihi, H., et al., 2014. Maternal exposure to titanium dioxide nanoparticles during pregnancy; impaired memory and decreased hippocampal cell proliferation in rat offspring. Environ. Toxicol. Pharmacol. 37 (2), 617–625.

Muhlfeld, C., Rothen-Rutishauser, B., Blank, F., Vanhecke, D., Ochs, M., Gehr, P., 2008. Interactions of nanoparticles with pulmonary structures and cellular responses. Am. J. Physiol. Lung Cell Mol. Physiol. 294 (5), L817–829.

Murphy, F.A., Schinwald, A., Poland, C.A., Donaldson, K., 2012. The mechanism of pleural inflammation by long carbon nanotubes: interaction of long fibres with macrophages stimulates them to amplify pro-inflammatory responses in mesothelial cells. Part. Fibre Toxicol. 9, 8.

Naik, P., Cucullo, L., 2012. In vitro blood–brain barrier models: current and perspective technologies. J. Pharm. Sci. 101 (4), 1337–1354.

Nance, E.A., Woodworth, G.F., Sailor, K.A., Shih, T.-Y., Xu, Q., Swaminathan, G., et al., 2012. A dense poly(ethylene glycol) coating improves penetration of large polymeric nanoparticles within brain tissue. Sci. Transl. Med. 4 (149), 149ra119, 149 p.

Nance, E., Timbie, K., Miller, G.W., Song, J., Louttit, C., Klibanov, A.L., et al., 2014. Non-invasive delivery of stealth, brain-penetrating nanoparticles across the blood - brain barrier using MRI-guided focused ultrasound. J. Control. Release 189, 123–132.

Nel, A., Xia, T., Meng, H., Wang, X., Lin, S., Ji, Z., Zhang, H., 2013. Nanomaterial toxicity testing in the 21st century: use of a predictive toxicological approach and high-throughput screening. Acc. Chem. Res. 46 (3), 607–621.

Ni, Y., Hu, H., Malarkey, E.B., Zhao, B., Montana, V., Haddon, R.C., Parpura, V., 2005. Chemically functionalized water soluble single-walled carbon nanotubes modulate neurite outgrowth. J. Nanosci. Nanotechnol. 5 (10), 1707–1712, Available from: http://www.ncbi.nlm.nih.gov/pubmed/16245532.

Ntim, S.A., Sae-Khow, O., Desai, C., Witzmann, F.A., Mitra, S., 2012. Size dependent aqueous dispersibility of carboxylated multiwall carbon nanotubes. J. Environ. Monit. 14 (10), 2772–2779.

Nunes, A., Al-Jamal, K., Nakajima, T., Hariz, M., Kostarelos, K., 2012. Application of carbon nanotubes in neurology: clinical perspectives and toxicological risks. Arch. Toxicol. 86 (7), 1009–1020.

Oberdorster, G., Oberdorster, E., Oberdorster, J., 2005. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environ. Health Perspect. 113 (7), 823–839.

Oh, N., Park, J.H., 2014. Endocytosis and exocytosis of nanoparticles in mammalian cells. Int. J. Nanomed. 9 (Suppl. 1), 51–63.

Olivier, J.C., 2005. Drug transport to brain with targeted nanoparticles. NeuroRx 2 (1), 108–119.

Oszlanczi, G., Papp, A., Szaboo, A., Nagymajtenyi, L., Sapi, A., Konya, Z., et al., 2011. Nervous system effects in rats on subacute exposure by lead-containing nanoparticles via the airways. Inhal. Toxicol. 23 (4), 173–181.

Oszlanczi, G., Vezer, T., Sarkoezi, L., Horvath, E., Konya, Z., Papp, A., 2010a. Functional neurotoxicity of Mn-containing nanoparticles in rats. Ecotoxicol. Environ. Saf. 73 (8), 2004–2009.

Oszlanczi, G., Vezer, T., Sarkoezi, L., Horvath, E., Szabo, A., Horvath, E., et al., 2010b. Metal deposition and functional neurotoxicity in rats after 3-6 weeks nasal exposure by two physicochemical forms of manganese. Environ. Toxicol. Pharmacol. 30 (2), 121–126.

Oyabu, T., Myojo, T., Morimoto, Y., Ogami, A., Hirohashi, M., Yamamoto, M., et al., 2011. Biopersistence of inhaled MWCNT in rat lungs in a 4-week well-characterized exposure. Inhal. Toxicol. 23 (13), 784–791.

Palacios, N., Fitzgerald, K.C., Hart, J.E., Weisskopf, M.G., Schwarzschild, M.A., Ascherio, A., Laden, F., 2014. Particulate matter and risk of Parkinson disease in a large prospective study of women. Environ. Health 13, 80.

Papp, A., Oszlanczi, G., Horvath, E., Paulik, E., Kozma, G., Sapi, A., et al., 2012. Consequences of subacute intratracheal exposure of rats to cadmium oxide nanoparticles: electrophysiological and toxicological effects. Toxicol. Ind. Health 28 (10), 933–941.

Park, J.B., 2003. Phagocytosis induces superoxide formation and apoptosis in macrophages. Exp. Mol. Med. 35 (5), 325–335.

Parveen, A., Rizvi, S.H.M., Mahdi, A.A., Sushma, Ahmad, I., Mahdi, F., Singh, P.P., 2015. Intranasal exposure to silica nanoparticles induce alterations in pro-inflammatory environment of rat brain: Involvement of oxidative stress. Toxicol. Ind. Health, (Epub ahead of print).

Parveen, A., Rizvi, S.H.M., Mahdi, F., Tripathi, S., Ahmad, I., Shukla, R.K., et al., 2014. Silica nanoparticles mediated neuronal cell death in corpus striatum of rat brain: implication of mitochondrial, endoplasmic reticulum and oxidative stress. J. Nanopart. Res. 16 (11), 1–15.

Pauluhn, J., 2009. Pulmonary toxicity and fate of agglomerated 10 and 40 nm aluminum oxyhydroxides following 4-week inhalation exposure of rats: toxic effects are determined by agglomerated, not primary particle size. Toxicol. Sci. 109 (1), 152–167.

Pauluhn, J., 2010. Subchronic 13-week inhalation exposure of rats to multiwalled carbon nanotubes: toxic effects are determined by density of agglomerate structures, not fibrillar structures. Toxicol. Sci. 113 (1), 226–242.

Phillips, C.L., Yah, C.S., Iyuke, S.E., Pillay, V., Rumbold, K., Choonara, Y., 2013. The response effect of pheochromocytoma (PC12) cell lines to oxidized multi-walled carbon nanotubes (o-MWCMTs). Afr. Health Sci. 13 (4), 947–954.

Pinkernelle, J., Calatayud, P., Goya, G.F., Fansa, H., Keilhoff, G., 2012. Magnetic nanoparticles in primary neural cell cultures are mainly taken up by microglia. BMC Neurosci. 13, 32.

Pisani, C., Gaillard, J.C., Nouvel, V., Odorico, M., Armengaud, J., Prat, O., 2015. High-throughput, quantitative assessment of the effects of low-dose silica nanoparticles on lung cells: grasping complex toxicity with a great depth of field. BMC Genomics 16, 315.

Poland, C.A., Duffin, R., Kinloch, I., Maynard, A., Wallace, W.A., Seaton, A., et al., 2008. Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study. Nat. Nanotechnol. 3 (7), 423–428, Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18654567.

Prabhakar, P.V., Reddy, U.A., Singh, S.P., Balasubramanyam, A., Rahman, M.F., Indu Kumari, S., et al., 2012. Oxidative stress induced by aluminum oxide nanomaterials after acute oral treatment in Wistar rats. J. Appl. Toxicol. 32 (6), 436–445.

Privalova, L.I., Katsnelson, B.A., Loginova, N.V., Gurvich, V.B., Shur, V.Y., Valamina, I.E., et al., 2014. Subchronic toxicity of copper oxide nanoparticles and its attenuation with the help of a combination of bioprotectors. Int. J. Mol. Sci. 15 (7), 12379–12406, 12328.
Qiao, R., Jia, Q., Huwel, S., Xia, R., Liu, T., Gao, F., et al., 2012. Receptor-mediated delivery of magnetic nanoparticles across the blood–brain barrier. ACS Nano 6 (4), 3304–3310.
Ranft, U., Schikowski, T., Sugiri, D., Krutmann, J., Kramer, U., 2009. Long-term exposure to traffic-related particulate matter impairs cognitive function in the elderly. Environ. Res. 109 (8), 1004–1011.
Reddy, U.A., Prabhakar, P.V., Mahboob, M., 2015. Biomarkers of oxidative stress for in vivo assessment of toxicological effects of iron oxide nanoparticles. Saudi J. Biol. Sci., (Epub ahead of print).
Reimold, I., Domke, D., Bender, J., Seyfried, C.A., Radunz, H.-E., Fricker, G., 2008. Delivery of nanoparticles to the brain detected by fluorescence microscopy. Eur. J. Pharm. Biopharm. 70 (2), 627–632.
Rejman, J., Oberle, V., Zuhorn, I., Hoekstra, D., 2004. Size-dependent internalization of particles via the pathways of clathrin- and caveolae-mediated endocytosis. Biochem. J. 377, 159–169.
Rodriguez-Fragoso, P., Reyes-Esparza, J., Leon-Buitimea, A., Rodriguez-Fragoso, L., 2012. Synthesis, characterization and toxicological evaluation of maltodextrin capped cadmium sulfide nanoparticles in human cell lines and chicken embryos. J. Nanobiotechnol. 10, 47.
Rungby, J., Danscher, G., 1983. Localization of exogenous silver in brain and spinal cord of silver exposed rats. Acta Neuropathol. 60 (1–2), 92–98.
Rustom, A., Saffrich, R., Markovic, I., Walther, P., Gerdes, H.H., 2004. Nanotubular highways for intercellular organelle transport. Science 303 (5660), 1007–1010.
Sahay, G., Alakhova, D.Y., Kabanov, A.V., 2010. Endocytosis of nanomedicines. J. Control. Release 145 (3), 182–195.
Sato, Y., Yokoyama, A., Nodasaka, Y., Kohgo, T., Motomiya, K., Matsumoto, H., Tohji, K., 2013. Long-term biopersistence of tangled oxidized carbon nanotubes inside and outside macrophages in rat subcutaneous tissue. Sci. Rep. 3, 2516.
Sayes, C.M., Smith, P.A., Ivanov, I.V., 2013. A framework for grouping nanoparticles based on their measurable characteristics. Int. J. Nanomed. 8 (Suppl. 1), 45–56.
Schoelermann, J., Burtey, A., Allouni, Z.E., Gerdes, H.H., Cimpan, M.R., 2015. Contact-dependent transfer of TiO nanoparticles between mammalian cells. Nanotoxicology 1–12, 204–215.
Shah, S.A., Yoon, G.H., Ahmad, A., Ullah, F., Amin, F.U., Kim, M.O., 2015. Nanoscale-alumina induces oxidative stress and accelerates amyloid beta (Aβ) production in ICR female mice. Nanoscale 7 (37), 15225–15237.
Shang, L., Nienhaus, K., Nienhaus, G.U., 2014. Engineered nanoparticles interacting with cells: size matters. J. Nanobiotechnol. 12, 5.
Shang, S., Yang, S.-Y., Liu, Z.-M., Yang, X., 2015. Oxidative damage in the kidney and brain of mice induced by different nano-materials. Front. Biol. 10 (1), 91–96.
Sharma, A., Muresanu, D.F., Lafuente, J.V., Patnaik, R., Tian, Z.R., Buzoianu, A.D., Sharma, H.S., 2015. Sleep deprivation-induced blood–brain barrier breakdown and brain dysfunction are exacerbated by size-related exposure to Ag and Cu nanoparticles neuroprotective effects of a 5-HT3 receptor antagonist ondansetron. Mol. Neurobiol. 52 (2), 867–881.
Sharma, A., Muresanu, D.F., Patnaik, R., Sharma, H.S., 2013. Size- and age-dependent neurotoxicity of engineered metal nanoparticles in rats. Mol. Neurobiol. 48 (2), 386–396.
Shcharbin, D., Janaszewska, A., Klajnert-Maculewicz, B., Ziemba, B., Dzmitruk, V., Halets, I., et al., 2014. How to study dendrimers and dendriplexes III. Biodistribution, pharmacokinetics and toxicity in vivo. J. Control. Release 181, 40–52.
Sheng, L., Ze, Y., Wang, L., Yu, X., Hong, J., Zhao, X., et al., 2015. Mechanisms of TiO2 nanoparticle-induced neuronal apoptosis in rat primary cultured hippocampal neurons. J. Biomed. Mater. Res. A 103 (3), 1141–1149.
Shilo, M., Motiei, M., Hana, P., Popovtzer, R., 2014. Transport of nanoparticles through the blood–brain barrier for imaging and therapeutic applications. Nanoscale 6 (4), 2146–2152.
Shimizu, M., Tainaka, H., Oba, T., Mizuo, K., Umezawa, M., Takeda, K., 2009. Maternal exposure to nanoparticulate titanium dioxide during the prenatal period alters gene expression related to brain development in the mouse. Part. Fibre Toxicol. 6, 20.
Shrivastava, R., Raza, S., Yadav, A., Kushwaha, P., Flora, S.J.S., 2014. Effects of sub-acute exposure to TiO_2, ZnO and Al_2O_3 nanoparticles on oxidative stress and histological changes in mouse liver and brain. Drug Chem. Toxicol. 37 (3), 336–347.
Simkó, M., Mattsson, M.O., 2010. Risks from accidental exposures to engineered nanoparticles and neurological health effects: a critical review. Part. Fibre Toxicol. 7, 42, Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=21176150.

Simkó, M., Mattsson, M.O., 2014. Interactions between nanosized materials and the brain. Curr. Med. Chem. 21 (37), 4200–4214, Available from: http://www.ncbi.nlm.nih.gov/pubmed/25039776.

Simkó, M., Nosske, D., Kreyling, W.G., 2014. Metrics, dose, and dose concept: the need for a proper dose concept in the risk assessment of nanoparticles. Int. J. Environ. Res. Public Health 11 (4), 4026–4048, Available from: http://www.scopus.com/inward/record.url?eid=2-s2.0-84898870623&partnerID=40&md5=fe4b8d8685002d9e8b967a41dba45aa6.

Singh, S.P., Kumari, M., Kumari, S.I., Rahman, M.F., Kamal, S.S.K., Mahboob, M., Grover, P., 2013. Genotoxicity of nano- and micron-sized manganese oxide in rats after acute oral treatment. Mutat. Res., Genet. Toxicol. Environ. Mutagen. 754 (1–2), 39–50.

Skalska, J., Frontczak-Baniewicz, M., Struzynska, L., 2015. Synaptic degeneration in rat brain after prolonged oral exposure to silver nanoparticles. Neurotoxicology 46, 145–154.

Soenen, S.J., Manshian, B., Doak, S.H., De Smedt, S.C., Braeckmans, K., 2013. Fluorescent non-porous silica nanoparticles for long-term cell monitoring: cytotoxicity and particle functionality. Acta Biomater. 9 (11), 9183–9193.

Strickland, J.D., Lefew, W.R., Crooks, J., Hall, D., Ortenzio, J.N., Dreher, K., Shafer, T.J., 2016. In vitro screening of metal oxide nanoparticles for effects on neural function using cortical networks on microelectrode arrays. Nanotoxicology 10, 619–628.

Su, C.-K., Cheng, T.-Y., Sun, Y.-C., 2015. Selective chemical vaporization of exogenous tellurium for characterizing the time-dependent biodistribution and dissolution of quantum dots in living rats. J. Anal. At. Spectrom. 30 (2), 426–434.

Sun, C., Yin, N., Wen, R., Liu, W., Jia, Y., Hu, L., et al., 2016. Silver nanoparticles induced neurotoxicity through oxidative stress in rat cerebral astrocytes is distinct from the effects of silver ions. Neurotoxicology 52, 210–221.

Takeda, K., Suzuki, K.-i., Ishihara, A., Kubo-Irie, M., Fujimoto, R., Tabata, M., et al., 2009. Nanoparticles transferred from pregnant mice to their offspring can damage the genital and cranial nerve systems. J. Health Sci. 55, 95–102, 2012 American Chemical Society (ACS). All Rights Reserved.

Tang, M., Li, Z., Chen, L., Xing, T., Hu, Y., Yang, B., et al., 2009. The effect of quantum dots on synaptic transmission and plasticity in the hippocampal dentate gyrus area of anesthetized rats. Biomaterials 30 (28), 4948–4955.

Tang, M., Wang, M., Xing, T., Zeng, J., Wang, H., Ruan, D.Y., 2008a. Mechanisms of unmodified CdSe quantum dot-induced elevation of cytoplasmic calcium levels in primary cultures of rat hippocampal neurons. Biomaterials 29 (33), 4383–4391.

Tang, M., Xing, T., Zeng, J., Wang, H., Li, C., Yin, S., et al., 2008b. Unmodified CdSe quantum dots induce elevation of cytoplasmic calcium levels and impairment of functional properties of sodium channels in rat primary cultured hippocampal neurons. Environ. Health Perspect. 116 (7), 915–922.

Tang, J., Xiong, L., Wang, S., Wang, J., Liu, L., Li, J., et al., 2008c. Influence of silver nanoparticles on neurons and blood–brain barrier via subcutaneous injection in rats. Appl. Surf. Sci. 255 (2), 502–504.

Teeguarden, J.G., Hinderliter, P.M., Orr, G., Thrall, B.D., Pounds, J.G., 2006. Particokinetics in vitro: dosimetry considerations for in vitro nanoparticle toxicity assessments. Toxicol. Sci. 95 (2), 300–312.

Tiwari, S.B., Amiji, M.M., 2006. A review of nanocarrier-based CNS delivery systems. Curr. Drug Deliv. 3 (2), 219–232.

Tosi, G., Vilella, A., Chhabra, R., Schmeisser, M.J., Boeckers, T.M., Ruozi, B., et al., 2014. Insight on the fate of CNS-targeted nanoparticles. Part II: Intercellular neuronal cell-to-cell transport. J Control. Release 177, 96–107.

Trickler, W.J., Lantz-McPeak, S.M., Robinson, B.L., Paule, M.G., Slikker, Jr., W., Biris, A.S., et al., 2014. Porcine brain microvessel endothelial cells show pro-inflammatory response to the size and composition of metallic nanoparticles. Drug Metab. Rev. 46 (2), 224–231.

van der Zande, M., Vandebriel, R.J., Van Doren, E., Kramer, E., Herrera Rivera, Z., Serrano-Rojero, C.S., et al., 2012. Distribution, elimination, and toxicity of silver nanoparticles and silver ions in rats after 28-day oral exposure. ACS Nano 6 (8), 7427–7442.

Varela, J.A., Bexiga, M.G., Aberg, C., Simpson, J.C., Dawson, K.A., 2012. Quantifying size-dependent interactions between fluorescently labeled polystyrene nanoparticles and mammalian cells. J. Nanobiotechnol. 10, 39.

Walczyk, D., Bombelli, F.B., Monopoli, M.P., Lynch, I., Dawson, K.A., 2010. What the cell "sees" in bionanoscience. J. Am. Chem. Soc. 132 (16), 5761–5768.

Wang, X., Gerdes, H.H., 2015. Transfer of mitochondria via tunneling nanotubes rescues apoptotic PC12 cells. Cell Death Differ. 22 (7), 1181–1191.

Wang, J., Liu, Y., Jiao, F., Lao, F., Li, W., Gu, Y., et al., 2008. Time-dependent translocation and potential impairment on central nervous system by intranasally instilled TiO_2 nanoparticles. Toxicology 254 (1-2), 82–90.

Wang, F., Jiao, C., Liu, J., Yuan, H., Lan, M., Gao, F., 2011a. Oxidative mechanisms contribute to nanosize silican dioxide-induced developmental neurotoxicity in PC12 cells. Toxicol. In Vitro 25 (8), 1548–1556.
Wang, Y., Wang, B., Zhu, M.T., Li, M., Wang, H.J., Wang, M., et al., 2011b. Microglial activation, recruitment and phagocytosis as linked phenomena in ferric oxide nanoparticle exposure. Toxicol. Lett. 205 (1), 26–37.
Wang, Y., Wu, Q., Sui, K., Chen, X.X., Fang, J., Hu, X., et al., 2013. A quantitative study of exocytosis of titanium dioxide nanoparticles from neural stem cells. Nanoscale 5 (11), 4737–4743.
Wen, R., Yang, X., Hu, L., Sun, C., Zhou, Q., Jiang, G., 2016. Brain-targeted distribution and high retention of silver by chronic intranasal instillation of silver nanoparticles and ions in Sprague-Dawley rats. J. Appl. Toxicol. 36 (3), 445–453.
Willhite, C.C., Karyakina, N.A., Yokel, R.A., Yenugadhati, N., Wisniewski, T.M., Arnold, I.M.F., et al., 2014. Systematic review of potential health risks posed by pharmaceutical, occupational and consumer exposures to metallic and nanoscale aluminum, aluminum oxide, aluminum hydroxide and its soluble salts. Crit. Rev. Toxicol. S4, 1–80.
Winkler, D.A., Mombelli, E., Pietroiusti, A., Tran, L., Worth, A., Fadeel, B., McCall, M.J., 2013. Applying quantitative structure-activity relationship approaches to nanotoxicology: current status and future potential. Toxicology 313 (1), 15–23.
Win-Shwe, T.T., Fujimaki, H., 2011. Nanoparticles and neurotoxicity. Int. J. Mol. Sci. 12 (9), 6267–6280.
Win-Shwe, T.-T., Sone, H., Kurokawa, Y., Zeng, Y., Zeng, Q., Nitta, H., Hirano, S., 2014. Effects of PAMAM dendrimers in the mouse brain after a single intranasal instillation. Toxicol. Lett. 228 (3), 207–215.
Wong, L.L., Hirst, S.M., Pye, Q.N., Reilly, C.M., Seal, S., McGinnis, J.F., 2013. Catalytic nanoceria are preferentially retained in the rat retina and are not cytotoxic after intravitreal injection. PLoS One 8, e58431.
World Economic Forum, 2011. The Global Economic Burden of Non-communicable Diseases. Available from: http://www3.weforum.org/docs/WEF_Harvard_HE_GlobalEconomicBurdenNonCommunicableDiseases_2011.pdf
Wu, J., Liu, W., Xue, C., Zhou, S., Lan, F., Bi, L., et al., 2009. Toxicity and penetration of TiO_2 nanoparticles in hairless mice and porcine skin after subchronic dermal exposure. Toxicol. Lett. 191 (1), 1–8.
Wu, D., Pak, E.S., Wingard, C.J., Murashov, A.K., 2012. Multi-walled carbon nanotubes inhibit regenerative axon growth of dorsal root ganglia neurons of mice. Neurosci. Lett. 507 (1), 72–77.
Wu, J., Sun, J., Xue, Y., 2010. Involvement of JNK and P53 activation in G2/M cell cycle arrest and apoptosis induced by titanium dioxide nanoparticles in neuron cells. Toxicol. Lett. 199 (3), 269–276.
Wu, J., Yu, C., Tan, Y., Hou, Z., Li, M., Shao, F., Lu, X., 2015. Effects of prenatal exposure to silver nanoparticles on spatial cognition and hippocampal neurodevelopment in rats. Environ. Res. 138, 67–73.
Xu, H., Bai, J., Meng, J., Hao, W., Xu, H., Cao, J.M., 2009. Multi-walled carbon nanotubes suppress potassium channel activities in PC12 cells. Nanotechnology 20 (28), 285102.
Xu, L., Dan, M., Shao, A., Cheng, X., Zhang, C., Yokel, R.A., et al., 2015a. Silver nanoparticles induce tight junction disruption and astrocyte neurotoxicity in a rat blood–brain barrier primary triple coculture model. Int. J. Nanomedicine. 10, 6105–6118.
Xu, F., Piett, C., Farkas, S., Qazzaz, M., Syed, N.I., 2013. Silver nanoparticles (AgNPs) cause degeneration of cytoskeleton and disrupt synaptic machinery of cultured cortical neurons. Mol. Brain 6 (1), 29.
Xu, L., Shao, A., Zhao, Y., Wang, Z., Zhang, C., Sun, Y., et al., 2015b. Neurotoxicity of silver nanoparticles in rat brain after intragastric exposure. J. Nanosci. Nanotechnol. 15 (6), 4215–4223.
Xue, Y., Wu, J., Sun, J., 2012. Four types of inorganic nanoparticles stimulate the inflammatory reaction in brain microglia and damage neurons in vitro. Toxicol. Lett. 214 (2), 91–98.
Xu, G., Yong, K.T., Roy, I., Mahajan, S.D., Ding, H., Schwartz, S.A., Prasad, P.N., 2008. Bioconjugated quantum rods as targeted probes for efficient transmigration across an in vitro blood–brain barrier. Bioconjug. Chem. 19 (6), 1179–1185.
Yan, F., Wang, Y., He, S., Ku, S., Gu, W., Ye, L., 2013. Transferrin-conjugated, fluorescein-loaded magnetic nanoparticles for targeted delivery across the blood–brain barrier. J. Mater. Sci. 24 (10), 2371–2379.
Yang, S., Ye, R., Cai, W., Xiang, X., Yang, X., 2013. Oxidative damage of brain induced by nano-CdSeS in mice. Adv. Mater. Res. 669 (316–324), 310.
Yin, N.Y., Liu, Q., Liu, J.Y., He, B., Cui, L., Li, Z.N., et al., 2013. Silver nanoparticle exposure attenuates the viability of rat cerebellum granule cells through apoptosis coupled to oxidative stress. Small 9 (9-10), 1831–1841.
Yin, N., Yao, X., Zhou, Q., Faiola, F., Jiang, G., 2015a. Vitamin E attenuates silver nanoparticle-induced effects on body weight and neurotoxicity in rats. Biochem. Biophys. Res. Commun. 458 (2), 405–410.
Yin, N., Zhang, Y., Yun, Z., Liu, Q., Qu, G., Zhou, Q., et al., 2015b. Silver nanoparticle exposure induces rat motor dysfunction through decrease in expression of calcium channel protein in cerebellum. Toxicol. Lett. 237 (2), 112–120.

Yokel, R.A., Au, T.C., MacPhail, R., Hardas, S.S., Butterfield, D.A., Sultana, R., et al., 2012. Distribution, elimination and biopersistence to 90 days of a systemically-introduced 30 nm ceria engineered nanomaterial in rats. Toxicol. Sci. 127 (1), 256–268.

Yokel, R.A., Unrine, J.M., Wu, P., Wang, B., Grulke, E., 2014. Nanoceria biodistribution and retention in the rat are not greatly influenced by dosing schedule, dose, or shape. Environ. Sci. 1 (6), 549–560.

Yu, Y., Duan, J., Yu, Y., Li, Y., Liu, X., Zhou, X., et al., 2014. Silica nanoparticles induce autophagy and autophagic cell death in HepG2 cells triggered by reactive oxygen species. J. Hazard. Mater. 270, 176–186.

Zanobetti, A., Dominici, F., Wang, Y., Schwartz, J.D., 2014. A national case-crossover analysis of the short-term effect of PM2.5 on hospitalizations and mortality in subjects with diabetes and neurological disorders. Environ. Health 13 (1), 38.

Ze, Y., Hu, R., Wang, X., Sang, X., Xiao, Z., Li, B., et al., 2014. Neurotoxicity and gene-expressed profile in brain-injured mice caused by exposure to titanium dioxide nanoparticles. J. Biomed. Mater. Res. A 102A (2), 470–478.

Zensi, A., Begley, D., Pontikis, C., Legros, C., Mihoreanu, L., Buechel, C., Kreuter, J., 2010. Human serum albumin nanoparticles modified with apolipoprotein A-I cross the blood–brain barrier and enter the rodent brain. J. Drug Target. 18 (10), 842–848.

Zensi, A., Begley, D., Pontikis, C., Legros, C., Mihoreanu, L., Wagner, S., et al., 2009. Albumin nanoparticles targeted with Apo E enter the CNS by transcytosis and are delivered to neurones. J. Control. Release 137 (1), 78–86.

Zhang, L., Bai, R., Liu, Y., Meng, L., Li, B., Wang, L., et al., 2012. The dose-dependent toxicological effects and potential perturbation on the neurotransmitter secretion in brain following intranasal instillation of copper nanoparticles. Nanotoxicology 6 (5), 562–575.

Zhang, J., Song, W., Guo, J., Zhang, J., Sun, Z., Li, L., et al., 2013. Cytotoxicity of different sized TiO_2 nanoparticles in mouse macrophages. Toxicol. Ind. Health 29 (6), 523–533.

Zhang, D., Zhang, Z., Liu, Y., Chu, M., Yang, C., Li, W., et al., 2015. The short- and long-term effects of orally administered high-dose reduced graphene oxide nanosheets on mouse behaviors. Biomaterials 68, 100–113.

Zheng, G., Chen, J., Li, H., Glickson, J.D., 2005. Rerouting lipoprotein nanoparticles to selected alternate receptors for the targeted delivery of cancer diagnostic and therapeutic agents. Proc. Natl. Acad. Sci. USA 102 (49), 17757–17762.

Zheng, W., McKinney, W., Kashon, M., Salmen, R., Kan, H., Castranova, V., Kan, H., 2016. The influence of inhaled multi-walled carbon nanotubes on the autonomic nervous system. Part. Fibre Toxicol. 13 (1), 8.

Zhu, M.-T., Feng, W.-Y., Wang, Y., Wang, B., Wang, M., Ouyang, H., et al., 2009. Particokinetics and extrapulmonary translocation of intratracheally instilled ferric oxide nanoparticles in rats and the potential health risk assessment. Toxicol. Sci. 107 (2), 342–351.

Zieminska, E., Stafiej, A., Struzynska, L., 2014. The role of the glutamatergic NMDA receptor in nanosilver-evoked neurotoxicity in primary cultures of cerebellar granule cells. Toxicology 315, 38–48.

CHAPTER

13

Immune System

Diana Boraschi, Bengt Fadeel**, Albert Duschl†*

*Institute of Protein Biochemistry of the National Research Council, Napoli, Italy;
**Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden;
†University of Salzburg, Salzburg, Austria

OUTLINE

Introduction 313
Innate Immune Responses 315
The Immune System Strikes Back 320
Complement Activation 321
Adaptive Immune Responses 322
Nanomaterials and Allergies 324
Immunosuppression by Nanomaterials 326
Definitely Not Innocent Bystanders 328
Turning the Tables: Biomedical Applications 329
Take-Home Messages 330
Acknowledgments 331
References 331

INTRODUCTION

This chapter deals with adverse effects of nanomaterials on the immune system. We shall describe both general and specific effects of nanoparticles on the immune system. The immune system can be considered as a disseminated organ that is present throughout the body. Professional immune cells, such as T cells and B cells, act specifically in the immune system, but "nonprofessional" cells can also play important roles, for example, by producing factors that stimulate local inflammation. Immunity, therefore, encompasses essentially all tissues and organs.

However, particular attention will be paid to immune cells present in peripheral blood. The blood is the environment in which many professional immune cells reside in their resting states and from which they migrate to sites of infection. Blood is also the compartment

Adverse Effects of Engineered Nanomaterials. http://dx.doi.org/10.1016/B978-0-12-809199-9.00013-6
Copyright © 2017 Elsevier Inc. All rights reserved.

that nanoparticles reach once they enter into the body and have crossed biological barriers, such as the air–blood barrier in the lungs. In addition, blood from human subjects is the main source of immune cells available for study, so from a practical point of view, when considering general immune responses, the literature on immune cells derived from blood serve as a good starting point.

Giving special attention to the immune system is well justified in the context of nanosafety (Shvedova et al., 2010). Immune cells are enriched close to body surfaces (e.g., in skin, airways, gastrointestinal tract), where pathogenic microorganisms, but also nanomaterials, are most likely to enter the body. Immunity is specialized to recognize nonself entities and to distinguish whether they pose a threat to the integrity of the organism. Furthermore, evaluating the risk posed by foreign or engineered nanoparticles is, in a nutshell, the core task of nanosafety research. Understanding how the immune system interacts with nanoparticles is also important for nanomedicine, since immunotoxicity may limit certain applications, while some nanodrugs target immune functions (Dobrovolskaia et al., 2016). Of course, even professional cells of the immune system may sometimes overreact or react erroneously, as in the case of allergies, or in the case of autoimmune diseases. Understanding whether nanoparticles are perceived as a threat and whether exposure to these materials leads to immune activation or immunosuppression is of critical importance (Dobrovolskaia et al., 2016; Ilinskaya and Dobrovolskaia, 2016).

We are aware of immunity mostly when the immune system battles an infection, but in reality the most common outcome of encounters with nonself entities is *tolerance*. Most entities surrounding us, even most bacteria, are harmless, and ignoring them is the correct response. The immune system is specialized to fight off pathogenic viruses, microorganisms, and parasites. If the immune system mounts a defense reaction against entities that are not pathogens, as in the case of allergies and autoimmunity, diseases may ensue. The question is whether nanoparticles are viewed by the immune system as dangerous pathogens or harmless. Toxicity, in general, certainly has a connection to immunity, since wound healing is the final stage of an inflammation, and inflammation with its tissue-reconstruction activity may be required after toxic agents have damaged a tissue. In addition, activation of cellular stress mechanisms may occur if nanoparticles do act as stressors. However, full-blown immune responses like those involving inflammation, complement activation or antibody production may not be useful for getting rid of nanoparticles, while they could be detrimental to the integrity of the organism itself, so the induction of tolerance is presumably more appropriate. It should be noted that tolerance is not the same as failure to recognize nonself. The induction of tolerance is an active process and is reinforced by immune mechanisms preventing future response, which include making reactive T cells permanently unresponsive (anergic) (Chappert and Schwartz, 2010) or inducing regulatory T cells (Treg), which maintain tolerance by secreting immunosuppressive cytokines (Sakaguchi, 2011). Furthermore, "activation" of the immune system is strictly speaking not an unambiguous indicator of danger. If the activation leads, for example, to induction of Treg, immune activation indicates that the agent has been perceived as harmless. The induction of defensive reactions like inflammation, however, does indicate that the agent has been perceived as potentially dangerous. In most cases, the defensive reaction is successful, that is, the immune system rapidly and successfully eliminates the potentially dangerous agents and reestablishes tissue homeostasis, thereby preserving the physical and functional integrity of the tissue. Thus, in the context of nanosafety assessment,

it is of paramount importance to consider that inflammatory parameters [reactive oxygen species (ROS) production, production of inflammatory cytokines, complement activation, etc.] are not definitive markers of nanotoxicity, but they simply indicate that the immune system has initiated a defensive reaction. Thus, the presence of inflammatory markers does not allow us to distinguish a toxic effect/pathological reaction from a normal and successful immune response. The key elements in the identification of a nanoparticle-induced immunological hazard are in the duration of an inflammatory reaction (a successful inflammation resolves rapidly and reestablishes homeostasis, while a pathological inflammation can become chronic), and in its deregulation (e.g., excessive production/activation of inflammatory factors versus impaired or anomalous activation of antiinflammatory and repair mechanisms). Thus, it is obvious that reliable and informative immunotoxicity testing requires relevant in vivo and in vitro models, which can distinguish between normal and prepathological immune responses. In the following sections, we discuss the innate and adaptive immune responses, and how nanoparticles may interact with immune cells.

INNATE IMMUNE RESPONSES

Innate immunity or inflammation (the two terms are almost synonymous) is the earliest defense mechanism of the body. At variance with adaptive immunity, which develops only after a first encounter with the pathogen, inflammation is an immediate response that is practically always ready to come into action and does not need to "learn." The advantages of innate reactions are in their rapidity and potency, which is life-saving in most circumstances, while the disadvantage is in their limited specificity. The major side effects of innate immunity/inflammation are indeed due to its potency and its lack of specificity, as the inflammatory reaction, in addition to killing pathogens, can cause unwanted damage to the body itself. The concluding steps of the inflammatory response, once the danger is eliminated, encompass a complex series of reactions that restore the physical and functional integrity of the damaged tissue (tissue repair and remodeling, with cell proliferation and angiogenesis) (Fig. 13.1). Danger signals are the best inducers of inflammation. These signals may either derive from pathogens (pathogen associated molecular patterns, or PAMPs) or from the body itself, following necrotic cell death (danger associated molecular patterns, or DAMPs). Numerous danger signals have a hydrophobic character, so it not surprising that increased hydrophobicity makes nanoparticles stronger inducers of inflammation (Moyano et al., 2012; Shima et al., 2015). In fact, it has been proposed that nanoparticles, or nanoparticles coated with proteins, may be seen as nanomaterial associated molecular patterns or NAMPs (Farrera and Fadeel, 2015).

Each phase of the inflammatory response is an integral part of the overall defense reaction, and is finely regulated in order to avoid pathological derangements. Variations in the timing, amplitude, and duration of the various phases may transform the defensive response into a pathological reaction. This can happen, for example, in the case of persistence of the inflammatory stimuli, which leads to a persistent inflammatory reaction that would cause the unresolved destruction of the surrounding tissues. Indeed, in most chronic inflammatory diseases, for instance, in rheumatoid arthritis, the chronic inflammatory reaction is accompanied by a chronic anomalous resolution phase, in which the body tries to shutdown inflammation and

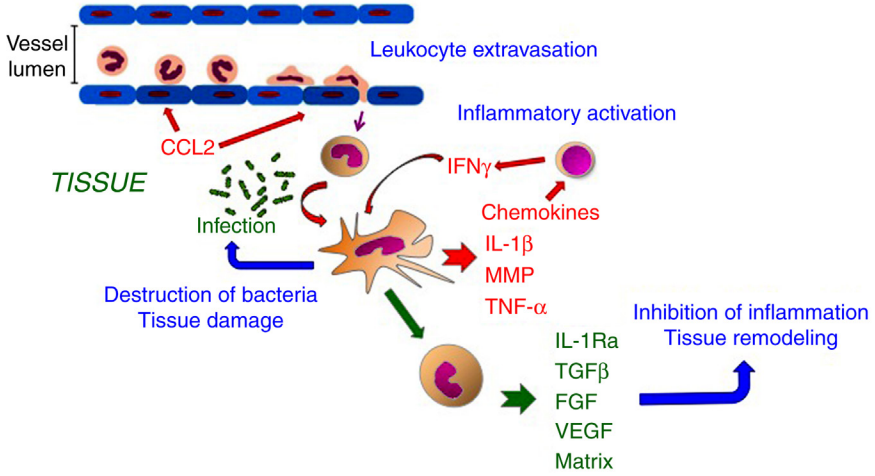


FIGURE 13.1 The development of the innate/inflammatory defense reaction. A tissue injury, such as an infection leads to the release of chemotactic factors (e.g., CCL2) that recruit monocytes from the circulation to the tissues, where they meet the infectious agents and become activated to produce inflammatory factors. These are both involved in pathogen destruction and in recruiting other cells that amplify the defensive response (e.g., T lymphocytes that produce IFN-γ). Upon elimination of danger, monocytes stop producing inflammatory factors, and contribute to tissue repair and remodeling by producing angiogenic and proliferation-inducing factors.

reconstruct the damaged tissue without succeeding. Tissue (pannus) formation in parallel to bone and cartilage destruction is the result. Some types of nanoparticles may have the characteristics of a persistent foreign stimulus, as they could cause permanent inflammatory activation when they are made of materials that cannot be digested by the scavenger cells. The inflammatory effects of nanoparticles have been described in several instances, both in vivo and in vitro (Castranova et al., 2013; Nagai and Toyokuni, 2010). However, since the size of nanoparticles is such that they may easily escape the particle-clearing defensive mechanisms (e.g., phagocytosis), it is not surprising that in many cases nanomaterials do not trigger a direct inflammatory response (Kunzmann et al., 2011). Inflammatory effects that have been observed by many researchers may have been caused by concurring events. For instance, the different state of aggregation or agglomeration of nanoparticles upon contact with the biological environment may change the size of the entities coming in contact with the immune cells, which tend to recognize larger particles (>0.5 μm) more effectively and readily clear or sequester them, with less pathological/toxic consequences as compared to the smaller particles (Geiser, 2010; Groves et al., 2008). In addition, inflammation may be due to molecules, such as bacterial components adsorbed to the nanomaterial rather than by the nanomaterial itself.

It is generally accepted that nanoparticles entering the body are rapidly coated with proteins and other biomolecules, forming a biocorona on the surface of the nanoparticle, and that this interaction can cause changes both in the proteins and in the particles (discussed in detail in Chapter 1). It is known that the particulate shape of an antigen (as in the case of microorganisms or complex antigens as opposed to haptens) allows much better recognition by dendritic cells (DCs), which can then process and present it, thereby initiating an immune response. If this happens with self-proteins (i.e., autologous proteins that are adsorbed onto

nanoparticles and display altered characteristics upon interaction with the nanoparticle surface), then an autoimmune response may ensue. Thus, the alteration which may be caused to self proteins by the interaction with nanoparticles and the persistence of these new entities may provide several of the conditions for the initiation of chronic and autoimmune pathologies, that is, recognition of self antigens and persistent stimulation.

Another factor contributing to the development of autoimmunity is the defective clearance of apoptotic cells by scavenger phagocytes (Munoz et al., 2010). Although there is no direct evidence of a role for nanoparticles in the pathogenesis of autoimmune diseases, it should be noted that human macrophages exposed to carbon nanotubes have impaired capacity for clearance of apoptotic cells in vitro (Witasp et al., 2009), in spite of the low cytotoxicity of the nanotubes. Similarly, nanoparticle loaded macrophages were shown to have a diminished capacity for phagocytosis of certain pathogens (Kodali et al., 2013). Nonetheless, autoimmune diseases are complex diseases, and multiple risk factors contribute to the pathogenesis (genetic, environmental, nutritional, stress, inflammation, infection). Further studies are required to assess whether or not any specific types of nanoparticles, and specifically whether nanoparticle-induced inflammation, may be among the risk factors.

The inflammasome is a cytosolic protein complex in macrophages and other myeloid cells that detects infection and stress-associated signals and promotes inflammatory responses through activation of caspase-1 which in turns initiates the processing of pro-IL-1β leading to secretion of proinflammatory IL-1β (Franchi et al., 2009; Sutterwala et al., 2007) (Fig. 13.2). Inflammatory caspases and inflammasomes have been described as the master switches of inflammation (Martinon and Tschopp, 2007). In addition, the activation of the NLRP3 inflammasome can also lead to caspase-1-dependent cell death of macrophages, a phenomenon called pyroptosis. The inflammasome activation requires signals from Toll-like receptors (TLR), localized in the plasma membrane or in endosomal/lysosomal membranes, or from cytoplasmic NOD-like receptors (NLR) and RIG-like receptors (RLR). These receptor classes, which belong to the so-called pattern recognition receptors (PRR), recognize a wide range of bacterial and viral compounds. TLRs are often the first receptors coming in contact with foreign agents (due to their location on the plasma membrane) and also able to recognize some self-compounds, such as heat shock proteins and defensins, which act as "alarmins" or danger signals. A variety of molecules can induce NLRP3 inflammasome activation, either independently of receptor signals or as part of the receptor-mediated activation pathway, including ROS (Martinon, 2010; Shvedova et al., 2005). It is known that inflammasome activation can be triggered by naturally occurring particles produced within the body, such as cholesterol or uric acid crystals. Thus, it is reasonable to assume that the mechanisms that are used in response to uric acid crystals may be able to respond to synthetic nanosized particles as well. Several mechanisms can be considered. Rupture of lysosomes, as shown for alum and silica particles, leads to release of the protease cathepsin B, which has been proposed to mediate inflammasome activation (Hornung et al., 2008). Furthermore, a drop in cytosolic K^+ was reported to be the common step that is necessary and sufficient for caspase-1 activation in response to bacterial toxins and particulate matter (Muñoz-Planillo et al., 2013) and it will be of interest to understand whether this also plays a general role in nanoparticle-triggered inflammasome activation. Direct recognition of nanoparticles by TLRs remains possible and may not even require uptake of nanoparticles into cells, since several members of the TLR family are localized in the plasma membrane. However, binding of nanoparticles to extracellular

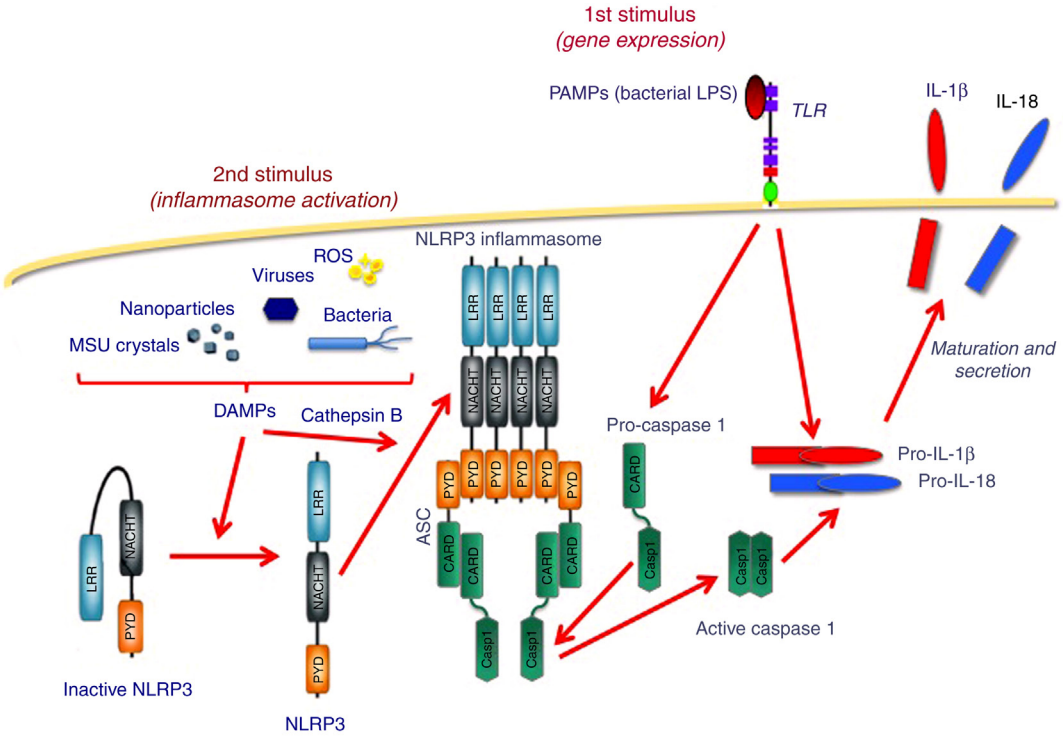


FIGURE 13.2 **Nanoparticles and inflammasome activation.** Release of active inflammatory cytokines IL-1β and IL-18 is consequence of a first stimulus (usually TLR activation) that upregulates gene expression and synthesis of the proforms of cytokines and caspase-1. Inflammasome activation depends on different stimuli, including intracellular nanoparticles, that can activate the inactive form of an NLR (here the NLRP3 molecule) leading to the formation of the inflammasome that matures and activates procaspase 1. Active caspase 1 cleaves inactive pro-IL-1β and pro-IL-18 and promotes the extracellular release of the active cytokines.

TLRs would by itself not be sufficient to induce an inflammatory response, since it is believed that more than one signal is required for inflammasome activation. Hence, two signals are usually required for inflammasome activation in vitro; the additional signal (often referred to as signal 1) is often furnished by adding a defined amount of endotoxin or LPS. However, it is noted that endotoxin is unlikely to play a major role in vivo. In a recent study, extracellular high-mobility group box 1 (HMGB1)—a known danger signal—was shown to act as signal 1 and neutralization of HMGB1 reduced carbon nanotube-induced IL-1β secretion in mice (Jessop and Holian, 2015). Overall, the discovery that nanoparticles are able to activate the inflammasome implies that common pathways are deployed by the innate immune system in response to a range of different stimuli, including not only microorganisms, and endogenous crystal or fibre-like materials, but also to exogenous nanoparticles of various composition and shapes.

Neutrophils or polymorphonuclear granulocytes are granular leukocytes that play a key role in inflammation (Bhattacharya et al., 2013). They are the most short-lived cells in the

bloodstream. The turnover of neutrophils is tremendous; it has been estimated that more than half of the hematopoietic activity in the bone marrow is directed toward neutrophil production. Neutrophils are specialized in the phagocytosis of bacteria and other pathogens and are capable of phagocytosis on their own (i.e., without the participation of any other immune mechanism) or through antibody- or complement-mediated opsonization of pathogens. Neutrophils are equipped with oxidative and proteolytic enzymes for the destruction of ingested pathogens. Neutrophils also release so-called neutrophil extracellular traps or NETs that contribute to the defense against extracellular bacterial and fungal pathogens (Brinkmann et al., 2004). NETs consist of chromatin fibers decorated with antimicrobial proteins, including neutrophil elastase (NE) and myeloperoxidase (MPO). Neutrophils are known to sense microbe size and to selectively release NETs in response to large pathogens, such as *Candida albicans*, but not in response to small yeast or single bacteria (Branzk et al., 2014). Size-tailored neutrophil responses are thus utilized to clear pathogens. Interestingly, several types of nanoparticles, including carbon nanotubes, have been shown to be captured in NETs, at least in vitro (Farrera et al., 2014; Hwang et al., 2015). There is some evidence for inflammasome activation also in neutrophils. However, in contrast to macrophages, the NLRP3 inflammasome in neutrophils responds to soluble, but not to particulate or crystalline agonists (Chen et al., 2016a), underscoring that myeloid cell identity, and the nature of the danger signal, can strongly influence signaling by the same PRR.

Neutrophils are key players in inflammation and numerous studies have documented increased numbers of neutrophils in the lungs of animals exposed to nanomaterials (Shvedova et al., 2012). In one of the earliest studies performed on carbon nanotubes, an early neutrophil accumulation (day 1), followed by a sequential appearance of lymphocytes (day 3) and macrophages (day 7), was demonstrated in mice after pharyngeal aspiration of single-walled carbon nanotubes (SWCNT) and this was accompanied by early elevation of proinflammatory cytokines (TNF-α, IL-1β; day 1) followed by elevated levels of the profibrogenic cytokine TGF-β1 (peaked on day 7). Functional respiratory deficiencies and decreased bacterial clearance (*Listeria monocytogenes*) were also noted in mice exposed to SWCNT (Shvedova et al., 2005). In another illustrative study, a panel of nanoparticles, including cerium oxide (CeO_2), titanium dioxide (TiO_2), carbon black (CB), silicon dioxide (SiO_2), nickel oxide (NiO), zinc oxide (ZnO), copper oxide (CuO) nanoparticles, was instilled into the lungs of rats. Only the CeO_2, NiO, ZnO, and CuO nanoparticles triggered inflammation. Furthermore, each of these nanoparticles was found to induce a unique inflammatory "footprint", both acutely (24 h) and chronically (at 4 weeks). Acutely, the patterns of neutrophil and eosinophil infiltrates differed for the CeO_2, NiO, ZnO, and CuO nanoparticles. Chronic inflammatory responses also differed, with neutrophilic, neutrophilic/lymphocytic, eosinophilic/fibrotic/granulomatous, and fibrotic/granulomatous inflammation being caused, respectively, by the CeO_2, NiO, ZnO, and CuO nanoparticles (Cho et al., 2010). These in vivo studies imply that different metal/metal oxide nanoparticles have different risk profiles, but also remind us that inflammation is not a static response (the response may resolve once the harmful agent has been dealt with). Granuloma formation and fibrosis, on the other hand, certainly indicates that a more progressive and chronic response has occurred in response to the nanoparticles. Recent studies in mice have implied that alveolar epithelial cells (also a component of the innate immune system in the lung) and not alveolar macrophages might be the drivers of acute neutrophilic inflammation upon exposure to carbonaceous nanoparticles (Chen et al., 2016b).

Mast cells also belong to the innate immune system and have a function in host defense against parasites, tissue repair, and angiogenesis. Mast cells are activated by a variety of stimuli through various receptors including receptors for IgE. Once activated, the mast cell produces histamine, leukotrienes, proteases, cytokines, chemokines, and other substances that cause airway inflammation. IL-33 was identified as a danger signal released by necrotic cells capable of activating mast cells, thus suggesting a role for mast cells as sensors of cell injury and recent studies have provided evidence for a role of IL-33 and its receptor (ST2) in pulmonary and cardiovascular responses to multiwalled carbon nanotubes (MWCNT) (Katwa et al., 2012). Furthermore, pharyngeal instillation of MWCNT in mice resulted in elevated levels of IL-33 and enhanced airway hyper-reactivity (AHR), and recruitment of so-called innate lymphoid cells to the airways (Beamer et al., 2013). The authors proposed a model in which innate lymphoid cells, acting in response to IL-33, stimulated AHR and eosinophil recruitment through the release of IL-13. Altogether, these studies illustrate the intricate orchestration of different inflammatory cell types.

Finally, a very interesting aspect of the mechanism of inflammation, in which nanoparticles might have a role, is the recently revamped concept of "innate memory." The concept is based on a wealth of old evidence that also innate immune cells, such as macrophages can retain memory of previous exposure thereby responding differently to subsequent challenges with the same or unrelated agents. Innate memory can be broadly defined as induction of tolerance, of which the best studied is the endotoxin tolerance (Biswas and Lopez-Collazo, 2009), and the induction of enhanced response, or "trained immunity" (Netea et al., 2011). Based on this concept, strategies are being designed for improving resistance to infections and diseases by targeting the memory capacity of innate immunity. While this is presently being attempted with the use of particulate vaccines (Toepfer et al., 2015), these findings open the way to the controlled use of nanoparticles for modulating innate memory in disease prevention.

THE IMMUNE SYSTEM STRIKES BACK

There has been a widespread concern that certain carbon nanotubes may exhibit "asbestos-like" pathogenicity, in part due to the fiber-like morphology (Poland et al., 2008), but also due to the assumption that CNTs are biopersistent, like asbestos fibers. However, several groups have reported in recent years that carbonaceous nanoparticles (fullerenes, SWCNTs, MWCNTs, and graphene oxide or GO) may undergo enzymatic biodegradation (Bhattacharya et al., 2016). These observations suggest that the potentially detrimental effects of these materials may be mitigated thereby allowing these materials to be applied in a safe manner, if degradation is controlled. Indeed, these data suggest that the innate immune system can "strike back" and also imply that similar strategies are deployed to destroy microorganisms and nanomaterials (at least as shown for carbon-based nanoparticles).

The first proof-of-principle studies on enzymatic degradation of carbon nanotubes were performed using the plant-derived enzyme, horseradish peroxidase (Allen et al., 2008, 2009). In subsequent studies, neutrophil MPO and eosinophil peroxidase were both shown to "digest" carbon nanotubes and in a more recent study, lactoperoxidase, a secreted enzyme in the airways, was also shown to be capable of enzymatic degradation of SWCNTs (Andón et al., 2013; Bhattacharya et al., 2015; Kagan et al., 2010). Thus, while neutrophils

and eosinophils are capable of efficient biodegradation of SWCNTs, these cells—not least neutrophils—are short-lived with a lifespan of days. In contrast, macrophages may persist over weeks of chronic inflammation elicited, for instance, by pulmonary exposure to SWCNTs (Shvedova et al., 2005). Indeed, macrophages have emerged as a likely candidate cell type involved in the clearance of CNTs from the lungs, but these cells express relatively low levels of MPO in comparison to neutrophils and a different mechanism has been invoked for degradation of nanotubes, involving a superoxide/peroxynitrite oxidative pathway (Elgrabli et al., 2015; Kagan et al., 2014).

COMPLEMENT ACTIVATION

The complement system encompasses a group of serum proteins that, in a cascade of events, interact with each other as well as with antibodies and with immune cells. Complement is an important part of the immune system and can "tag" pathogens in a process called opsonization. Opsonized pathogens are quickly attacked by antibodies and eliminated by phagocytic immune cells. In addition, complement can induce immune attack on pathological cells (e.g., virus-infected cells and tumor cells), and it can also by itself destroy the body's own cells. Complement is activated via relatively unspecific mechanisms and it has the ability to severely damage body tissues. For this reason, complement activities need to be tightly controlled and any complement activating effect of nanoparticles (not least nanomedicines) should be ruled out.

There are three main ways for microorganisms to stimulate complement activation, the "classical," the "lectin," and the "alternative" pathway, all of which can be activated by SWCNTs and MWCNTs (Salvador-Morales et al., 2006). Thus, it appears that nanomaterials may be recognized and treated like a pathogenic microorganism by the immune system. A number of nanoparticle types have now been reported to activate complement, and charged nanomaterials appear to be more efficient activators of the complement system compared to their neutral counterparts (Dobrovolskaia et al., 2008, 2009).

Appropriate coating seems to be the method of choice for eliminating unwanted complement activation properties of nanoparticles, even though it may be difficult to produce nanomaterials with sufficient solubility in blood without incurring the risks of recognition by complement factors (Sim and Wallis, 2011). Lipid nanocapsules displayed a very low capacity of complement activation when they contain polyethylene glycol at high density (Mosqueira et al., 2001; Vonarbourg et al., 2006). Apart from the material used for coating, its density may have surprising effects. Block copolymer polyaxamine 908 can lead to reduced complement activation when used for coating (Al-Hanbali et al., 2006), but more recent investigations showed that different complement activation pathways were triggered depending on the density of the polymers (Hamad et al., 2010). The need to minimize complement activation is particularly relevant for nanomedicine, since delivery of large amounts of nanoparticles into the blood of patients may pose a direct risk of adverse reactions mediated by complement. It was shown that hypersensitivity reactions toward Doxil (a liposome-encapsulated doxorubicin formulation used in cancer chemotherapy) were due to complement activation (Chanan-Khan et al., 2003). In the area of nanomedicine, scientists are working since many years to develop coatings that mask nanoparticles from complement recognition and attack,

including the use of complement inhibitors, such as heparin or the modulation of the surface characteristics (Inturi et al., 2015; Passirani et al., 1998). As already mentioned, avoiding complement activation has indeed a dual advantage, that is, avoiding possible adverse effects including shock caused by activated complement, and avoiding rapid removal of the nanoparticles by phagocytic cells due to opsonization with complement components.

ADAPTIVE IMMUNE RESPONSES

There is extensive literature on dendritic cells (DCs) and nanoparticles, from both the immunology and nanomedicine fields. DCs are the major antigen-presenting cells in the body. Typically, these cells are in a resting state in tissues close to body surfaces, where they become activated upon contact with nonself materials perceived as dangerous, migrate to local lymph nodes and interact there with specific T cells. B cells, also enriched in lymph nodes, need to interact both with T cells and with DCs to produce antibodies that mediate major mechanisms for attacking pathogens. The DC-T-B axis describes the normal mechanism of adaptive immune activation and it immediately suggests an application for nanoparticles: they are nonself and can even be modified to boost immune responses, so they may be useful for vaccination where a strong immune response is desired. Nanoparticles coated with either protein antigens or with antigenic DNA have both the potential to act as adjuvants, by stimulation a localized inflammatory reaction that enhances the induction of specific immunity, and, by acting as delivery vehicles that facilitate efficient uptake of the vaccine antigens. Cancer antigens have been a particular focus of the field, since it is usually difficult to raise an efficient immune response against them. For reviews on the application of nanoparticles for vaccine delivery, see Boraschi and Italiani (2015); De Koker et al. (2011); Xiang et al. (2010).

It is clear that nanomedicine requires a thorough understanding of toxicological properties of nanoparticles used for diagnosis or therapy (Fadeel and Garcia-Bennett, 2010). A striking example of important insights for nanosafety questions derived from medicine is the finding that nanosized alum particles act by stimulating the NLRP3 inflammasome, probably through intracellular induction of ROS (Eisenbarth et al., 2008; Li et al., 2008a) (Fig. 13.2). The mechanism of the adjuvant alum, which has been in clinical use for a long time, was found to depend on a specific proinflammatory signaling pathway in antigen presenting cells. The ability of some nanoparticles to rupture lysosomal membranes allows them to access the cytoplasmic compartment. This behavior can be exploited in vaccination regimes for induction of optimal vaccine efficacy, since uptake of antigen-carrying nanoparticles in lysosomes would allow MHC-II dependent antigen presentation, while lysosomal destabilization and release of the antigen in the cytoplasmic compartment will induce MHC-I cross-presentation, a type of immunity of particular efficacy against viruses and tumors (Firdous et al., 2014). Lysosomal rupture would also allow nanoparticles to activate the inflammasome (discussed earlier), thereby acting as adjuvants, but also as danger signals (Hornung et al., 2008).

The most crucial function of DCs is to take up protein antigens, process them, and present to T cells, bound to their MHC molecules, the peptides derived from these nonself proteins. The interaction between MHC/peptide complexes and the T cell receptor can stimulate the activation of the T cell, leading to an antigen-specific immune response. The uptake, processing

and presentation of proteins may in principle be subjected to interference by nanoparticles present on the surface of or within the antigen-presenting cell, but there is another possibility related to the so-called bio-corona. Proteins are known to readily attach to nanoparticles, and the impact of this coating on the nanoparticle fate and distribution and subsequent biological activities has received significant interest. Indeed, experiments performed by mixing nanoparticles with human plasma have shown that the bio-corona forms very rapidly (<1 min) (Tenzer et al., 2013). Numerous in vitro studies have shown that the composition of the cell culture medium and percentage of animal serum determines adsorption of different amounts and types of proteins to nanoparticles (Maiorano et al., 2010; Monopoli et al., 2011; Saptarshi et al., 2013), and that the protein coating evolves over time from a "soft" layer which can be removed by centrifugation to a "hard" shell, which is firmly attached (Casals et al., 2010; Dell'Orco et al., 2010). Even though in vivo experiments are more challenging, a recent study succeeded in comparing the in vivo and in vitro formed protein coronas on clinically relevant lipid nanoparticles in terms of morphology and composition, and found that while the total amount of protein attached on circulating liposomes correlated with that observed from in vitro incubations, the range of molecular species present in the in vivo corona was greater (Hadjidemetriou et al., 2015). It also seemed that the in vivo formed corona did not coat the liposome surface entirely. Notwithstanding, corona formation seems inevitable, and therefore, when cells (for instance, phagocytes) get into contact with nanoparticles, they will not see a pristine nanoparticle, but rather a nanoparticle-protein complex (discussed in detail in Chapter 1). This has profound consequences both for the properties of the nanomaterials and for the cellular responses toward them. The corona of human plasma proteins was found to impact on hemolysis (lysis of red blood cells), thrombocyte activation, and cellular uptake and toxicity in endothelial cells (Tenzer et al., 2013). In addition, it is important to also consider the formation of different protein coronas in different biological compartments. Indeed, while most studies to date have focused on the blood, the corona that is formed in the lungs upon inhalation of nanoparticles may differ markedly. In a recent study, the corona of nanoparticles that had transited through respiratory tract lining fluid was determined and an enrichment for innate immunity proteins, including complement factors, was noted (Kumar et al., 2016). Functional protein classification supported the hypothesis that the corona formation constituted opsonization, preparing particles for phagocytosis and clearance from the lungs.

It is noted that polymer coating of nanoparticles, a popular strategy in nanomedicine, does not necessarily prevent corona formation; in fact, recent studies have shown that the adsorption of certain proteins (presumably acting as "dysopsonins" or in some way masking the particles) was required for the "stealthiness" of poly(ethylene glycol) coated polystyrene nanoparticles (Schöttler et al., 2016).

The consequences of this interaction for the proteins involved are less clear. The formation of a "hard" protein shell depends on nanoparticle–protein and on protein–protein interactions, which contribute to a dense packing of proteins in a single layer (Casals et al., 2010; Rocker et al., 2009). Bovine serum albumin bound to dextran-poly(isobutylcyanoacrylate) nanoparticles was shown to exist in a specific configuration (Vauthier et al., 2009). In the case of self proteins (e.g., serum proteins in the case of intravenous nanomedicines), alterations in the protein folding or aggregation state due to protein adsorption on the nanoparticle surface can induce uptake and processing of the self proteins by the antigen presenting

cells, in particular macrophages (which have the role of detecting and eliminating misfolded proteins), and could ultimately lead the induction of an immune response against self antigens. In the case of proteins delivered with nanocarriers for vaccination, it is known that small posttranslational modifications of proteins can have a strong impact on the peptides presented (Hill et al., 2003; Moss et al., 2005; Qiao et al., 2005; Wallberg et al., 2007), implying that protein alterations due to adsorption on a nanoparticle surface may lead to qualitative and quantitative differences in peptide presentation to T cells and type of subsequent immune response. A recent study used nanoparticles composed of poly(γ-glutamic acid) functionalized with different hydrophobic segments (Shima et al., 2015). Hydrophobicity increased particle/antigen uptake, as expected, but this could be modulated. Mixing hydrophobic nanoparticles with the model antigen ovalbumin had a significant effect in inducing humoral immunity (possibly MHC II-dependent), while encapsulation of ovalbumin within the nanoparticles induced a much stronger cellular response, presumably MHC I-dependent (Shima et al., 2015). The fibre-shaped protein fibrinogen, when bound to negatively charged poly(acrylic acid)-conjugated gold nanoparticles, becomes partially unfolded and is able to interact with the integrin receptor Mac-1, inducing inflammatory cytokine secretion, while the particles alone or fibrinogen alone did not trigger such effects, suggesting a true "corona" effect (Deng et al., 2011). Subsequent studies from the same group showed that this effect is dependent on the size of the particles (Deng et al., 2013). Similar unfolding effects have been described for the globular protein albumin bound to layered silicate nanoparticles. Upon formation of a hard corona on the surface of the nanomaterial, an epitope of albumin is revealed, which usually forms during heat denaturation, and which binds to scavenger receptors on macrophages thereby inducing uptake (Mortimer et al., 2014). These examples are in line with the basic principle that unfolded proteins are recognized as "altered" by the immune system. An experimental approach to photothermal anticancer therapy has used gold nanorods to induce a localized partial unfolding of collagen under heat, so as to make collagen a better target for fibronectin-decorated drug-loaded nanoparticles (Lo et al., 2013), which shows that this seemingly random effect could be harnessed for specific (clinical) applications.

These observations lead to the question as to whether nanoparticle-corona complexes can elicit the induction of antibodies. Nanoparticles are, in general, not believed to be antigenic (Ilinskaya and Dobrovolskaia, 2016). However, structural changes following adsorption of proteins to nanoparticles may reveal "cryptic" epitopes (Lynch et al., 2006), either by different processing in DCs or by production of a different antibody repertoire. In addition to the potential recognition of antigenic nanoparticles or nanoparticle-associated molecules, antigen-independent effects of antibodies on nanoparticles may be observed. Both IgG antibodies and fibrinogen were shown to modify aggregation of polystyrene nanoparticles, most likely by adsorbing on the nanoparticle surface, with low concentrations leading to high aggregation, while high concentrations reduced the aggregation (Cukalevski et al., 2015).

NANOMATERIALS AND ALLERGIES

The rise in allergic diseases observed in association with a "Western lifestyle" during the last century (Heinrich, 2011; Holgate and Lack, 2005) has prompted an intensive search for causes. One factor suspected to contribute to the dramatic rise in allergies is environmental

pollution, since combustion-derived particles and ultrafine particles are associated with inhaling lung diseases, including allergy and asthma (Nel et al., 1998; van Zijverden and Granum, 2000). This history has led to two lasting legacies in the field of nanosafety research: (1) due to the overlap in size, combustion-derived ultrafine particles and engineered nanoparticles are often discussed together, since at least size-dependent effects may be similar in both types of materials (Oberdörster et al., 2005), and (2) the paradigm of toxicity mediated by oxidative stress, originally developed for combustion-generated particles, has become an important consideration for engineered nanoparticles as well (Li et al., 2008b) (for more details on pulmonary responses to nanomaterials, see Chapters 9 and 10). There are few indications that engineered nanoparticles can misdirect immunity specifically toward T_H2 responses, which are associated with type 1 allergies, such as hay fever, animal hair allergy, house dust mite allergy, or allergic asthma. Some studies in mouse models, however, have reported an enhancement of allergic parameters induced by nanomaterials. Nanosized TiO_2 increased allergic lung inflammation, T_H2 cytokine production and IgE levels in mice sensitized to ovalbumin (Larsen et al., 2010; Rossi et al., 2010), and enhanced allergy to the same allergen was also reported following exposure to SWCNT and MWCNT (Nygaard et al., 2009). On the other hand, loading the major birch pollen allergen Bet v 1 onto nanosized poly(D,L-lactic-*co*-glycolic acid) (PLGA) reduced allergy parameters in previously Bet v 1-sensitized mice, as compared to exposure to Bet v 1 alone (Scholl et al., 2004). Studies on allergic mice are usually done using the BALB/c strain, which readily develops allergy, with ovalbumin (OVA) being the most widely used allergen. However, BALB/c mice are so extremely prone to (allergic) T_H2 responses that an increased response may simply indicate increased immune activation, which in another mouse might manifest in different form, in particular as T_H1 inflammation. Mouse models are nevertheless the in vivo system of choice for developing antiallergic therapies.

The only clinically available causal therapies for allergy are various regimens of specific immunotherapy, in which large and increasing doses of allergen are applied to desensitize the patient. Numerous studies have reported the use of nanoparticles to improve therapeutic success in mouse models (Gomez et al., 2009; Licciardi et al., 2014; Nouri et al., 2015; Pali-Scholl et al., 2013; Salem, 2014). While these studies as well as clinical treatments usually involve injection of allergens, PLGA-encapsulated allergen was also effective in a mouse model of sublingual application (Salari et al., 2015). This form of application is also available for humans. Thus, nanomedical formulations may offer improved therapeutic success for allergy patients.

Recently, it was reported that allergens noncovalently bound to gold nanoparticles elicit different reactivity in blood cells from allergy patients depending on the allergen used (Radauer-Preiml et al., 2016). For the grass pollen allergen Php p 5, there was no difference between free allergen and allergen bound to nanoparticles. For the birch pollen allergen Bet v 1, responses increased or decreased for different patients, which can be explained assuming nonrandom binding to the particles surface, since some allergic epitopes may be shielded while others are exposed. For the house dust mite allergen Der p 1, the response was consistently increased. Of note, Der p 1 is a protease. Binding to nanoparticles increased the proteolytic activity and led to stronger lung epithelial layer disruption in an in vitro model (Radauer-Preiml et al., 2016). These findings suggest that exposure to allergens presented on the surface of nanoparticles could be a possible risk factor for sensitive persons.

On the other hand, antiallergic effects of silver nanoparticles have been described after treatment of OVA-sensitized allergic C57BL/6 mice with nanosized silver particles (Park et al., 2010); and calcium carbonate or calcium phosphate nanoparticles were reported to reduce nickel allergy by binding nickel ions (Vemula et al., 2011). It should be noted that nickel allergy is based on completely different mechanisms than most common allergies, and neither T_H2 cytokines nor IgE antibodies are involved. In contrast, in a very recent study, mice pretreated with silver nanoparticles and bacterial LPS, but not with the silver ions that are thought to cause allergies, developed allergic inflammation in response to the silver (Hirai et al., 2016). Nickel nanoparticles also caused sensitization, whereas gold and silica nanoparticles, which are minimally ionizable, did not. These results suggest that metal nanoparticles served as ion carriers to enable metal sensitization. Overall, however, the literature does not support the hypothesis of a major contribution of engineered nanoparticles to the current allergy epidemic, and comparisons to the much more complex combustion-derived ultrafine particles have to be made with care. Given the increasing exposure and intense consumer awareness, specific assays for detecting allergy-promoting effects will nevertheless have to be considered for products where this issue is particularly relevant, as in the cosmetics industry (Robertson et al., 2010; Wiechers and Musee, 2010). Indoor allergens are present in many work place environments and their inhalation should be considered in nanosafety assessment.

IMMUNOSUPPRESSION BY NANOMATERIALS

Immunosuppression is a flourishing medical field, in which various nanoformulated drugs are in the pipeline and some are already in the clinic. The field of immunosuppressive nanomedicines is covered in recent reviews (Boraschi and Penton-Rol, 2014; Ilinskaya and Dobrovolskaia, 2016). Nanoparticle platforms are mainly used to deliver well-known immunosuppressive drugs, for improving their pharmacokinetics and for decreasing their side effects. However, we will focus here on the issue of immunosuppression triggered by the nanomaterials themselves, either through direct or indirect mechanisms. While such responses may highlight new modes of action that can be exploited for drug development, the main concern addressed here is that exposure to nanomaterials could lead to unwanted immunosuppression.

The most direct cause of immunosuppression caused by nanomaterials is direct toxicity causing immune cell death. Cell death occurs mainly by two mechanisms, necrosis and apoptosis (Fink and Cookson, 2005). Apoptosis is a genetically regulated mechanism for deleting a cell without inducing inflammation and is as such a normal, continuously occurring process that is required for eliminating senescent or anomalous cells and in all processes of tissue maintenance and regeneration. More recently, other forms of programmed cell death have been described, which may involve release of inflammatory factors (Andón and Fadeel, 2013). Nanomaterials can be involved in these processes. For instance, pyroptosis is induced in THP-1 macrophage-like cells by certain Ag particles (Simard et al., 2015), and ZnO nanoparticles lead to cell death associated with autophagy in Jurkat and SupT1 lymphocyte-like cells, due to release of Zn^{2+} ions that induce excessive production of ROS (Johnson et al., 2015).

Among the immune cells, phagocytes are the professional defense cells that patrol the body for potential dangers. From this perspective, it is likely that immune cells meet and handle nanomaterials more frequently than many other cell types (with the exception of nonprofessional defense cells, such as skin and mucosal epithelial cells). Phagocytic immune cells are also often the first to come in contact with nanomaterials, either in blood (for instance, with injected nanomedicines), at the body surfaces (in particular respiratory mucosa, gastrointestinal tract, skin), and in internal tissues (e.g., in the case of wounds or after extravasation). There are two main types of phagocytic cells, as discussed previously, namely polymorphonuclear leukocytes or neutrophils and mononuclear phagocytes (blood monocytes and tissue resident macrophages). Uptake of nanoparticles, even at high doses, does not necessarily kill monocytes/macrophages (Reisetter et al., 2011), fibroblasts (Petrache Voicu et al., 2015), or lung-derived epithelial cells (Herd et al., 2013). In this situation, cells can use autophagy as a mechanism to maintain survival and functionality (Andón and Fadeel, 2013). Due to their physical characteristics, nanoparticles (in particular small spherical ones) may also enter cells by nonphagocytic mechanisms. Indeed, in a recent study on C_{60} fullerenes, passive diffusion into membranes provided a significant source of biologically available nanomaterial (Russ et al., 2016). This implies that particles may also be found, although to a lesser extent, in nonphagocytic immune cells, including T and B lymphocytes. ZnO nanoparticles were reported to have substantial cytotoxicity for monocytes, less for NK cells, and even more modest for T cells and B cells (Hanley et al., 2009). In this study, cytotoxicity was linked to contact of nanoparticles with cell membranes, phagocytosis, and consequent ROS production, which are indeed all expected to be high for the strongly phagocytic monocytes. Another study found monocyte-derived dendritic cells to be particularly sensitive to cytotoxicity by ZnO but not TiO_2 nanoparticles (Andersson-Willman et al., 2012). The cytotoxicity of ZnO nanoparticles has been associated with particle dissolution in the endosomal compartment, which leads to release of Zn^{++} ions and ultimately to programmed cell death (Johnson et al., 2015; Kao et al., 2012).

Even in the absence of overt toxicity, nanoparticle-loaded immune cells may be less fit for exerting their defensive and scavenging functions. Although not many data have been published on this topic, nanoparticle-loaded immune cells may undergo apoptosis sooner than normal, or be subjected to anomalous autophagic mechanisms that affect immunological fitness (Stern et al., 2012). Even more relevant is the possibility that nanoparticle-loaded immune cells may be less able to tackle incoming danger in an adequate fashion. For example, when a pathogenic virus enters the body, the immune system recognizes the virus as dangerous and initiates a complex reaction that leads to viral clearance and reestablishment of the normal healthy conditions. If immune cells are loaded with nanoparticles, will they be able to initiate and develop the defensive response, to produce interferons, kill virus-infected cells, and later to repair the damages produced to the infected tissue? As discussed in the section on inflammation (see section "Innate Immune Responses"), some studies suggest that immune function is impaired following concomitant exposure to nanoparticles and pathogens. Here, we emphasize the concept that immunosuppression can rest on much subtler mechanisms than on the direct killing of immune cells, and that nanomaterials may have unforeseeable modulatory effects in the absence of any overt sign of toxicity. Indeed, it should also be considered that nanomaterials may affect immune responses by

abnormally activating immunosuppressive mechanisms. These mechanisms usually come into play during the final phases of defensive responses, that is, when the infection/danger has been eliminated and the immune activation must be reconducted to baseline (Valledor et al., 2010). Among downregulatory mechanisms of innate immune reactions, a series of inflammatory cytokine inhibitors are abundantly expressed and released in the resolution phase of the response (e.g., IL-1Ra, IL-18BP, sIL-1RII, TIR8, TGFβ, IL-10). Other molecules act as antiinflammatory transcriptional regulators (IL-33, IL-37). Besides directly inhibiting the innate inflammatory response, several of these factors can also create the microenvironmental conditions for the development of regulatory T cells (Treg) that are responsible for the downregulation of specific T cell responses (Shevach, 2009). Concomitantly, the same factors/microenvironments promote tissue remodeling for reestablishing tissue integrity. Recent studies have shown that SWCNT are able to promote metastatic growth of lung carcinoma in C57BL6/J mice and that this is mediated by an increased accumulation of myeloid-derived suppressor cells (MDSC) (Shvedova et al., 2013). The same authors reported that SWCNTs trigger MDSC-derived production of TGFβ in the lungs, resulting in upregulated tumor burden, using the same mouse model (Shvedova et al., 2015). Taken together, these results indicate that an acute pulmonary exposure to carbon nanotubes changes the interaction between myeloid regulatory cells and malignant cells to benefit tumor growth and metastasis. It remains to be understood whether this is seen for other nanomaterials or whether CNTs are particularly harmful.

DEFINITELY NOT INNOCENT BYSTANDERS

An issue of major relevance when assessing the possible effects of nanoparticles on the immune system is the fact that nanoparticles are not "naked." As discussed already, proteins and other endogenous molecules are rapidly adsorbed on the nanoparticle surface when the particles are introduced into the body. However, nanoparticles may also interact with other biologically active entities in the environment, most frequently microorganisms and molecules derived thereof. In these circumstances, it can be difficult to attribute a possible effect to the nanoparticle rather than to the associated contaminating molecules. For immunological effects this observation is particularly relevant, because the possible contaminating agents (microorganisms) are major inducers of inflammation. Thus, the activity displayed by nanoparticles in in vivo or in vitro assays of immune response in many published studies may be due to contaminating bioactive bacterial products rather than to the nanoparticles themselves (Li and Boraschi, 2016).

The best-known example is that of bacterial lipopolysaccharide (LPS, or endotoxin). LPS is a component of the cell wall of gram-negative bacteria and is one of the most potent activators of innate immunity by interacting with TLR4 receptors on the surface of monocytes/macrophages and other cells (Jerala, 2007). Even minute amounts of LPS can fully activate an inflammatory reaction in human monocytes, for example, by upregulating the expression of the inflammatory cytokine IL-1β (Guha and Mackman, 2001; Oostingh et al., 2011). Primary dendritic cells are also especially sensitive to LPS (Schwarz et al., 2014). Trace amounts of LPS are found practically everywhere, even in the absence of living bacteria, due to its molecular structure that makes it very resistant to cleaning and sterilization procedures (solvents,

heat, etc.) and to its "stickiness" to surfaces. Thus, glassware and reagents must be confirmed as "endotoxin-free" before being used for any immune assays. When testing the immunosafety of nanoparticles, both in vitro and in vivo, it is also necessary that the test nanomaterial is completely free of endotoxin contamination, to avoid the risk of testing the effects of LPS on the immune responses rather than those of the nanoparticles (Shi et al., 2010). An issue that may hamper appropriate evaluation of the presence of LPS in nanomaterial preparations is that nanoparticles can physically interfere with standard methods for endotoxin evaluation, thus risking to providing misleading results (Dobrovolskaia et al., 2010; Neun and Dobrovolskaia, 2011). Indeed, in a recent study, graphene-based materials were shown to elicit ambiguous results when applying the standard limulus amebocyte lysate (LAL) assay while a macrophage activation test for endotoxin was found to yield unequivocal results (Mukherjee et al., 2016). The latter results are important since previous work has suggested that graphene oxide triggers necroptosis in macrophages via TLR4 (Qu et al., 2013); it is important to delineate the intrinsic effects of a nanomaterial versus the (trivial) consequences of endotoxin contamination. Two types of approaches can be followed in order to evaluate the immunosafety of nanomaterials while avoiding the biological interference by LPS. The first, which can be applied to engineered nanoparticles, is to perform the entire synthetic process in endotoxin-free conditions, a procedure expensive in terms of facilities and equipment but leading to excellent results (Vallhov et al., 2006). The second, suitable for all kinds of nanoparticles, is that of devising assays in which LPS does not interfere (for instance, by using LPS-resistant mice for in vivo experiments, deliberate activation with LPS in vitro). This latter experimental setting, that is, an assay in which cells are deliberately activated so as to simulate the reaction to an infectious agent, has the advantage of allowing researchers to use nanoparticles as produced, including their environmentally induced changes and contaminations, a situation closer to real life (Boraschi et al., 2011, 2012).

TURNING THE TABLES: BIOMEDICAL APPLICATIONS

The focus of the current chapter is on the immunological effects of nanomaterials from a safety assessment perspective. Nevertheless, while it is abundantly clear that nanomaterials have the potential to either suppress or to activate immune functions, which could account for adverse effects in a living organism, such immunomodulatory effects could also be exploited for medical applications, provided that other toxic effects (cytotoxicity, genotoxicity, etc.) are controlled. Indeed, activation of the immune system is highly desirable in cancer therapy, and in cases of chronic microbial infections that cannot be cleared by normal mechanisms (Pardoll, 2012). Moreover, the success of vaccination is predicated on the activation of specific immune responses. On the other hand, suppression of immune responses is key to treatment of autoimmune diseases and allergies (Boraschi and Pentol-Rol, 2016). The nanomedicine community would therefore strongly benefit from a deeper interaction with the nanosafety research community. Thus, although beyond the scope of this chapter, a thorough characterization of the immunomodulatory effects of nanomaterials may open the way for their exploitation in the treatment of many human diseases, to achieve either immunostimulation (infections, tumors) or immunosuppression (allergies, autoimmunity, chronic, and degenerative diseases).

TAKE-HOME MESSAGES

The immune system has evolved to recognize whether nonself or anomalous entities pose a threat to the body. Furthermore, as discussed in the present chapter, immune cells are able to sense and respond to nanoparticles. Importantly, the immune system is able to sense threats that are indirect and not immediately apparent, as opposed, for instance, to the induction of cell death, making immunotoxicity assessment a "sentinel" form of toxicity assessment of nanomaterials. In other words, immune responses may be viewed as a warning sign and this can be exploited for the design of safer nanoparticles. Here, we summarize some of the lessons learned from studies of the immune system, and how these may guide and inform nanosafety assessment:

- Immune cells are specialized for recognizing dangerous nonself entities, and they are concentrated close to body surfaces where pathogens, but also engineered nanoparticles, are most likely to intrude. The sentinel function of immunity can be used to investigate health risks of nanomaterials. Powerful complement activation or a strong inflammatory response would label a nanomaterial as a potential threat to human health. However, it is worth noting that an inflammatory reaction is only sign of a defensive response, and does not per se indicate whether the consequences of the reaction would turn out to be detrimental to the organism or not. On the other hand, if no immune activation is observed, the chances are good that the nanomaterial is quite harmless, since it is treated as harmless by the immune system.
- There is an extensive body of knowledge regarding how immune cells interact with nonself entities including microorganisms. This is useful to elucidate the mechanisms of interaction between nanomaterials and biological systems. Conversely, the use of precisely tunes libraries of engineered nanomaterials could also inform us on how immune responses are shaped.
- Immune activation follows various pathways, but in a rather foreseeable manner. Processes like cellular stress, inflammation or allergy can be followed using a limited number of markers. Since these processes arrive, independent of the initiating event, to specified endpoints, it is possible to define general endpoints that characterize the activation process. For example, cellular stress tends to involve NF-κB activation, inflammation involves inflammasome activation, and type 1 allergy involves the production of IL-4 and IL-13. Therefore, such endpoints are well suited to address immunosafety, independently of the test agent.
- While immune activation processes are part of our defense system, the endpoints described earlier are not markers of pathology, but simply markers of immune activation. Thus, the context in which such activation occurs and its characteristics (in terms of extent and duration of the response) determines the difference between a normal defensive response, which resolves, and a pathological reaction that persists and causes tissue damage. Evaluation of the kinetics of the immune response in the presence of nanoparticles is therefore needed.
- Redundancy is a major feature of the immune system, where many molecules and molecular pathways may be used to reach the same result, which ensures an efficacious response to danger. However, this also means that different organisms are likely to use

different mechanisms. Concerning mice, the most frequently used animal model for studying immunity, many defense processes, including the regulation of inflammation, are different from those preferentially active in humans. Thus, immunosafety assays should be designed so that pathways and mechanisms relevant to human immunity are considered.

- For safety evaluation, models and assays are generally designed having as target the healthy organism. However, not all people are in perfect health. For example, many workers have chronic lung conditions, such as allergic asthma or chronic obstructive pulmonary disease (COPD). Other people are old, with an immune system less able to react to many challenges, or babies/young children, with a still immature immune system. Immunotoxicology has excellent tools to simulate disease states and design models representing different conditions of immunological frailty and such models should also be applied for the study of nanomaterials.

Acknowledgments

The authors are supported by the European Commission (FP7-NANOREG, grant agreement no. 310584; FP7-NANOVALID, grant agreement No: 263147; H2020 PANDORA, grant agreement no. 671881); the Italian Ministry of Education, University and Research (cluster project MEDINTECH); and the FWF doctoral program Immunity in Cancer and Allergy. The authors are grateful to Paola Italiani (CNR) for assistance with the design and preparation of figures.

References

Al-Hanbali, O., Rutt, K.J., Sarker, D.K., Hunter, A.C., Moghimi, S.M., 2006. Concentration dependent structural ordering of poloxamine 908 on polystyrene nanoparticles and their modulatory role on complement consumption. J. Nanosci. Nanotechnol. 6, 3126–3133.

Allen, B.L., Kichambare, P.D., Gou, P., Vlasova, I.I., Kapralov, A.A., Konduru, N., et al., 2008. Biodegradation of single-walled carbon nanotubes through enzymatic catalysis. Nano Lett. 8, 3899–3903.

Allen, B.L., Kotchey, G.P., Chen, Y., Yanamala, N.V., Klein-Seetharaman, J., Kagan, V.E., et al., 2009. Mechanistic investigations of horseradish peroxidase-catalyzed degradation of single-walled carbon nanotubes. J. Am. Chem. Soc. 131, 17194–17205.

Andersson-Willman, B., Gehrmann, U., Cansu, Z., Buerki-Thurnherr, T., Krug, H.F., Gabrielsson, S., et al., 2012. Effects of subtoxic concentrations of TiO2 and ZnO nanoparticles on human lymphocytes, dendritic cells and exosome production. Toxicol. Appl. Pharm. 264, 94–103.

Andón, F.T., Fadeel, B., 2013. Programmed cell death: molecular mechanisms and implications for safety assessment of nanomaterials. Acc. Chem. Res. 46 (3), 733–742.

Andón, F.T., Kapralov, A.A., Yanamala, N., Feng, W., Baygan, A., Chambers, B.J., et al., 2013. Biodegradation of single-walled carbon nanotubes by eosinophil peroxidase. Small 9 (16), 2721–2729.

Beamer, C.A., Girtsman, T.A., Seaver, B.P., Finsaas, K.J., Migliaccio, C.T., Perry, V.K., et al., 2013. IL-33 mediates multi-walled carbon nanotube (MWCNT)-induced airway hyper-reactivity via the mobilization of innate helper cells in the lung. Nanotoxicology 7 (6), 1070–1081.

Bhattacharya, K., Andón, F.T., El-Sayed, R., Fadeel, B., 2013. Mechanisms of carbon nanotube-induced toxicity: focus on pulmonary inflammation. Adv. Drug. Deliv. Rev. 65 (15), 2087–2097.

Bhattacharya, K., El-Sayed, R., Andón, F.T., Mukherjee, S.P., Gregory, J., Li, H., et al., 2015. Lactoperoxidase-mediated degradation of single-walled carbon nanotubes in the presence of pulmonary surfactant. Carbon 91, 506–517.

Bhattacharya, K., Mukherjee, S.P., Gallud, A., Burkert, S.C., Bistarelli, S., Bellucci, S., Bottini, M., Star, A., Fadeel, B., 2016. Biological interactions of carbon-based nanomaterials: from coronation to degradation. Nanomedicine 12 (2), 333–351.

Biswas, S.K., Lopez-Collazo, E., 2009. Endotoxin tolerance: new mechanisms, molecules and clinical significance. Trends Immunol. 30, 475–487.
Boraschi, D., Italiani, P., 2015. From antigen delivery system to adjuvanticy: the broad application of nanoparticles in vaccinology. Vaccines (Basel) 3, 930–939.
Boraschi, D., Pentol-Rol, G. (Eds.), 2016. Immune Rebalancing: The Future of Immunosuppression. Academic Press/Elsevier, London/San Diego/Cambridge/Oxford.
Boraschi, D., Penton-Rol, G., 2014. Perspectives in immunopharmacology: the future of immunosuppression. Immunol. Lett. 161, 211–215.
Boraschi, D., Oostingh, G.J., Casals, E., Italiani, P., Nelissen, I., Puntes, V.F., Duschl, A., 2011. Nano-immunosafety: issues in assay validation. J. Phys. Conf. 304, 012077.
Boraschi, D., Costantino, L., Italiani, P., 2012. Interaction of nanoparticles with immunocompetent cells: nanosafety considerations. Nanomedicine (Future Med) 7, 121–131.
Branzk, N., Lubojemska, A., Hardison, S.E., Wang, Q., Gutierrez, M.G., Brown, G.D., et al., 2014. Neutrophils sense microbe size and selectively release neutrophil extracellular traps in response to large pathogens. Nat. Immunol. 15 (11), 1017–1025.
Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D.S., et al., 2004. Neutrophil extracellular traps kill bacteria. Science 303 (5663), 1532–1535.
Casals, E., Pfaller, T., Duschl, A., Oostingh, G.J., Puntes, V., 2010. Time evolution of the nanoparticle protein corona. ACS Nano 4, 3623–3632.
Castranova, V., Schulte, P.A., Zumwalde, R.D., 2013. Occupational nanosafety considerations for carbon nanotubes and carbon nanofibers. Acc. Chem. Res. 46 (3), 642–649.
Chanan-Khan, A., Szebeni, J., Savay, S., Liebes, L., Rafique, N.M., Alving, C.R., et al., 2003. Complement activation following first exposure to pegylated liposomal doxorubicin (Doxil): possible role in hypersensitivity reactions. Ann. Oncol. 14, 1430–1437.
Chappert, P., Schwartz, R.H., 2010. Induction of T cell anergy: integration of environmental cues and infectious tolerance. Curr. Opin. Immunol. 22, 552–559.
Chen, K.W., Bezbradica, J.S., Groß, C.J., Wall, A.A., Sweet, M.J., Stow, J.L., et al., 2016a. The murine neutrophil NLRP3 inflammasome is activated by soluble but not particulate or crystalline agonists. Eur. J. Immunol. 46 (4), 1004–1010.
Chen, S., Yin, R., Mutze, K., Yu, Y., Takenaka, S., Königshoff, M., et al., 2016b. No involvement of alveolar macrophages in the initiation of carbon nanoparticle induced acute lung inflammation in mice. Part. Fibre Toxicol. 13 (1), 33.
Cho, W.S., Duffin, R., Poland, C.A., Howie, S.E., MacNee, W., Bradley, M., et al., 2010. Metal oxide nanoparticles induce unique inflammatory footprints in the lung: important implications for nanoparticle testing. Environ. Health Perspect. 118 (12), 1699–1706.
Cukalevski, R., Ferreira, S.A., Dunning, C.J., Berggard, T., Cedervall, T., 2015. IgG and fibrinogen driven nanoparticle aggregation. Nano Res. 8, 2733–2743.
De Koker, S., Lambrecht, B.N., Willart, M.A., van Kooyk, Y., Grooten, J., Vervaet, C., et al., 2011. Designing polymeric particles for antigen delivery. Chem. Soc. Rev. 40, 320–339.
Dell'Orco, D., Lundqvist, M., Oslakovic, C., Cedervall, T., Linse, S., 2010. Modeling the time evolution of the nanoparticle-protein corona in a body fluid. PLoS One 5, e10949.
Deng, Z.J., Liang, M., Monteiro, M., Toth, I., Minchin, R.F., 2011. Nanoparticle-induced unfolding of fibrinogen promotes Mac-1 receptor activation and inflammation. Nat. Nanotechnol. 6, 39–44.
Deng, J., Sun, M., Zhu, J., Gao, C., 2013. Molecular interactions of different size AuNP-COOH nanoparticles with human fibrinogen. Nanoscale 5, 8130–8137.
Dobrovolskaia, M.A., Aggarwal, P., Hall, J.B., McNeil, S.E., 2008. Preclinical studies to understand nanoparticle interaction with the immune system and its potential effects on nanoparticle biodistribution. Mol. Pharm. 5, 487–495.
Dobrovolskaia, M.A., Patri, A.K., Zheng, J., Clogston, J.D., Ayub, N., Aggarwal, P., et al., 2009. Interaction of colloidal gold nanoparticles with human blood: effects on particle size and analysis of plasma protein binding profiles. Nanomedicine 5, 106–117.
Dobrovolskaia, M.A., Neun, B.W., Clogston, J.D., Ding, H., Ljubimova, J., McNeil, S.E., 2010. Ambiguities in applying traditional Limulus amebocyte lysate tests to quantify endotoxin in nanoparticle formulations. Nanomedicine (Lond) 5, 555–562.
Dobrovolskaia, M.A., Shurin, M., Shvedova, A.A., 2016. Current understanding of interactions between nanoparticles and the immune system. Toxicol. Appl. Pharmacol. 299, 78–89.

Eisenbarth, S.C., Colegio, O.R., O'Connor, W., Sutterwala, F.S., Flavell, R.A., 2008. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. Nature 453, 1122–1126.
Elgrabli, D., Dachraoui, W., Ménard-Moyon, C., Liu, X.J., Bégin, D., Bégin-Colin, S., et al., 2015. Carbon nanotube degradation in macrophages: live nanoscale monitoring and understanding of biological pathway. ACS Nano 9 (10), 10113–10124.
Fadeel, B., Garcia-Bennett, A.E., 2010. Better safe than sorry: understanding the toxicological properties of inorganic nanoparticles manufactured for biomedical applications. Adv. Drug Deliv. Rev. 62, 362–374.
Farrera, C., Fadeel, B., 2015. It takes two to tango: understanding the interactions between engineered nanomaterials and the immune system. Eur. J. Pharm. Biopharm. 95 (Pt. A), 3–12.
Farrera, C., Bhattacharya, K., Lazzaretto, B., Andón, F.T., Hultenby, K., Kotchey, G.P., et al., 2014. Extracellular entrapment and degradation of single-walled carbon nanotubes. Nanoscale 6 (12), 6974–6983.
Fink, S.L., Cookson, B.T., 2005. Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. Infect. Immun. 73, 1907–1916.
Firdous, J., Islam, M.A., Park, S.-M., Cheon, I.-S., Shim, B.-S., Yoon, H.-S., et al., 2014. Induction of long-term immunity against respiratory syncytial virus glycoprotein by an osmotic polymeric nanocarrier. Acta Biomaterialia 10, 4606–4617.
Franchi, L., Eigenbrod, T., Nunez, G., 2009. Cutting edge: TNF-alpha mediates sensitization to ATP and silica via the NLRP3 inflammasome in the absence of microbial stimulation. J. Immunol. 183, 792–796.
Geiser, M., 2010. Update on macrophage clearance of inhaled micro- and nanoparticles. J. Aerosol. Med. Pulm. Drug Deliv. 23, 207–217.
Gomez, S., Gamazo, C., San Roman, B., Grau, A., Espuelas, S., Ferrer, M., et al., 2009. A novel nanoparticulate adjuvant for immunotherapy with Lolium perenne. J. Immunol. Methods 348, 1–8.
Groves, E., Dart, A.E., Covarelli, V., Caron, E., 2008. Molecular mechanisms of phagocytic uptake in mammalian cells. Cell Mol. Life Sci. 65, 1957–1976.
Guha, M., Mackman, N., 2001. LPS induction of gene expression in human monocytes. Cell Signal 13, 85–94.
Hadjidemetriou, M., Al-Ahmady, Z., Mazza, M., Collins, R.F., Dawson, K., Kostarelos, K., 2015. In vivo biomolecule corona around blood-circulating, clinically used and antibody-targeted lipid bilayer nanoscale vesicles. ACS Nano 9 (8), 8142–8156.
Hamad, I., Al-Hanbali, O., Hunter, A.C., Rutt, K.J., Andresen, T.L., Moghimi, S.M., 2010. Distinct polymer architecture mediates switching of complement activation pathways at the nanosphere-serum interface: implications for stealth nanoparticle engineering. ACS Nano 4, 6629–6638.
Hanley, C., Thurber, A., Hanna, C., Punnoose, A., Zhang, J.H., Wingett, D.G., 2009. The influences of cell type and ZnO nanoparticle size on immune cell cytotoxicity and cytokine induction. Nanoscale Res. Lett. 4, 1409–1420.
Heinrich, J., 2011. Influence of indoor factors in dwellings on the development of childhood asthma. Int. J. Hyg. Environ. Health 214, 1–25.
Herd, H., Daum, N., Jones, A.T., Huwer, H., Ghandehari, H., Lehr, C.M., 2013. Nanoparticle geometry and surface orientation influence mode of cellular uptake. ACS Nano 7, 1961–1973.
Hill, J.A., Southwood, S., Sette, A., Jevnikar, A.M., Bell, D.A., Cairns, E., 2003. Cutting edge: the conversion of arginine to citrulline allows for a high-affinity peptide interaction with the rheumatoid arthritis-associated HLA-DRB1*0401 MHC class II molecule. J. Immunol. 171, 538–541.
Hirai, T., Yoshioka, Y., Izumi, N., Ichihashi, K.I., Handa, T., Nishijima, N., et al., 2016. Metal nanoparticles in the presence of lipopolysaccharides trigger the onset of metal allergy in mice. Nat. Nanotechnol. 11 (9), 808–816.
Holgate, S.T., Lack, G., 2005. Improving the management of atopic disease. Arch. Dis. Child 90, 826–831.
Hornung, V., Bauernfeind, F., Halle, A., Samstad, E.O., Kono, H., Rock, K.L., et al., 2008. Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. Nat. Immunol. 9, 847–856.
Hwang, T.L., Aljuffali, I.A., Hung, C.F., Chen, C.H., Fang, J.Y., 2015. The impact of cationic solid lipid nanoparticles on human neutrophil activation and formation of neutrophil extracellular traps (NETs). Chem. Biol. Interact. 235, 106–114.
Ilinskaya, A.N., Dobrovolskaia, M.A., 2016. Understanding the immunogenicity and antigenicity of nanomaterials: past, present and future. Toxicol. Appl. Pharmacol. 299, 70–77.
Inturi, S., Wang, G., Chen, F., Banda, N.K., Holers, V.M., Wu, L., et al., 2015. Modulatory role of surface coating of superparamagnetic iron oxide nanoworms in complement opsonization and leukocyte uptake. ACS Nano 9 (11), 10758–10768.
Jerala, R., 2007. Structural biology of the LPS recognition. Int. J. Med. Microbiol. 297, 353–363.

Jessop, F., Holian, A., 2015. Extracellular HMGB1 regulates multi-walled carbon nanotube-induced inflammation in vivo. Nanotoxicology 9 (3), 365–372.
Johnson, B.M., Fraietta, J.A., Gracias, D.T., Hope, J.L., Stairiker, C.J., Patel, P.R., et al., 2015. Acute exposure to ZnO nanoparticles induces autophagic immune cell death. Nanotoxicology 9, 737–748.
Kagan, V.E., Konduru, N.V., Feng, W., Allen, B.L., Conroy, J., Volkov, Y., et al., 2010. Carbon nanotubes degraded by neutrophil myeloperoxidase induce less pulmonary inflammation. Nat. Nanotechnol. 5, 354–359.
Kagan, V.E., Kapralov, A.A., St. Croix, C.M., Watkins, S.C., Kisin, E.R., Kotchey, G.P., et al., 2014. Lung macrophages "digest" carbon nanotubes using a superoxide/peroxynitrite oxidative pathway. ACS Nano 8, 5610–5621.
Kao, Y.Y., Chen, Y.C., Cheng, T.J., Chiung, Y.M., Liu, P.S., 2012. Zinc oxide nanoparticles interfere with zinc ion homeostasis to cause cytotoxicity. Toxicol. Sci. 125, 462–472.
Katwa, P., Wang, X., Urankar, R.N., Podila, R., Hilderbrand, S.C., Fick, R.B., et al., 2012. A carbon nanotube toxicity paradigm driven by mast cells and the IL-33/ST2 axis. Small 8, 2904–2912.
Kodali, V., Littke, M.H., Tilton, S.C., Teeguarden, J.G., Shi, L., Frevert, C.W., et al., 2013. Dysregulation of macrophage activation profiles by engineered nanoparticles. ACS Nano 7 (8), 6997–7010.
Kumar, A., Bicer, E.M., Morgan, A.B., Pfeffer, P.E., Monopoli, M., Dawson, K.A., et al., 2016. Enrichment of immunoregulatory proteins in the biomolecular corona of nanoparticles within human respiratory tract lining fluid. Nanomedicine 12 (4), 1033–1043.
Kunzmann, A., Andersson, B., Vogt, C., Feliu, N., Ye, F., Gabrielsson, S., et al., 2011. Efficient internalization of silica-coated iron oxide nanoparticles of different sizes by primary human macrophages and dendritic cells. Toxicol. Appl. Pharmacol. 253, 81–93.
Larsen, S.T., Roursgaard, M., Jensen, K.A., Nielsen, G.D., 2010. Nano titanium dioxide particles promote allergic sensitization and lung inflammation in mice. Basic Clin. Pharmacol. Toxicol. 106, 114–117.
Li, Y., Boraschi, D., 2016. Endotoxin contamination: a key element in the interpretation of nanosafety studies. Nanomedicine (Lond) 11 (3), 269–287.
Li, H., Willingham, S.B., Ting, J.P., Re, F., 2008a. Cutting edge: inflammasome activation by alum and alum's adjuvant effect are mediated by NLRP3. J. Immunol. 181, 17–21.
Li, N., Xia, T., Nel, A.E., 2008b. The role of oxidative stress in ambient particulate matter-induced lung diseases and its implications in the toxicity of engineered nanoparticles. Free Radic. Biol. Med. 44, 1689–1699.
Licciardi, M., Montana, G., Bondi, M.L., Bonura, A., Scialabba, C., Melis, M., et al., 2014. An allergen-polymeric nanoaggregate as a new tool for allergy vaccination. Int. J. Pharm. 465, 275–283.
Lo, J.H., von Maltzahn, G., Douglass, J., Park, J.H., Sailor, M.J., Ruoslahti, E., et al., 2013. Nanoparticle amplification photothermal unveiling of cryptic collagen binding sites. J. Mater. Chem. B Mater. Biol. Med. 1, 5235–5240.
Lynch, I., Dawson, K.A., Linse, S., 2006. Detecting cryptic epitopes created by nanoparticles. Sci. STKE 2006, pe14.
Maiorano, G., Sabella, S., Sorce, B., Brunetti, V., Malvindi, M.A., Cingolani, R., et al., 2010. Effects of cell culture media on the dynamic formation of protein-nanoparticle complexes and influence on the cellular response. ACS Nano 4, 7481–7491.
Martinon, F., 2010. Signaling by ROS drives inflammasome activation. Eur. J. Immunol. 40, 616–619.
Martinon, F., Tschopp, J., 2007. Inflammatory caspases and inflammasomes: master switches of inflammation. Cell Death Differ. 14, 10–22.
Monopoli, M.P., Walczyk, D., Campbell, A., Elia, G., Lynch, I., Bombelli, F.B., et al., 2011. Physical-chemical aspects of protein corona: relevance to in vitro and in vivo biological impacts of nanoparticles. J. Am. Chem. Soc. 133, 2525–2534.
Mortimer, G.M., Butcher, N.J., Musumeci, A.W., Deng, Z.J., Martin, D.J., Minchin, R.F., 2014. Cryptic epitopes of albumin determine mononuclear phagocyte system clearance of nanomaterials. ACS Nano 8, 3357–3366.
Mosqueira, V.C., Legrand, P., Gulik, A., Bourdon, O., Gref, R., Labarre, D., et al., 2001. Relationship between complement activation, cellular uptake and surface physicochemical aspects of novel PEG-modified nanocapsules. Biomaterials 22, 2967–2979.
Moss, C.X., Matthews, S.P., Lamont, D.J., Watts, C., 2005. Asparagine deamidation perturbs antigen presentation on class II major histocompatibility complex molecules. J. Biol. Chem. 280, 18498–18503.
Moyano, D.F., Goldsmith, M., Solfiell, D.J., Landesman-Milo, D., Miranda, O.R., Peer, D., et al., 2012. Nanoparticle hydrophobicity dictates immune response. J. Am. Chem. Soc. 134, 3965–3967.
Mukherjee, S.P., Lozano, N., Kucki, M., Del Rio-Castillo, A.E., Newman, L.D.D., Vázquez, E., et al., 2016. Detection of endotoxin contamination of graphene based materials using the TNF-α expression test and guidelines for endotoxin-free graphene oxide production. PLoS One. 11 (11), e0166816.

Munoz, L.E., Lauber, K., Schiller, M., Manfredi, A.A., Herrmann, M., 2010. The role of defective clearance of apoptotic cells in systemic autoimmunity. Nat. Rev. Rheumatol. 6, 280–289.
Muñoz-Planillo, R., Kuffa, P., Martínez-Colón, G., Smith, B.L., Rajendiran, T.M., Núñez, G., 2013. K^+ efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. Immunity 38 (6), 1142–1153.
Nagai, H., Toyokuni, S., 2010. Biopersistent fiber-induced inflammation and carcinogenesis: lessons learned from asbestos toward safety of fibrous nanomaterials. Arch. Biochem. Biophys. 502, 1–7.
Nel, A.E., Diaz-Sanchez, D., Ng, D., Hiura, T., Saxon, A., 1998. Enhancement of allergic inflammation by the interaction between diesel exhaust particles and the immune system. J. Allergy Clin. Immunol. 102, 539–554.
Netea, M.G., Quintin, J., van der Meer, J.W.M., 2011. Trained immunity: a memory for innate host defense. Cell Host Microb. 9, 355–361.
Neun, B.W., Dobrovolskaia, M.A., 2011. Detection and quantitative evaluation of endotoxin contamination in nanoparticle formulations by LAL-based assays. Methods Mol. Biol. 697, 121–130.
Nouri, H.R., Varasteh, A., Jaafari, M.R., Davies, J.M., Sankian, M., 2015. Induction of a Th1 immune response and suppression of IgE via immunotherapy with a recombinant hybrid molecule encapsulated in liposome-protamine-DNA nanoparticles in a model of experimental allergy. Immunol. Res. 62, 280–291.
Nygaard, U.C., Hansen, J.S., Samuelsen, M., Alberg, T., Marioara, C.D., Lovik, M., 2009. Single-walled and multi-walled carbon nanotubes promote allergic immune responses in mice. Toxicol. Sci. 109, 113–123.
Oberdörster, G., Oberdörster, E., Oberdörster, J., 2005. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environ. Health Perspect. 113, 823–839.
Oostingh, G.J., Casals, E., Italiani, P., Colognato, R., Stritzinger, R., Ponti, J., et al., 2011. Problems and challenges in the development and validation of human cell-based assays to determine nanoparticle-induced immunomodulatory effects. Part. Fibre Toxicol. 8, 8.
Pali-Scholl, I., Szollosi, H., Starkl, P., Scheicher, B., Stremnitzer, C., Hofmeister, A., et al., 2013. Protamine nanoparticles with CpG-oligodeoxynucleotide prevent an allergen-induced Th2-response in BALB/c mice. Eur. J. Pharm. Biopharm. 85, 656–664.
Pardoll, D.M., 2012. The blockade of immune checkpoints in cancer immunotherapy. Nat. Rev. Cancer 12, 252–263.
Park, H.S., Kim, K.H., Jang, S., Park, J.W., Cha, H.R., Lee, J.E., et al., 2010. Attenuation of allergic airway inflammation and hyperresponsiveness in a murine model of asthma by silver nanoparticles. Int. J. Nanomed. 5, 505–515.
Passirani, C., Barratt, G., Devissaguet, J.P., Labarre, D., 1998. Interactions of nanoparticles bearing heparin or dextran covalently bound to poly(methyl methacrylate) with the complement system. Life Sci. 62 (8), 775–785.
Petrache Voicu, S.N., Dinu, D., Sima, C., Hermenean, A., Ardelean, A., Codrici, E., et al., 2015. Silica nanoparticles induce oxidative stress and autophagy but not apoptosis in the MRC-5 cell line. Int. J. Mol. Sci. 16, 29398–29416.
Poland, C.A., Duffin, R., Kinloch, I., Maynard, A., Wallace, W.A., Seaton, A., et al., 2008. Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study. Nat. Nanotechnol. 3 (7), 423–428.
Qiao, S.W., Bergseng, E., Molberg, O., Jung, G., Fleckenstein, B., Sollid, L.M., 2005. Refining the rules of gliadin T cell epitope binding to the disease-associated DQ2 molecule in celiac disease: importance of proline spacing and glutamine deamidation. J. Immunol. 175, 254–261.
Qu, G., Liu, S., Zhang, S., Wang, L., Wang, X., Sun, B., et al., 2013. Graphene oxide induces toll-like receptor 4 (TLR4)-dependent necrosis in macrophages. ACS Nano 7 (7), 5732–5745.
Radauer-Preiml, I., Andosch, A., Hawranek, T., Luetz-Meindl, U., Wiederstein, M., Horejs-Hoeck, J., et al., 2016. Nanoparticle-allergen interactions mediate human allergic responses: protein corona characterization and cellular responses. Part. Fibre Toxicol. 13, 3.
Reisetter, A.C., Stebounova, L.V., Baltrusaitis, J., Powers, L., Gupta, A., Grassian, V.H., et al., 2011. Induction of inflammasome dependent pyroptosis by carbon black nanoparticles. J. Biol. Chem. 286 (24), 21844–21852.
Robertson, T.A., Sanchez, W.Y., Roberts, M.S., 2010. Are commercially available nanoparticles safe when applied to the skin? J. Biomed. Nanotechnol. 6, 452–468.
Rocker, C., Potzl, M., Zhang, F., Parak, W.J., Nienhaus, G.U., 2009. A quantitative fluorescence study of protein monolayer formation on colloidal nanoparticles. Nat. Nanotechnol. 4, 577–580.
Rossi, E.M., Pylkkanen, L., Koivisto, A.J., Nykasenoja, H., Wolff, H., Savolainen, K., et al., 2010. Inhalation exposure to nanosized and fine TiO2 particles inhibits features of allergic asthma in a murine model. Part. Fibre Toxicol. 7, 35.
Russ, K.A., Elvati, P., Parsonage, T.L., Dews, A., Jarvis, J.A., Ray, M., et al., 2016. C60 fullerene localization and membrane interactions in RAW 264.7 immortalized mouse macrophages. Nanoscale 8 (7), 4134–4144.

Sakaguchi, S., 2011. Regulatory T cells: history and perspective. Methods Mol. Biol. 707, 3–17.
Salari, F., Varasteh, A.R., Vahedi, F., Hashemi, M., Sankian, M., 2015. Down-regulation of Th2 immune responses by sublingual administration of poly (lactic-co-glycolic) acid (PLGA)-encapsulated allergen in BALB/c mice. Int. Immunopharmacol. 29, 672–678.
Salem, A.K., 2014. A promising CpG adjuvant-loaded nanoparticle-based vaccine for treatment of dust mite allergies. Immunotherapy 6, 1161–1163.
Salvador-Morales, C., Flahaut, E., Sim, E., Sloan, J., Green, M.L., Sim, R.B., 2006. Complement activation and protein adsorption by carbon nanotubes. Mol. Immunol. 43, 193–201.
Saptarshi, S.R., Duschl, A., Lopata, A.L., 2013. Interaction of nanoparticles with proteins: relation to bio-reactivity of the nanoparticle. J. Nanobiotechnol. 11, 26.
Scholl, I., Weissenbock, A., Forster-Waldl, E., Untersmayr, E., Walter, F., Willheim, M., et al., 2004. Allergen-loaded biodegradable poly(D,L-lactic-co-glycolic) acid nanoparticles down-regulate an ongoing Th2 response in the BALB/c mouse model. Clin. Exp. Allergy 34, 315–321.
Schöttler, S., Becker, G., Winzen, S., Steinbach, T., Mohr, K., Landfester, K., et al., 2016. Protein adsorption is required for stealth effect of poly(ethylene glycol)- and poly(phosphoester)-coated nanocarriers. Nat. Nanotechnol. 11 (4), 372–377.
Schwarz, H., Schmittner, M., Duschl, A., Horejs-Hoeck, J., 2014. Residual endotoxin contaminations in recombinant proteins are sufficient to activate human CD1c+ dendritic cells. PLoS One 9, e113840.
Shevach, E.M., 2009. Mechanisms of foxp3+ T regulatory cell-mediated suppression. Immunity 30, 636–645.
Shi, Y., Yadav, S., Wang, F., Wang, H., 2010. Endotoxin promotes adverse effects of amorphous silica nanoparticles on lung epithelial cells in vitro. J. Toxicol. Environ. Health A 73, 748–756.
Shima, F., Akagi, T., Akashi, M., 2015. Effect of hydrophobic side chains in the induction of immune responses by nanoparticle adjuvants consisting of amphiphilic poly(gamma-glutamic acid). Bioconjug. Chem. 26, 890–898.
Shvedova, A.A., Kisin, E.R., Mercer, R., Murray, A.R., Johnson, V.J., Potapovich, A.I., et al., 2005. Unusual inflammatory and fibrogenic pulmonary responses to single-walled carbon nanotubes in mice. Am. J. Physiol. Lung Cell Mol. Physiol. 289 (5), L698–L708.
Shvedova, A.A., Kagan, V.E., Fadeel, B., 2010. Close encounters of the small kind: adverse effects of man-made materials interfacing with the nano-cosmos of biological systems. Annu. Rev. Pharmacol. Toxicol. 50, 63–88.
Shvedova, A.A., Pietroiusti, A., Fadeel, B., Kagan, V.E., 2012. Mechanisms of carbon nanotube-induced toxicity: focus on oxidative stress. Toxicol. Appl. Pharmacol. 261 (2), 121–133.
Shvedova, A.A., Tkach, A.V., Kisin, E.R., Khaliullin, T., Stanley, S., Gutkin, D.W., et al., 2013. Carbon nanotubes enhance metastatic growth of lung carcinoma via up-regulation of myeloid-derived suppressor cells. Small 9 (9-10), 1691–1695.
Shvedova, A.A., Kisin, E.R., Yanamala, N., Tkach, A.V., Gutkin, D.W., Star, A., et al., 2015. MDSC and TGFβ are required for facilitation of tumor growth in the lungs of mice exposed to carbon nanotubes. Cancer Res. 75 (8), 1615–1623.
Sim, R.B., Wallis, R., 2011. Surface properties: Immune attack on nanoparticles. Nat. Nanotechnol. 6, 80–81.
Simard, J.C., Vallieres, F., de Liz, R., Lavastre, V., Girard, D., 2015. Silver nanoparticles induce degradation of the endoplasmic reticulum stress sensor activating transcription factor-6 leading to activation of the NLRP-3 inflammasome. J. Biol. Chem. 290, 5926–5939.
Stern, S.T., Adiseshaiah, P.P., Crist, R.M., 2012. Autophagy and lysosomal dysfunction as emerging mechanisms of nanomaterial toxicity. Part. Fibre Toxicol. 9, 20.
Sutterwala, F.S., Ogura, Y., Flavell, R.A., 2007. The inflammasome in pathogen recognition and inflammation. J. Leukoc. Biol. 82, 259–264.
Tenzer, S., Docter, D., Kuharev, J., Musyanovych, A., Fetz, V., Hecht, R., et al., 2013. Rapid formation of plasma protein corona critically affects nanoparticle pathophysiology. Nat. Nanotechnol. 8 (10), 772–781.
Toepfer, E., Italiani, P., Boraschi, D., 2015. Innate immune memory: the latest frontier of adjuvanticity. J. Immunol. Res. 2015, 748448.
Valledor, A.F., Comalada, M., Santamaria-Babi, L.F., Lloberas, J., Celada, A., 2010. Macrophage proinflammatory activation and deactivation: a question of balance. Adv. Immunol. 108, 1–20.
Vallhov, H., Qin, J., Johansson, S.M., Ahlborg, N., Muhammed, M.A., Scheynius, A., et al., 2006. The importance of an endotoxin-free environment during the production of nanoparticles used in medical applications. Nano Lett. 6, 1682–1686.

van Zijverden, M., Granum, B., 2000. Adjuvant activity of particulate pollutants in different mouse models. Toxicology 152, 69–77.

Vauthier, C., Lindner, P., Cabane, B., 2009. Configuration of bovine serum albumin adsorbed on polymer particles with grafted dextran corona. Colloids Surf. B 69, 207–215.

Vemula, P.K., Anderson, R.R., Karp, J.M., 2011. Nanoparticles reduce nickel allergy by capturing metal ions. Nat. Nanotechnol. 6, 291–295.

Vonarbourg, A., Passirani, C., Saulnier, P., Simard, P., Leroux, J.C., Benoit, J.P., 2006. Evaluation of pegylated lipid nanocapsules versus complement system activation and macrophage uptake. J. Biomed. Mater. Res. A 78, 620–628.

Wallberg, M., Bergquist, J., Achour, A., Breij, E., Harris, R.A., 2007. Malondialdehyde modification of myelin oligodendrocyte glycoprotein leads to increased immunogenicity and encephalitogenicity. Eur. J. Immunol. 37, 1986–1995.

Wiechers, J.W., Musee, N., 2010. Engineered inorganic nanoparticles and cosmetics: facts, issues, knowledge gaps and challenges. J. Biomed. Nanotechnol. 6, 408–431.

Witasp, E., Shvedova, A.A., Kagan, V.E., Fadeel, B., 2009. Single-walled carbon nanotubes impair human macrophage engulfment of apoptotic cell corpses. Inhal. Toxicol. 21 (Suppl. 1), 131–136.

Xiang, S.D., Selomulya, C., Ho, J., Apostolopoulos, V., Plebanski, M., 2010. Delivery of DNA vaccines: an overview on the use of biodegradable polymeric and magnetic nanoparticles. Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol. 2, 205–218.

CHAPTER

14

Endocrine System

Xuefei Lu, Yong Zhu, Tao Zhu

University of Science and Technology of China, Hefei, Anhui, China

OUTLINE

Introduction 339

Which Endocrine Organs are Disrupted by NMs? 340

Hypothalamus 341
Pituitary Gland 341
Pineal Gland 341
Thyroid Gland 342
Parathyroid Gland 342
Gonads 343
Adrenal Gland 346
Pancreas 346
Thymus 347

How Do NMs Functionally Disrupt Endocrine Organs? 348

The Physicochemical Properties of NMs 349
The Exposure Patterns of NMs 349
The Tissue Distribution of NMs 351

The Biological Mechanisms of Effects on Organs 351

Take-Home Messages 353

References 353

INTRODUCTION

The two major systems responsible for body control and communication are the neurological system and endocrine system. The endocrine system consists of endocrine organs called endocrine glands, which are located in many parts of the body. They release chemical messengers named hormones, which travel through the circulatory system and distribute pervasively in the body (Rushton and Cooley, 2009). The endocrine system uses hormones to control responses that do not need to be immediate, but have to be long-lasting. These responses include growth, metabolic rate, sexual development, and reproduction. Hence, the

Adverse Effects of Engineered Nanomaterials. http://dx.doi.org/10.1016/B978-0-12-809199-9.00014-8
Copyright © 2017 Elsevier Inc. All rights reserved.

possible hazardous effects on the endocrine system may not be observed immediately but may have long-term impacts on our health. Indeed, the cases of diseases associated with endocrine disorders, such as reproductive disorders, obesity, diabetes, and cancers have increased significantly in recent years (Darbre, 2015).

During the last few decades, experimental, clinical, and epidemiological studies have focused on effects of environmental endocrine disrupters (EDs) on animals and humans. EDs have been defined as "an exogenous substance that causes adverse health effects in an intact organism, and/or its progeny, consequent to changes in endocrine function" (Waring and Harris, 2005). They may be naturally occurring or industrial chemicals in our food, water, and air. Researchers have demonstrated that they can disrupt normal hormone regulation in mammals, fishes, birds, reptiles, amphibians, aquatic invertebrates, and even human beings. It is reported that most EDs are organic, with some key structural similarities to steroid and thyroid hormone (TH) molecules and thereby can compete for binding to the hormone receptors (Waring and Harris, 2005).

Nanomaterials (NMs), which possess at least one diameter between 1 and 100 nm (Roco, 2003) have been engineered and introduced into our industries and daily life in recent years. These NMs may enter the body through the lung, skin, or intestinal tract and then translocate into many other organs (Wang et al., 2007). Hence, more and more studies have focused on their possible toxicity, as evidenced in other chapters in this volume. Recent in vivo and in vitro studies have suggested that some NMs exert adverse effects on different organs, such as lung, liver, kidney (Sharma et al., 2012; Wang et al., 2007), and even the central nervous system (Wang et al., 2008) (see Chapter 12). Additionally, some types of NMs can impair the development and function of certain endocrine organs with resultant altered expression of certain hormones (Iavicoli et al., 2013; Lu et al., 2013). The potential endocrine-disrupting effects of NMs on animals and human beings are emerging as an important part of nanotoxicity. However, the endocrine system is the aggregate of multiple, highly interconnected endocrine organs, the acting pattern of which are usually slow and long-lasting. Thus, it is a formidable challenge to collect data of a certain NM toward a single endocrine organ within human body. The use of animal models would be warranted for the exploration of NMs on the endocrine system. This chapter will seek to summarize the current studies on the diversified effects of NMs on different endocrine organs and their functions. Moreover, we will discuss the determinants and mechanisms of these effects. These evidences may link exposure to some NMs with specific human endocrine diseases.

WHICH ENDOCRINE ORGANS ARE DISRUPTED BY NMs?

Endocrine systems in different vertebrates are similarly composed and located (Norris and Carr, 2013). They mainly include hypothalamus, pituitary gland, and pineal gland in brain; thyroid and parathyroid glands located below the jaw; thymus gland in the chest; and the adrenal glands just above the kidney. In addition, there are other important endocrine organs, such as the pancreas which produces insulin and glucagon, ovaries in women, and testes in men (Wilson, 2009). Here, we present evidence of the adverse effects of NMs on these organs.

Hypothalamus

Hypothalamus is the "master" endocrine organ. Depending on the body needs, it produces "releasing" or "inhibiting" hormones. These hormones travel to and act on the neighboring pituitary gland (Wilson, 2009). However, until now, the endocrine-disrupting effects of NMs on the hypothalamus remain unclear, or have not been studied yet.

Pituitary Gland

Pituitary gland receives signals from the hypothalamus and then produces many hormones, such as growth hormone (GH) which stimulates cells to grow, divide, and multiply, thyroid-stimulating hormone (TSH) which stimulates thyroid gland to release THs, and follicle-stimulating hormone (FSH) and luteinizing hormone (LH) which are important to reproduction and development. In addition, this gland also secretes adrenocorticotropic hormone (ACTH) and antidiuretic hormone (ADH) (Wilson, 2009).

It is proven to be extremely difficult for NMs to enter the brain when they are administered to adult animals, due to the blood–brain barrier (BBB) which protects the brain from foreign matters (Tosi et al., 2008) (see Chapter 12). However, polymeric nanoparticles (NPs) including poly(ethylene glycol)-block polylactide methyl ether (PEG-*b*-PLA) NPs can cross the BBB without appreciable damage. Therefore, these NPs are one of the most promising carriers for delivering drugs into central nervous system (CNS) (Tosi et al., 2008). In the study on delayed effects of neonatal exposure to PEG-*b*-PLA NPs on pubertal development and reproductive function in female Wistar rats from postnatal day (PND) 4 to PND 176, female pups were intraperitoneally injected daily from PND 4 to PND 7 a dose of 20 or 40 mg/kg body weight (BW). Exposure at both doses accelerated the onset of vaginal opening compared with the control group. More important, in the low-dose group, numbers of regular estrous cycles reduced significantly, pituitary weight increased due to hyperemia, hypothalamic gonadotropin-releasing hormone stimulated inappropriate LH secretion, serum levels of progesterone increased, and vascular dilatation and congestion was observed (Rollerova et al., 2015).

Pineal Gland

The pineal gland, also known as pineal body, is a small endocrine gland in the vertebrate brain. It secretes melatonin to regulate the body's rhythms, including the reproductive cycles and circadian rhythm (Wilson, 2009). Notably, there has already been evidence that NMs may impose adverse effects on pineal gland (Jovanovic et al., 2011). In a study of NP microinjection into the optic vesicle of zebrafish embryos, the final concentration of titanium dioxide (TiO_2)NPs and hydroxylated fullerenes [$C_{60}(OH)_{24}$] was 8.5 μg/g BW and 2 ng/g BW, respectively, which are both sublethal doses (Jovanovic et al., 2011). After the microinjections, embryos were incubated for 48 h at 28.5°C. At 96 h postfertilization, it was found that exposure to TiO_2NPs and $C_{60}(OH)_{24}$ caused shifts in response patterns of gene regulation. The downregulated genes included genes related to circadian rhythm, for example, period homolog 2. In addition, exposure to TiO_2NPs or $C_{60}(OH)_{24}$ also leads to downregulated thyrotroph embryonic factor involved in the development of the pituitary gland. This is the first paper

reporting circadian rhythm gene deregulation by NPs in aquatic animals, indicating that NPs have potential effects on circadian rhythm (Jovanovic et al., 2011).

For these endocrine organs in the brain, polymeric NPs are most likely to have endocrine-disrupting effects due to the ability to cross the BBB. However, other NMs can cross the blood–placental barrier (BPB) and enter the fetal brain (Kulvietis et al., 2011). It is thus necessary to assess the effects of different kinds of NMs on the development of fetal endocrine organs. Finally, we should be mindful of the combined endocrine toxicity with neurotoxicity, which may be referred to as neuroendocrine toxicity.

Thyroid Gland

The thyroid gland is stimulated by pituitary gland to release THs called triiodothyronine (T3) and thyroxine (T4). These two hormones are necessary for the body to turn food into energy. The thyroid also produces calcitonin, which plays a role in calcium homeostasis (Wilson, 2009). A study assessed the effects of three NMs, AgNPs, quantum dots (QDs), and zinc oxide (ZnO)NPs having a size of about 10 nm, on TH signaling in frog tissue using cultured tail fin biopsy (C-fin) assay derived from *Rana catesbeiana* tadpoles (Hinther et al., 2010). The C-fin assay maintains the tissue complexity and biological replication while multiple chemical responses can be assessed from the same individual. The biopsies were pretreated with AgNPs (0.06 μg/L–5.5 mg/L), QDs comprised of cadmium telluride (0.25 μg/L–22 mg/L) and ZnONPs (0.19–10 mg/L), respectively in cultured media for 2 h prior to T3 supplement (Hinther et al., 2010), added into media at a final concentration of 10 nM. The biopsies were then incubated for *a* further 48 h. Researchers found that AgNPs (≥2.75 mg/L) and QDs (≥0.22 mg/L) altered the mRNA expression of TH β (TRβ) and Rana larval keratin I (RLKI) in the presence or absence of T3, while ZnONPs had no effect. TRβ transcript levels increased (1.4-fold) when exposed to 0.1 and 1 nM AgNPs compared to T3 alone, while the highest AgNP concentration (10 nM) attenuated the T3-induced increase in TRβ transcript levels compared to T3 alone. In contrast, the T3-dependent decrease in RLKI transcripts was magnified threefold by exposure with both 10 nM AgNPs and T3 compared to T3 alone. The observed effects occurred at or below the current North American water quality guidelines for metals, suggesting that NMs may alter TH signaling in vivo (Hinther et al., 2010). More recently, cobalt ferrite ($CoFe_2O_4$) magnetic NPs were reported to cause the impairment of thyroid endocrine system in zebrafish larvae. Therein, NPs were microinjected into the fertilized and normal embryos at different doses (0, 10, 62.5, 125, 250, and 500 μM). After 168 h postfertilization, the amounts of T4 and T3 hormones increased in the group exposed to doses of 62.5 and 125 μM (Ahmad et al., 2015). By contrast, in another study, after male Sprague-Dawley rats were exposed to chromium NPs in basal diet supplemented at final concentration of 150, 300, or 450 μg/kg BW for 8 weeks under heat stress condition, no significant alteration in T3 and T4 levels was observed (Zha et al., 2009). Clearly, more studies on NM effects on thyroid function in various animal models are required.

Parathyroid Gland

The parathyroid gland secretes parathyroid hormone (PTH) to control calcium concentration in the blood (Wilson, 2009). However, to our knowledge, there has been no report to date assessing the potential effects of NMs on the parathyroid gland.

Gonads

Gonads, ovaries in females and testes in males, release sex hormones. These hormones play important roles in sexual development and reproduction (Wilson, 2009). Many studies have focused on the adverse effects of NM exposure on gonads. It is remarkable that many types of NMs lead to gonad damages and abnormal reproductive functions in different animal models (Kong et al., 2014; Moridian et al., 2015; Pietroiusti et al., 2011; Yamashita et al., 2011). Effects of NMs on fertility and pregnancy are discussed in Chapter 17. Here we discuss effects of NMs on gonads in mammals and other organisms.

Gonads in Mammals

Metal-based NMs, including metal NPs, QDs, and metal oxide NMs, are able to induce toxic effects in ovaries (Kong et al., 2014; Yamashita et al., 2011). For example, researchers reported endocrine-disrupting effects and reproductive toxicity of nickel (Ni)NPs (Kong et al., 2014). The average diameter of NiNPs was 90 nm. There was slight agglomeration. Sprague-Dawley rats were exposed by gavage to NiNPs at the doses of 5, 15, and 45 mg/kg BW for 18 weeks. In female rats, NiNPs elevated serum levels of FSH and LH, and lowered estradiol (E2) at the doses of 15 and 45 mg/kg. Additionally, ovarian lymphocytosis, vascular dilatation and congestion, inflammatory cell infiltration, and increase in apoptotic cells were observed in ovary tissues after exposure to NiNPs (Kong et al., 2014).

More importantly, the adverse effects of NMs by exposure to pregnant mice and fetuses have been reported (Pietroiusti et al., 2011; Yamashita et al., 2011). Yamashita et al. (2011) demonstrated that TiO_2NPs, with diameters of 35 nm, resulted in pregnancy complications after intravenous injection at the dose of 0.8 mg per mouse through tail vein into pregnant BALB/c mice (8–10 weeks) on two consecutive days, namely gestational day (GD) 16 and GD 17. The effects of TiO_2NPs were compared with those of another kind of NMs, silica NPs with diameters of 70 or 300 nm (0.8 mg per mouse). After 24 h, these two NPs were observed in the placenta, fetal liver, and fetal brain. Mice treated with these two NPs had smaller uteri and smaller fetuses than untreated controls. Further, the placenta of mice treated with 70 nm silica NPs showed variable structural abnormalities when compared to control mice. However, these changes were not found in pregnant mice treated with silica NPs with diameters of 300 and 1000 nm (Yamashita et al., 2011).

In addition, carbon-based NMs are another type of NMs, which are commonly investigated for the reproductive toxicity in female mammals (Pietroiusti et al., 2011). Single-wall carbon nanotubes (SWCNTs) can cause damage to the uterus and embryotoxicity in pregnant mice. A study showed that after intravenous injection of pristine SWCNTs (pSWCNTs) and oxidized SWCNTs (oSWCNTs) at a dose of 30 μg per mouse at GD 5.5, both SWCNTs induced gross fetal morphological abnormalities at GD 15.5 (Pietroiusti et al., 2011). Further lower dosages (0.1 and 0.3 μg per mouse) still resulted in the gross malformations of fetuses. Extensive vascular lesions and increased production of reactive oxygen species (ROS) were detected in placentae of malformed fetuses but not in normal fetuses (Pietroiusti et al., 2011). Whether these reproductive anomalies are produced by, or associated with, endocrine dysfunction remains to be determined.

However, another recent study found no toxic effects on fetal and maternal mice after exposure of gold NPs (AuNPs) to pregnant mice (Yang et al., 2012). Pregnant mice were given 13 nm AuNPs with different surface modifications (ferritin, PEG, and citrate) by intravenous

injection at doses of 0.9–7.2 μg/g BW at different gestational ages between embryonic day (E) 5.5 and 15.5. Prior to E 11.5, all NPs were detected in fetal tissues in significant amounts, but fetal gold levels declined dramatically after E 11.5. On the contrary, AuNP accumulation in the extraembryonic tissues (EET), including placenta, amnion, and uterine wall, increased 6- to 15-fold with gestational age. No AuNP toxicity to maternal mice, fetus, and even offsprings after birth were observed (Yang et al., 2012). This study consisted in a long-term experiment that explored the effects on pregnant mice at different gestational ages, fetus, and offsprings.

In summary, in these studies, researchers estimated the endocrine-disrupting effects of NMs by detecting serum levels of FSH, LH, and E2, observing pathological changes in ovaries and finally testing reproductive ability after exposure to NMs through different routes. Moreover, gestation is a special stage for females as fetuses are known to be more sensitive to environmental toxins than adults (Hurst et al., 1998), so particular attention should be paid to embryotoxicity of NMs. However, whether these abnormal development of embryos are induced by endocrine dysfunction remains to be determined.

As far as male mammals are concerned, many NMs are also found to cause adverse effects on reproductive organs and functions (Kong et al., 2014; Li et al., 2013; Moridian et al., 2015; Yoshida et al., 2009, 2010). Consistently we will summarize the endocrine-disrupting effects according to the types of NMs, respectively.

For example, after NiNP exposure at the dose of 15 and 45 mg/kg BW, the body weights gradually decreased, the ratio of epididymis weight over body weight increased, the motility of rat sperm was altered, and serum levels of testosterone (T) and FSH diminished (Kong et al., 2014). Moreover, pathological changes, such as disordered arrangement of cells in the tube and appearance of cell apoptosis and death in the testes of the NiNP exposure group were observed. This study provided systemic and comprehensive evidences that NMs can disturb hormone regulation and cause pathological changes in both ovaries and testes. In addition, it has also been reported that modified AuNPs can induce changes in plasma testosterone levels in male mice without affecting fertility (Li et al., 2013). In this study, mPEG@AuNPs (methoxy poly(ethylene glycol)-capped AuNPs) or PEG-NH^2@AuNPs (co-aminoethyl poly(ethylene glycol)-capped AuNPs) were intravenously injected into male ICR (Institute of Cancer Research) mice at the dose of 45 or 225 mg/kg BW at day 0, 2, and 4. Both mPEG@AuNPs and PEG-NH^2@AuNPs accumulated and then remained in the testes. Furthermore, by using transmission electron microscopy (TEM), the authors demonstrated that mPEG@AuNPs penetrated through the blood–testis barrier and accumulated in vesicles of spermatogonia (Li et al., 2013). PEG-NH^2@AuNP exposure for 7, 14, 21, and 30 days induced testosterone (T) release and consequently increased aggressive behavior at all-time points, while mPEG@AuNPs did not (Kong et al., 2014). However, no apparent changes were observed in testicular morphology or germ cell apoptosis (Li et al., 2013).

In a study of carbon black (CB)NPs associated endocrine toxicity (Yoshida et al., 2009), CBs with three sizes (14, 56, and 95 nm) were intratracheally administered (0.1 mg per mouse for 10 times every week) to male mice to determine possible adverse effects on reproduction. Serum testosterone level was significantly elevated in the 14 and 56 nm CB-exposed groups but not in 95 nm CB group. Partial vacuolation of Sertoli cells, in which testosterone is produced in male animals, was observed in all of CB-exposed groups (14, 56, and 95 nm) (Yoshida et al., 2009). In addition, in another study, 14 nm CBNPs were injected through tail vein to pregnant mice on GD 7 and 14 and reproductive functions of male offsprings were

determined at the age of 5, 10, and 15 weeks after birth (Yoshida et al., 2010). Daily sperm production was significantly decreased in male offsprings at the age of 15 weeks. However, CB administration exerted no marked effects on body weight, testicular weight, epididymal weight, or serum testosterone levels (Yoshida et al., 2010).

It is found that the adverse effects of certain NMs on testes may be reversible (Bai et al., 2010; Xu et al., 2014). Silica NPs (diameters of 64 nm) were administered to 8-week-old male ICR mice via tail vein every 3 days, 5 times over 13 days. Each dose of amorphous silica NPs was 20 mg/kg (Xu et al., 2014). Silica NP exposure was shown to cause damages to the mitochondrial cristae and decrease the levels of ATP, resulting in oxidative stress in the testis by day 15 and 35. However, by day 60 these damages were recovered. In addition, DNA damages and decreases in the quality and quantity of epididymal sperm were observed by days 15 and 35; also these changes disappeared by day 60. There were no observed effects on acrosome integrity and fertility in epididymal sperm, the numbers of spermatogonia and sperm in the testes, and the levels of sex hormones throughout the whole 60-day period (Xu et al., 2014).

Gonads in Other Animals

In addition to mammals, there are also many studies on reproductive nanotoxicity in other kinds of animals including *Caenorhabditis elegans* (*C. elegans*) (Qu et al., 2011), *Chironomus riparius* (*C. riparius*) (Nair et al., 2011), and *Drosophila melanogaster* (Philbrook et al., 2011). It is equally imperative to investigate the endocrine toxicity in these animals as the endocrine toxicity of a single organism may lead to ecological impact.

It has been reported that CdSe@ZnS core–shell QDs or CdTe QDs accumulated in the reproductive system of *C. elegans* and induced reproductive dysfunction including difficulty in egg laying and damaged eggs after long-term exposure (16 days) at a dose of 200 nM, while no adverse reproductive effects were observed in either female or male *C. elegans* after exposure for less than 3 days (Qu et al., 2011). In the case of *Drosophila*, male and virgin female adults were collected within 8 h of eclosion. Each group contained three or four males and two females and was placed in a vial containing standard molasses culture medium in a removable cap (Philbrook et al., 2011). Caps were replaced every 12 h for 14 days and the number of eggs laid in a 24-h period was recorded beginning on the second day after eclosion, when they had reached sexual maturity. Each experiment contained seven groups: a vehicle control, a microparticle (MP) control [0.05% weight/volume (w/v)], and five different concentrations of TiO_2 or AgNPs (0.005, 0.01, 0.05, 0.1, 0.5% w/v) (Philbrook et al., 2011). TiO_2NPs ($\geq$0.005% w/v) dramatically affected female fecundity, but did not impact developmental success calculated from egg or first instar larvae to adulthood. In contrast, AgNP feeding led to no significant decline in fecundity, but caused a significant reduction in developmental success compared to control and TiO_2-exposed group (Philbrook et al., 2011).

In another study, the invertebrate, *C. riparius* was used to investigate the toxicity of AgNPs. Researchers observed that egg numbers per egg mass decreased significantly in reproduction upon exposure to AgNPs at the concentration of 1 mg/L for 25 days (Nair et al., 2011). Chronic exposure to AgNPs upregulated the expression of gonadotropin-releasing hormone gene (CrGnRHl), which is crucial to reproductive physiology, leading to reproductive failure. These data suggest that AgNP exposure toward aquatic organisms may enhance gonadotropin-releasing hormone–mediated signaling and its related reproductive failure, resulting in decreased rates of pupation and adult emergence (Nair et al., 2011). Moreover,

among emerged adults, the average number of males was less than that of females, showing an alteration in the sex ratio relative to control groups.

Adrenal Gland

The adrenal gland consists of two distinct parts, the outer adrenal cortex and the inner medulla, either of which produces different hormones. The adrenal cortex produces a variety of steroid hormones, such as aldosterone, cortisol, and androgens. On the other hand, in the medulla, there are chromaffin cells which are the main source of adrenaline and noradrenaline. These two hormones function to produce rapid response throughout the body under stress situation (Wilson, 2009).

Many NMs, such as AgNPs, QDs, and CNTs are reported to exert adverse effects on chromaffin cells in vitro. For instance, researchers used neurosecretory mouse chromaffin cells of adrenal gland to test the effects of CdSe-ZnS core–shell QDs (5–36 nM) on Ca^{2+} channels' functionality and Ca^{2+}-dependent neurosecretion. Prolonged exposure (24 h) to QDs (≥16 nM) showed that the semiconductor nanocrystal was efficiently intracellularly internalized without affecting cell integrity (Gosso et al., 2011). QDs significantly reduced the size of Ca^{2+} currents in a voltage-independent manner without affecting channel gating, the size of the readily releasable pool (RRP) of secretory vesicles, the frequency of release, and the overall quantity of released catecholamines (Gosso et al., 2011). These data suggest that exposure to CdSe-ZnS QDs impairs the overall catecholamine supply from chromaffin cells.

In addition, researchers found that serum levels of cortisol decreased remarkably after exposure by gavage with 150, 300, or 450 μg/kg BW. However, the functional effects of cortisol like the level of blood sugar were not tested (Kong et al., 2014).

Pancreas

The pancreas produces insulin and glucagon which work together to regulate sugar concentration in the blood (Wilson, 2009). In the study assessing the effects of chromium NPs, exposure by gavage with 150, 300, or 450 μg/kg BW significantly decreased serum levels of insulin in male in Sprague-Dawley rats. This may imply that chromium NPs can disrupt regulation of sugar concentration in the blood (Kong et al., 2014).

Meantime, there are in vitro evidences that NMs may influence insulin signaling (Gurevitch et al., 2012). TiO_2NPs might impair insulin response in Fao cells by indirect inflammatory activation of macrophages and/or direct interference with insulin signaling (Gurevitch et al., 2012). Fao cell line is a well-differentiated subclone derived from the rat hepatoma cell line. Conditioned medium (CM) from 100 μg/mL TiO_2NP-treated macrophages significantly attenuated tyrosine phosphorylation of the insulin receptor substrate proteins IRS1 and IRS2 and Ser phosphorylation of GSKβ, all critically involved in the insulin-signaling pathway. It is suggested that TiO_2NP exposure impaired the cellular response to insulin stimulation in Fao cells and produced insulin resistance (Gurevitch et al., 2012). Furthermore, direct exposure to TiO_2NPs (50 or 200 μg/mL) affected insulin responses in liver-derived cells and activated the stress kinases JNK and p38MAP kinase, which interfere with insulin signaling (Gurevitch et al., 2012). Hence, this study provides a proof-of-concept for the capacity of TiO_2NPs to induce insulin resistance which may eventually underlie diabetes.

Thymus

The thymus is an immune organ controlling the development of lymphocytes. It also produces thymopoietins and thymosins, which are hormones important to the immune system (Wilson, 2009). It is reported that NMs can be distributed from the systemic circulation to thymus and can also decrease the weight of the thymus (Zhang et al., 2010). However, there has been no single evidence that NMs can have endocrine-disrupting effects on thymus yet.

In conclusion, the endocrine-disrupting effects of NMs are summarized in Fig. 14.1. However, there is a lack of data on the potential effects of NMs on certain endocrine organs and further studies are warranted. Specifically, more in-depth, long-term, and systemic studies are warranted to investigate the serum hormone levels, pathological changes, the expression of genes involved in the development, and function of endocrine organs in adult or fetal animals. More importantly, the behavioral and functional effects on animals as well as their progenies should also be addressed. Since some NMs can spread throughout the whole body after exposure, they may exert effects on various endocrine organs and functions. Therefore, it is imperative to investigate endocrine functions of the whole body, not only a certain endocrine organ. Also, it would be desired to evaluate the endocrine-disrupting effects while assessing the genotoxicity, neurotoxicity, immunotoxicity, reproductive toxicity, embryotoxicity, and even ecotoxicity of NMs (Fig. 14.2). The endocrine-disrupting effects on a single organism may potentially disturb the balance of the whole population. The disturbance may

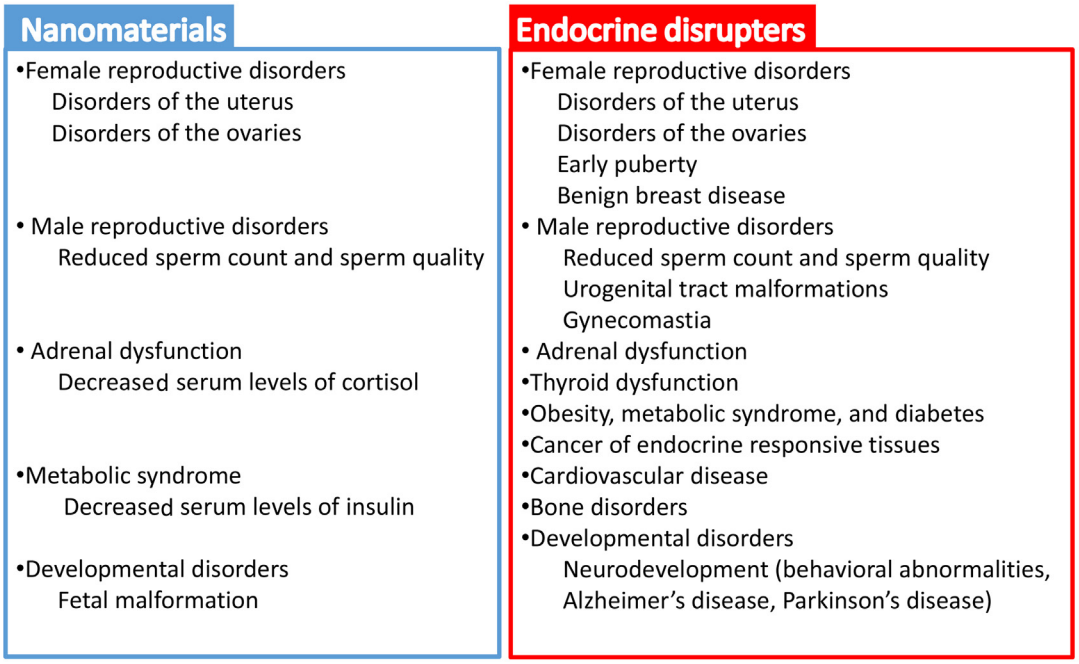


FIGURE 14.1 Endocrine disorders reported to be affected by nanomaterials (NMs) compared to those caused by classical endocrine disrupters (EDs). *Source: Modified from Darbre, P.D., 2015. Endocrine Disruption and Human Health. Elsevier Science.*

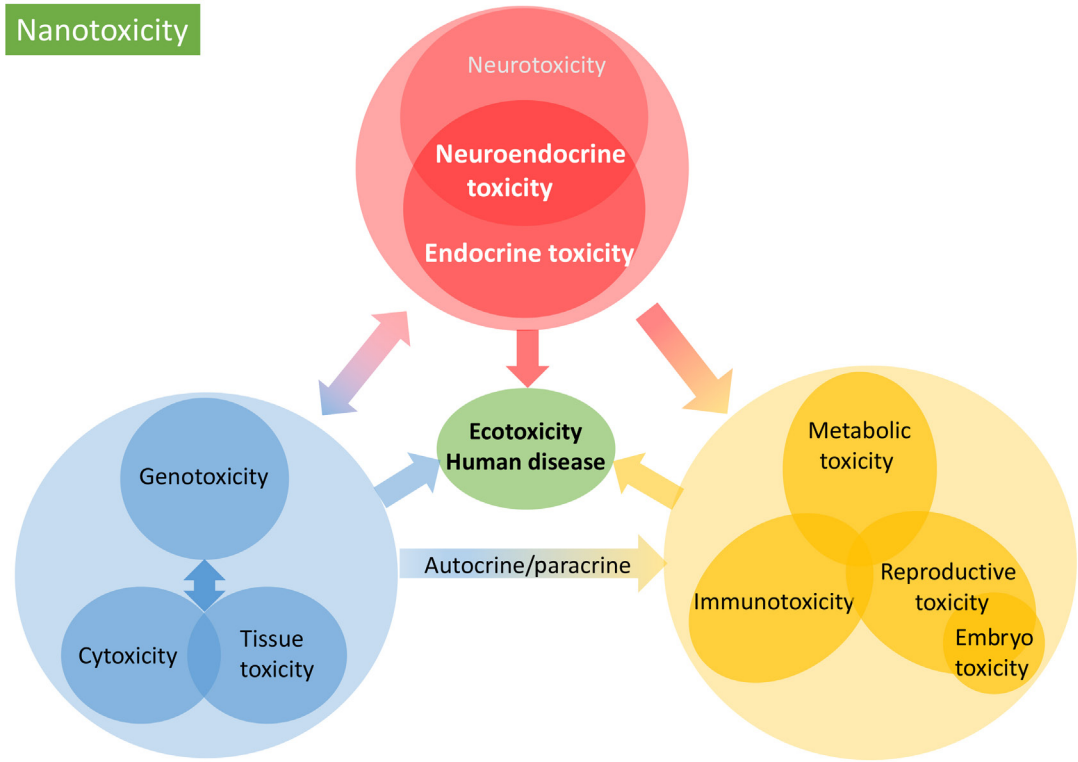


FIGURE 14.2 **The role of endocrine toxicity in the context of nanotoxicity.**

be disastrous for the whole ecology including human beings. Moreover, with mass production of certain NMs, for instance, TiO_2NPs, epidemiological studies are urgently required to determine the possible endocrine-disrupting impairment on wildlife and human.

HOW DO NMs FUNCTIONALLY DISRUPT ENDOCRINE ORGANS?

Endocrine organs regulate the body through hormones. First, they are secreted by endocrine glands and carried by the blood to target cells in the distant organ. Before transported in the blood, they are often both modified by conjugation and bound to carrier proteins. Finally, at target sites, the free (bioavailable) hormones bind to corresponding cellular receptors, which then alter the downstream signal in the target cells to regulate the functions in the target organs. Like EDs, some NMs may directly disturb any of these processes, including the hormone synthesis, secretion, conjugation, binding to carrier proteins, and the binding to receptors or modifying receptor levels. Moreover, the endocrine-disrupting effects through some pathways described earlier have been proved (Jain et al., 2012; Ramdhan et al., 2009). In addition, NMs may indirectly contribute to endocrine-disrupting effects

induced by pathological changes, such as inflammation and fibrosis, in other systems. In the following section, we will discuss the factors which may contribute to endocrine-disrupting effects of NMs.

The Physicochemical Properties of NMs

Each type of NMs may exert different endocrine-disrupting effects according to the specific physical and chemical properties (Powell and Kanarek, 2006). These properties include size, surface modification, shape, agglomeration state, and so on (Rivera Gil et al., 2010). For example, CBNPs with three sizes (14, 56, and 95 nm) were administered intratracheally (0.1 mg per mouse for 10 times every week) to male mice, and the authors found that serum testosterone levels were significantly elevated in the 14 and 56 nm CB-exposed groups, but not in the 95 nm CB group (Yoshida et al., 2010). Surface modification is another property that has attracted attention (Love et al., 2012). In a study discussed earlier, after intravenous injection of pSWCNTs and oSWCNTs, the percentage of early miscarriages and fetal malformations was significantly higher in females exposed to oSWCNTs than in those exposed to pristine materials (Pietroiusti et al., 2011). In another example, the endocrine-disrupting effects were observed in male mice exposed to PEG-NH^2@AuNPs while no adverse effects were observed in those exposed to mPEG@AuNPs (Li et al., 2013). Hence, surface modification influences or even determines the endocrine toxicity of NMs.

In addition to the physical and chemical properties of NMs themselves, impurities mixed with NMs like catalysts during synthesis may have a causal role in inducing the endocrine-disrupting effects. Undoubtedly, the physical and chemical properties of NMs are major determinants of their endocrine-disrupting effects. Thus, it is warranted to compare the endocrine-disrupting effects of the same kind of NMs while possessing different physicochemical properties.

The Exposure Patterns of NMs

To define whether NMs are harmful to the endocrine system, we need to consider the exposure patterns of NMs, including exposure routes, dose, and duration. Indeed, the same NMs may have different effects on endocrine functions depending upon the exposure.

The entry route is a major factor which affects the endocrine-disrupting effects of NMs. For vertebrate animals, the exposure routes used in experiments include inhalation, oral entry (water or food), intraperitoneal injection, and intravenous injection (Hoet et al., 2004). Among them, intravenous injection results in greater uptake than other routes (Crain and Guillette, 2000). Inhaled or oral NMs can be partially eliminated while some can be absorbed into the bloodstream. Then they may be transported to organs including endocrine organs via circulation (Hoet et al., 2004). EDs can pass through the liver, wherein they are metabolically detoxificated (Crain and Guillette, 2000). Since it is difficult for NMs to enter the body by dermal exposure (Hoet et al., 2004), few studies explore the adverse effects on endocrine organs or their functions after dermal exposure of NMs.

For invertebrate animals, NMs are often added into the environment. For example, aquatic animals are exposed to NMs in the water and flies are cultured in molasses culture medium supplemented with NMs.

Due to different usage, different NMs enter the bodies with different efficiency via different routes. For example, CBNPs are mainly taken into the body through inhalation, for example, from laser printer emission or through dermal uptake from cosmetics (Schmid and Riediker, 2008), so it is more meaningful to investigate the adverse effects of these NPs exposed by inhalation, intratracheal instillation (Yoshida et al., 2010), or dermal uptake. However, there are few studies which compare the endocrine-disrupting effects of the same kind of NMs at the same dose by different routes.

Endocrine-disrupting effects of NMs are induced in a dose-dependent manner. Many studies investigate the endocrine-disrupting effects of NMs at different doses and subsequently determine the lowest effective dose (Gavello et al., 2013; Gosso et al., 2011; Kong et al., 2014; Philbrook et al., 2011; Pietroiusti et al., 2011). For example, SWCNTs (from 10 ng to 30 μg per mouse) were administered to female mice soon after implantation (postcoital day 5.5); 10 days later, animals were sacrificed, and uteri, placentas, and fetuses examined. The lowest effective dose inducing miscarriage and fetal malformation was 100 ng per mouse (Pietroiusti et al., 2011).

However, there is a study showing that a relatively low dose of NP-rich diesel exhaust (NR-DE) induced endocrine-disrupting effects, while high dose exposure did not (Ramdhan et al., 2009). NR-DE exposure at low (22.5 ± 0.2 nm in diameter, 15.4 ± 1.0 mg/m^3 in mass weight, 2.27 × 10^5 cm^{-3} in mean number concentration) and medium (26.1 ± 0.5 nm, 36.4 ± 1.2 mg/m^3, 5.11 * 10^5 cm^{-3}) concentrations for 1 and 2 months (5 h/day, 5 days/week) significantly increased plasma testosterone in male Fischer 344 rats, whereas exposure to a high concentration (27.1 ± 0.5 nm, 168.8 ± 2.7 mg/m^3, 1.36 * 10^6 cm^{-3}) did not (Ramdhan et al., 2009). The reason why relatively low doses, but not high doses of NR-DEs induced endocrine-disrupting effects remains unclear, although a partial explanation is that EDs can act through receptor-mediated mechanisms, which allows them to act at very low doses and in a nonlinear manner. The dose–response curves can take many different forms (Crain and Guillette, 2000). Furthermore, for some EDs, effects at low doses may not be predictive of effects at high doses because of different mechanisms of action (Crain and Guillette, 2000).

The duration of exposure is also an important factor. For example, treatment with CdSe@ZnS core–shell QDs or CdTe QDs induced difficulty in egg laying and damaged eggs after long-term exposure (16 days) at a dose of 200 nM in female *C. elegans*, while no adverse reproductive effects were observed after exposure for less than 3 days (Qu et al., 2011). Since endocrine responses are often long-lasting and have a long latency, the relative long-term exposure may be required to evaluate the effect. In addition, since endocrine responses are often long-term and slow, there may be a latency period before NM-triggered effects are manifested. The latency period may be very short, such as 24 h, but sometimes it could be relatively long, up to weeks. For example, after 14 nm CBNP injection via tail vein to pregnant mice on GD 7 and 14, daily sperm production was significantly decreased in male offsprings of maternal mice exposed to CBNPs at the age of 15 weeks, but not at the age of 5 or 10 weeks (Yoshida et al., 2010). However, another report found that just 24 h after intravenous injection of TiO_2NPs (diameters of 35 nm) or silica NPs (diameters of 70 nm) at the dose of 0.8 mg per mouse via tail vein into pregnant BALB/c mice (8–10 weeks) on two consecutive days, GD 16 and GD 17, mice treated with NPs had smaller uteri and smaller fetuses compared to the untreated controls (Yamashita et al., 2011).

Nevertheless, endocrine-disrupting effects may also be reversible. In a recent study, silica NPs were injected at the dose of 20 mg/kg via tail vein into male ICR mice every 3 days,

5 times over 13 days (Xu et al., 2014). The study showed that silica NPs caused damages to mitochondrial cristae and decreased the levels of ATP, resulting in oxidative stress in the testis by days 15 and 35. However, the damage was repaired by day 60. Further, DNA damage and the decreases in the quantity and quality of epididymal sperm were found by days 15 and 35, but these changes were recovered by day 60 (Xu et al., 2014).

The critical windows of susceptibility to NMs should be carefully considered. Some NMs can induce endocrine-disrupting effects in adult animals (Yoshida et al., 2010), but other NMs exert adverse effects in fetal or neonatal animals, and not in adults (Pietroiusti et al., 2011; Yamashita et al., 2011). Hence, when endocrine-disrupting effects of NMs are investigated, the timing of the exposure should be prudently considered.

The Tissue Distribution of NMs

The translocation and biodistribution of NMs into endocrine organs suggest that these materials are potentially detrimental to these organs and their functions like hormone synthesis and release. The internal amount of NMs may indicate the severity of the endocrine-disrupting effects. Hence, NM measurement in endocrine tissues is useful to determine the endocrine-disrupting effects. Measurement of body burdens of NMs requires the collection of tissue samples. For AuNPs and magnetic NPs, one can make use of inductively coupled plasma mass spectrometry (ICP-MS) to measure the amounts in endocrine organs (Li et al., 2013; Yang et al., 2012). However, for other NMs it is more difficult to quantify the amount of NMs. In some experiments, researchers labeled NMs to track or examine their biodistribution (Bai et al., 2010; Yamashita et al., 2011). For example, the author used ^{64}Cu-labeled carboxylated carbon nanotubes to examine the accumulation of carbon nanotubes after they were intravenously injected into male mice and the material was found in the testes within 24 h (Bai et al., 2010). It is certain that NMs can be distributed into the gonads, while there are few studies yet to investigate the biodistribution of NMs in other endocrine organs.

THE BIOLOGICAL MECHANISMS OF EFFECTS ON ORGANS

The pathological alteration in endocrine organs was often observed after NM exposure in vivo (Kong et al., 2014; Yoshida et al., 2009, 2010). For example, ovarian lymphocytosis, vascular dilatation and congestion, inflammatory cell infiltration, and increase in apoptotic cells were found in ovary tissues after exposure to NiNPs (Kong et al., 2014). More importantly, NM exposure disturbs normal hormone biosynthesis and release (Nair et al., 2011; Ramdhan et al., 2009). AgNPs exposure in the fourth instar larvae *C. riparius* upregulated CrGnRHl expression and lead to reproductive failure (Nair et al., 2011). For mammals, after female Sprague-Dawley rats were exposed by gavage to NiNPs, serum levels of FSH and LH increased and E2 lowered (Kong et al., 2014). Another example is that inhaled exposure to NR-DE for 1 and 2 months (5 h/day, 5 days/week) significantly increased plasma testosterone in male Fischer 344 rats (Ramdhan et al., 2009). The authors found NR-DE exposure also significantly increased mRNA and protein expression of steroidogenic acute regulatory protein (StAR) and cytochrome P450 side-chain cleavage (P450scc) in the testis of rats, the elevation pattern of which was very similar to that of plasma testosterone levels. Interestingly, exposure

for 1 month significantly increased GH receptor expression in the testis, and exposure at low dose also increased expression of testicular insulin-like growth factor I and hepatic microsomal cytochrome P450 2C11 in rats. These two factors are thought to be related to GH secretion. Hence, disruption of testosterone levels by NR-DE exposure may, in part, be regulated by increasing StAR and P450scc expressions via GH signaling (Ramdhan et al., 2009). NMs can also alter the expression of genes involved in special endocrine functions like circadian rhythm. For instance, after microinjections of TiO_2NPs and hydroxylated fullerenes [$C_{60}(OH)_{24}$] into embryos, exposure to TiO_2NPs and $C_{60}(OH)_{24}$ caused shifts in gene regulatory response patterns involved in circadian rhythm in embryos (Jovanovic et al., 2011).

In addition to impairing endocrine organs and hormone biosynthesis and release, some NMs can disturb hormone binding with the receptor on the target cells or the downstream signaling (Jain et al., 2012). For instance, CdTe QDs induce estrogen receptor (ER) activation as well as nongenomic activation of MAPK and PI3K, similar to 17β-estradiol in the ER-positive MCF-7 breast cancer cell line (Jain et al., 2012). Green CdTe QDs (2.81 nm in diameter) were observed to elicit a more robust estrogenic response than orange QDs (3.10 nm in diameter). Green QDs were visualized within the nucleus, whereas larger orange QDs remain in the cytoplasm (Jain et al., 2012). The selective estrogen receptor antagonist, ICI182780, abolished the estrogenic effects of green or orange QDs, indicating that QD-induced estrogenic signaling was indeed mediated by estrogen receptors. In vivo, chronic treatment of mice with QDs, produced a two- to threefold increase in uterine weight compared to the control group (Jain et al., 2012).

Fig. 14.3 summarizes the confirmed biological mechanisms of NMs on endocrine functions. In addition, it is worth noting that EDs can compete with hormones for binding to carrier

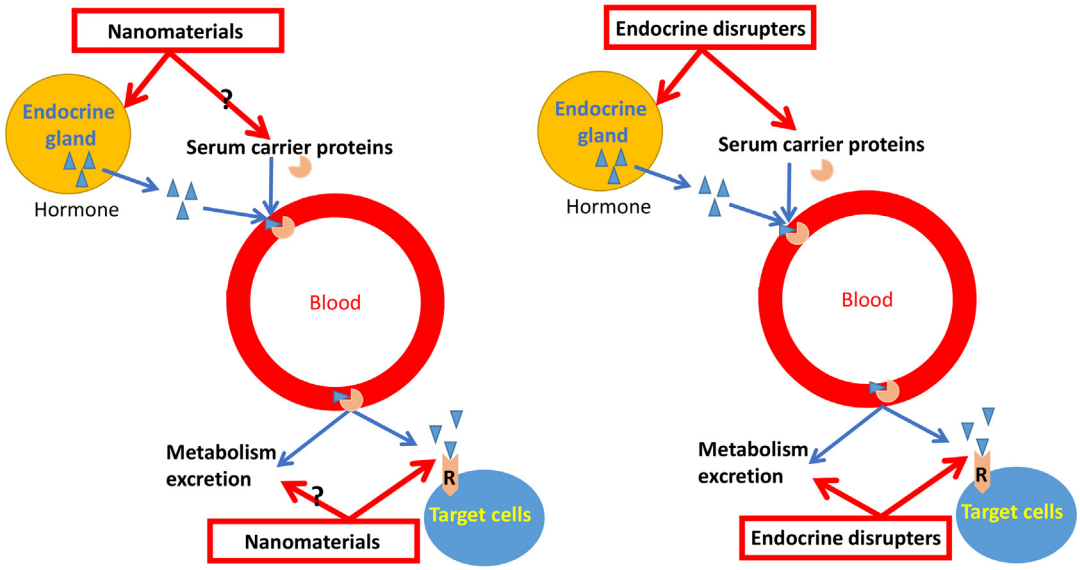


FIGURE 14.3 Biological mechanisms by which NM exposure cause endocrine-disrupting effects compared to those of classical EDs. *Source: The right part of this figure was modified from Darbre, P.D., 2015. Endocrine Disruption and Human Health. Elsevier Science.*

proteins, which alter the levels of unbound bioavailable steroid and can disturb hormone metabolism through conjugation of steroids (Crain and Guillette, 2000). NMs can also interact with serum proteins (Walkey et al., 2012). However, whether NMs can exert endocrine-disrupting effects through these two mechanisms remains unclear.

Finally, some studies referred to previously showed that NM exposure to animals can influence the offspring (Philbrook et al., 2011; Yoshida et al., 2010) These transgenerational effects may cause ecotoxicity of NMs, which could be profound and long-lasting.

TAKE-HOME MESSAGES

- NM exposure can alter serum level of hormones, disturb normal endocrine function, and potentially cause endocrine disorders.
- Exposure to some NMs have been reported to exert adverse effects on offsprings. Epidemiological studies are required to investigate the endocrine-disrupting effects on humans.
- Many factors should be considered when investigating the endocrine-disrupting effects of NMs. These include physicochemical properties, synthetic process, as well as the dose, route, and duration of exposure.
- Since the endocrine system plays an important role in regulating other systems through hormones, a thorough investigation of endocrine toxicity of NMs is highly warranted.

References

Ahmad, F., Liu, X., Zhou, Y., Yao, H., Zhao, F., Ling, Z., Xu, C., 2015. Assessment of thyroid endocrine system impairment and oxidative stress mediated by cobalt ferrite ($CoFe_2O_4$) nanoparticles in zebrafish larvae. Environ. Toxicol.doi: 10.1002/tox.22206.

Bai, Y.H., Zhang, Y., Zhang, J.P., Mu, Q.X., Zhang, W.D., Butch, E.R., Snyder, S.E., Yan, B., 2010. Repeated administrations of carbon nanotubes in male mice cause reversible testis damage without affecting fertility. Nat. Nanotechnol. 5, 683–689.

Crain, A., Guillette, L.J., 2000. Environmental Endocrine Disruptors: An Evolutionary Perspective. Taylor & Francis, New York, NY.

Darbre, P.D., 2015. Endocrine Disruption and Human Health. Elsevier Science.

Gavello, D., Fenoglio, I., Fubini, B., Cesano, F., Premoselli, F., Renna, A., Carbone, E., Carabelli, V., 2013. Inhibition of catecholamine secretion by iron-rich and iron-deprived multiwalled carbon nanotubes in chromaffin cells. Neurotoxicology 39, 84–94.

Gosso, S., Gavello, D., Giachello, C.N.G., Franchino, C., Carbone, E., Carabelli, V., 2011. The effect of CdSe-ZnS quantum dots on calcium currents and catecholamine secretion in mouse chromaffin cells. Biomaterials 32, 9040–9050.

Gurevitch, D., Shuster-Meiseles, T., Nov, O., Zick, Y., Rudich, A., Rudich, Y., 2012. TiO_2 nanoparticles induce insulin resistance in liver-derived cells both directly and via macrophage activation. Nanotoxicology 6, 804–812.

Hinther, A., Vawda, S., Skirrow, R.C., Veldhoen, N., Collins, P., Cullen, J.T., van Aggelen, G., Helbing, C.C., 2010. Nanometals induce stress and alter thyroid hormone action in amphibia at or below North American water quality guidelines. Environ. Sci. Technol. 44, 8314–8321.

Hoet, P.H., Bruske-Hohlfeld, I., Salata, O.V., 2004. Nanoparticles—known and unknown health risks. J. Nanobiotechnol. 2, 12.

Hurst, C.H., Abbott, B.D., DeVito, M.J., Birnbaum, L.S., 1998. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin in pregnant Long Evans rats: disposition to maternal and embryo/fetal tissues. Toxicol. Sci. 45, 129–136.

Iavicoli, I., Fontana, L., Leso, V., Bergamaschi, A., 2013. The effects of nanomaterials as endocrine disruptors. Int. J. Mol. Sci. 14, 16732–16801.

Jain, M.P., Vaisheva, F., Maysinger, D., 2012. Metalloestrogenic effects of quantum dots. Nanomedicine 7, 23–37.
Jovanovic, B., Ji, T., Palic, D., 2011. Gene expression of zebrafish embryos exposed to titanium dioxide nanoparticles and hydroxylated fullerenes. Ecotoxicol. Environ. Saf. 74, 1518–1525.
Kong, L., Tang, M., Zhang, T., Wang, D.Y., Hu, K., Lu, W.Q., Wei, C., Liang, G.Y., Pu, Y.P., 2014. Nickel nanoparticles exposure and reproductive toxicity in healthy adult rats. Int. J. Mol. Sci. 15, 21253–21269.
Kulvietis, V., Zalgeviciene, V., Didziapetriene, J., Rotomskis, R., 2011. Transport of nanoparticles through the placental barrier. Tohoku J. Exp. Med. 225, 225–234.
Li, W.Q., Wang, F., Liu, Z.M., Wang, Y.C., Wang, J., Sun, F., 2013. Gold nanoparticles elevate plasma testosterone levels in male mice without affecting fertility. Small 9, 1708–1714.
Love, S.A., Liu, Z., Haynes, C.L., 2012. Examining changes in cellular communication in neuroendocrine cells after noble metal nanoparticle exposure. Analyst 137, 3004–3010.
Lu, X., Liu, Y., Kong, X., Lobie, P.E., Chen, C., Zhu, T., 2013. Nanotoxicity: a growing need for study in the endocrine system. Small 9, 1654–1671.
Moridian, M., Khorsandi, L., Talebi, A.R., 2015. Morphometric and stereological assessment of the effects of zinc oxide nanoparticles on the mouse testicular tissue. Bratisl. Lek. Listy 116, 321–325.
Nair, P.M.G., Park, S.Y., Lee, S.W., Choi, J., 2011. Differential expression of ribosomal protein gene, gonadotrophin releasing hormone gene and Balbiani ring protein gene in silver nanoparticles exposed *Chironomus riparius*. Aquat. Toxicol. 101, 31–37.
Norris, D.O., Carr, J.A., 2013. Vertebrate Endocrinology. Academic Press.
Philbrook, N.A., Winn, L.M., Afrooz, A.R.M.N., Saleh, N.B., Walker, V.K., 2011. The effect of TiO_2 and Ag nanoparticles on reproduction and development of *Drosophila melanogaster* and CD-1 mice. Toxicol. Appl. Pharm. 257, 429–436.
Pietroiusti, A., Massimiani, M., Fenoglio, I., Colonna, M., Valentini, F., Palleschi, G., Camaioni, A., Magrini, A., Siracusa, G., Bergamaschi, A., Sgambato, A., Campagnolo, L., 2011. Low doses of pristine and oxidized single-wall carbon nanotubes affect mammalian embryonic development. ACS Nano 5, 4624–4633.
Powell, M.C., Kanarek, M.S., 2006. Nanomaterial health effects—part 1: background and current knowledge. WMJ 105, 16–20.
Qu, Y., Li, W., Zhou, Y.L., Liu, X.F., Zhang, L.L., Wang, L.M., Li, Y.F., Iida, A., Tang, Z.Y., Zhao, Y.L., Chai, Z.F., Chen, C.Y., 2011. Full assessment of fate and physiological behavior of quantum dots utilizing *Caenorhabditis elegans* as a model organism. Nano Lett. 11, 3174–3183.
Ramdhan, D.H., Ito, Y., Yanagiba, Y., Yamagishi, N., Hayashi, Y., Li, C.M., Taneda, S., Suzuki, A.K., Watanabe, G., Taya, K., Kamijima, M., Nakajima, T., 2009. Nanoparticle-rich diesel exhaust may disrupt testosterone biosynthesis and metabolism via growth hormone. Toxicol. Lett. 191, 103–108.
Rivera Gil, P., Oberdorster, G., Elder, A., Puntes, V., Parak, W.J., 2010. Correlating physico-chemical with toxicological properties of nanoparticles: the present and the future. ACS Nano 4, 5527–5531.
Roco, M.C., 2003. Nanotechnology: convergence with modern biology and medicine. Curr. Opin. Biotechnol. 14, 337–346.
Rollerova, E., Jurcovicova, J., Mlynarcikova, A., Sadlonova, I., Bilanicova, D., Wsolova, L., Kiss, A., Kovriznych, J., Kronek, J., Ciampor, F., Vavra, I., Scsukova, S., 2015. Delayed adverse effects of neonatal exposure to polymeric nanoparticle poly(ethylene glycol)-block-polylactide methyl ether on hypothalamic-pituitary-ovarian axis development and function in Wistar rats. Reprod. Toxicol. 57, 165–175.
Rushton, L., Cooley, D.A., 2009. The Endocrine System. Infobase Publishing, New York, NY.
Schmid, K., Riediker, M., 2008. Use of nanoparticles in Swiss industry: a targeted survey. Environ. Sci. Technol. 42, 2253–2260.
Sharma, V., Singh, P., Pandey, A.K., Dhawan, A., 2012. Induction of oxidative stress, DNA damage and apoptosis in mouse liver after sub-acute oral exposure to zinc oxide nanoparticles. Mutat. Res. 745, 84–91.
Tosi, G., Costantino, L., Ruozi, B., Forni, F., Vandelli, M.A., 2008. Polymeric nanoparticles for the drug delivery to the central nervous system. Expert Opin. Drug Deliv. 5, 155–174.
Walkey, C.D., Olsen, J.B., Guo, H., Emili, A., Chan, W.C., 2012. Nanoparticle size and surface chemistry determine serum protein adsorption and macrophage uptake. J. Am. Chem. Soc. 134, 2139–2147.
Wang, J., Chen, C., Liu, Y., Jiao, F., Li, W., Lao, F., Li, Y., Li, B., Ge, C., Zhou, G., Gao, Y., Zhao, Y., Chai, Z., 2008. Potential neurological lesion after nasal instillation of TiO_2 nanoparticles in the anatase and rutile crystal phases. Toxicol. Lett. 183, 72–80.

Wang, J., Zhou, G., Chen, C., Yu, H., Wang, T., Ma, Y., Jia, G., Gao, Y., Li, B., Sun, J., Li, Y., Jiao, F., Zhao, Y., Chai, Z., 2007. Acute toxicity and biodistribution of different sized titanium dioxide particles in mice after oral administration. Toxicol. Lett. 168, 176–185.

Waring, R.H., Harris, R.M., 2005. Endocrine disrupters: a human risk? Mol. Cell Endocrinol. 244, 2–9.

Wilson, M.R., 2009. The Endocrine System: Hormones, Growth, and Development. The Rosen Publishing Group, New York, NY.

Xu, Y., Wang, N., Yu, Y., Li, Y., Li, Y.B., Yu, Y.B., Zhou, X.Q., Sun, Z.W., 2014. Exposure to silica nanoparticles causes reversible damage of the spermatogenic process in mice. PLoS One 9, e101572.

Yamashita, K., Yoshioka, Y., Higashisaka, K., Mimura, K., Morishita, Y., Nozaki, M., Yoshida, T., Ogura, T., Nabeshi, H., Nagano, K., Abe, Y., Kamada, H., Monobe, Y., Imazawa, T., Aoshima, H., Shishido, K., Kawai, Y., Mayumi, T., Tsunoda, S., Itoh, N., Yoshikawa, T., Yanagihara, I., Saito, S., Tsutsumi, Y., 2011. Silica and titanium dioxide nanoparticles cause pregnancy complications in mice. Nat. Nanotechnol. 6, 321–328.

Yang, H., Sun, C.J., Fan, Z.L., Tian, X., Yan, L., Du, L.B., Liu, Y., Chen, C.Y., Liang, X.J., Anderson, G.J., Keelan, J.A., Zhao, Y.L., Nie, G.J., 2012. Effects of gestational age and surface modification on materno-fetal transfer of nanoparticles in murine pregnancy. Sci. Rep. 2, 847.

Yoshida, S., Hiyoshi, K., Ichinose, T., Takano, H., Oshio, S., Sugawara, I., Takeda, K., Shibamoto, T., 2009. Effect of nanoparticles on the male reproductive system of mice. Int. J. Androl. 32, 337–342.

Yoshida, S., Hiyoshi, K., Oshio, S., Takano, H., Takeda, K., Ichinose, T., 2010. Effects of fetal exposure to carbon nanoparticles on reproductive function in male offspring. Fertil. Steril. 93, 1695–1699.

Zha, L., Zeng, J., Sun, S., Deng, H., Luo, H., Li, W., 2009. Chromium(III) nanoparticles affect hormone and immune responses in heat-stressed rats. Biol. Trace Elem. Res. 129, 157–169.

Zhang, X.D., Wu, H.Y., Wu, D., Wang, Y.Y., Chang, J.H., Zhai, Z.B., Meng, A.M., Liu, P.X., Zhang, L.A., Fan, F.Y., 2010. Toxicologic effects of gold nanoparticles in vivo by different administration routes. Int. J. Nanomed. 5, 771–781.

CHAPTER

15

Skin

*Nancy A. Monteiro-Riviere**, *Francesca Larese Filon***

***Nanotechnology Innovation Center, Kansas State University, Manhattan, KS, United States; **University of Trieste, Trieste, Italy**

OUTLINE

Skin Anatomy and Functions 357
Functions of Skin 358
Skin Morphology 358

Nanoparticle Penetration 359
Barrier Modifications 361
Mechanical Action 361
UVB-Damaged Skin 362
Solvent Effects 363
Carbon-Based Nanomaterials 364
Quantum Dots 364
TiO_2 and ZnO 365
Gold (Au)NPs 367
Silver (Ag)NPs 367

Toxicity Studies 368
Irritation 368
Sensitization 368
Carbon-Based Nanomaterials 369
C_{60} Fullerenes 370
SWCNTs 371
MWCNTs 371
Quantum Dots 372
Silver (Ag)NPs 372
Other Metals 373

Take-Home Messages 374

References 374

SKIN ANATOMY AND FUNCTIONS

Skin is one of the largest organs of the body and is the primary barrier to absorption from the environment. Due to its accessibility and continuous exposure to a myriad of chemicals, both accidental and deliberate (e.g., cosmetics), the skin is a common route of exposure and target for nanomaterials and toxic chemicals. There are properties of a chemical/nanomaterial that will determine its propensity to cause dermatotoxicity, which is the ability to penetrate the skin and subsequent interactions with the biological components of skin that could elicit a toxicological response. Due to the recent advances in nanotechnology, this chapter will review nanomaterials used in biological applications, consumer products, as well as occupational

Adverse Effects of Engineered Nanomaterials. http://dx.doi.org/10.1016/B978-0-12-809199-9.00015-X
Copyright © 2017 Elsevier Inc. All rights reserved.

exposure and discuss their toxicity, mutagenicity, carcinogenicity, and irritation potential, as well as penetration in intact skin or skin cells (keratinocytes) (for a further discussion on genotoxicity, see Chapter 18). In addition, basic anatomical factors that contribute to the barrier properties of skin that may affect the processes of absorption or determine targets for toxicity, as well as how these factors determine whether a compound can be absorbed or can penetrate in skin to elicit a toxicological response will be presented.

Functions of Skin

Skin can act as an environmental barrier to protect major organs, as a diffusion barrier that minimizes insensible water loss that results in dehydration, and as a metabolic barrier that can metabolize a compound to eliminate more easily the products after absorption has occurred. Skin can function in temperature regulation, as an immunological effector axis by having Langerhans cells processing antigens and as an effector axis by initiating an inflammatory response to a foreign insult. Skin has a well-developed stroma, which supports all other organs and has neurosensory properties where receptors sense the modalities of touch, pain, and heat. Skin can act as an endocrine organ by synthesizing vitamin D, is a target for androgens that regulate sebum production, and is a target for insulin that regulates carbohydrate and lipid metabolism. In addition, it contains several sebaceous glands that secrete sebum, a complex mixture of lipids that function as antibacterial agents, and apocrine and eccrine sweat glands that produce a secretion that contains scent and functions in territorial demarcation. Skin also plays a role in the biosynthesis of keratin, collagen, melanin, lipids, carbohydrates, as well as in respiration and biotransformation of xenobiotics and vitamin D. Anatomically, skin can be divided into two principal and distinct components: a stratified, avascular, outer cellular epidermis, and an underlying dermis consisting of connective tissue with numerous cell types and special adnexal structures. The epidermis consists of stratified squamous keratinized epithelium that undergoes a programmed proliferation and differentiation that eventually results in the formation of the major barrier to chemical penetration: the stratum corneum. Two primary cell types, the keratinocytes (stratum basale, stratum spinosum, stratum granulosum, stratum lucidum, and stratum corneum) and the nonkeratinocytes [melanocytes, Merkel cells (tactile epithelioid cells), and Langerhans cells] are present. However, the stratum corneum is the primary anatomical barrier responsible for modulating chemical, drug, and particle absorption into the skin (Monteiro-Riviere, 1991, 2006, 2010).

Skin Morphology

The epidermis consists of a keratinized stratified squamous epithelium and undergoes an orderly pattern of proliferation, differentiation, and keratinization. The stratum corneum is the outermost superficial epidermal layer that serves as the first line of defense between the body and the environment, and the lower part of this layer is crucial in preventing the penetration of irritants and allergens (Cork et al., 2006). The stratum corneum can vary in thickness depending on the regions of the body and the species (Monteiro-Riviere et al., 1990). Each stratum corneum cell (corneocyte) is 30 μm in diameter and 0.5–0.8 μm in thickness (Holbrook and Odland, 1974). These cells are highly organized and stacked upon one another to form vertical interlocking columns having a flattened tetrakaidecahedron shape

(Menten, 1976a,b). This spatial arrangement helps facilitate the maintenance of the skin's efficient barrier function. Lipids between the stratum corneum are derived from lamellar granules (Odland bodies) of the stratum granulosum to form the intercellular lipid component of the stratum corneum barrier. These lipids are arranged into lamellar sheets that constitute the epidermal permeability barrier. The number of lamellae may vary within the same area of the stratum corneum and when viewed by transmission electron microscopy (TEM) consists of a pattern of alternating electron-dense and electron-lucent bands which represent paired bilayers formed from fused lamellar granule disks (Landmann, 1986; Madison et al., 1987; Menon et al., 1992; Swartzendruber et al., 1987, 1989). These lipids consist of ceramides, cholesterol, fatty acids, and small amounts of cholesterol esters, as well as hydrolytic enzymes, such as acid phosphates, proteases, lipases, and glycosidases. Each corneocyte is embedded in the lipid matrix produced by the lamellar granules. Cells are bathed in the lipid matrix to form the so-called "brick and mortar" structure where the nonviable corneocytes represent the "bricks" and the intercellular lipids represent the "mortar" (Elias, 1983, 2010). Most chemicals are absorbed through skin via this intercellular pathway, with partitioning into and diffusion through the lipid predictive of compound absorption. In atopic dermatitis, the skin barrier is impaired and allergens can penetrate through the skin causing IgE sensitization. Skin exposure to irritants in an occupational setting or environmental scenario may cause a disruption of the stratum corneum layers by protein denaturation agents, such as detergents or by extraction of lipids from stratum corneum with solvents (Elsner, 1994; Fluhr et al., 2002; Monteiro-Riviere, 2008; Monteiro-Riviere et al., 2001; Roberts et al., 2008). Lipid extraction studies conducted in the abdominal, inguinal, and back regions of pigs demonstrated relative proportions of individual lipids (ceramides, fatty acids, cholesterol, triglycerides, and cholesterol esters) were similar across all body regions (Monteiro-Riviere et al., 2001). Skin is permeable to some chemicals, but the permeability may increase 4–100 times in atopic subjects with damaged skin (Larese Filon et al., 2009, 2011), thereby increasing the risk for penetration, irritation, and sensitization. Regional and species differences in skin thickness, hair follicle density, blood flow, age, and disease states may all influence barrier function (Monteiro-Riviere, 1991, 2008).

NANOPARTICLE PENETRATION

People are in daily contact with many different substances and the dermal absorption of these substances needs to be considered for risk evaluation studies (Fiserova-Bergerova et al., 1990; Nielsen and Grandjean, 2004; Sartorelli et al., 2007). There is much information on skin absorption in relation to drugs, cosmetics, toxins, and metal salts and powders (Sartorelli et al., 2007), and many of these toxic substances are labeled with "skin notation" by the American Conference of Governmental Industrial Hygienists (ACGIH), which warns that cutaneous exposure to these compounds may significantly contribute to total systemic exposure (Sartorelli et al., 2007). However, there is very little information regarding nanoparticles (NPs) and their ability to enter through skin. Recently, the transdermal route has been investigated with a variety of results due to the fact that there are so many different types of nanomaterials (Monteiro-Riviere and Baroli, 2010). European projects, such as FP5-NANODERM stated that both titanium oxide and zinc oxide NPs are unable to permeate skin, yet many

investigators had shown permeation of NP through skin (Baroli et al., 2007; Lansdown and Taylor, 1997; Ryman-Rasmussen et al., 2006).

There are four potential pathways in which compounds can penetrate through healthy skin, depending on their physicochemical properties: intercellular (between cells), transcellular (through cells), through hair follicles, and through sweat glands (Monteiro-Riviere, 1991; Riviere, 1994; Scheuplein, 1967). Most dermal absorption literature is based on chemicals and drugs that are very small (<500 Da) and soluble in dosing formulations that move via passive diffusion across the stratum corneum intercellular lipids (Monteiro-Riviere and Riviere, 2009; Riviere and Monteiro-Riviere, 2010). Also, there are a variety of factors that can influence the extent of uptake in the skin including barrier integrity, a contaminated surface, the anatomical structure, and the presence of skin diseases, such as allergic and irritant contact dermatitis, atopic eczema, and psoriasis (Monteiro-Riviere, 2008). Moreover, mechanical flexion (Rouse et al., 2007; Tinkle et al., 2003), irritant detergents, and some chemicals (Filon et al., 2006; Nielsen et al., 2007) may also increase skin absorption.

It should be noted that NP may inhibit skin penetration of other components. In fact, a thin layer of glycerin containing NP of either calcium carbonate or calcium phosphate applied to isolated in vitro pig skin and to mice in vivo prevented the penetration of nickel ions into the skin. It is thought that the NP captured nickel ions by cation exchange which remained on the surface of the skin, thereby making them easily removed by simple washing techniques. Particles smaller than 500 nm added to topical creams may be an effective way to limit the exposure of metal ions that may cause skin irritation (Vermula et al., 2011).

The penetration of NP in the skin is a controversial subject (Bronaugh, 2008; Chen and Schluesener, 2008; EPA, 2007; Gwinn and Vallyathan, 2006; Kielhorn et al., 2006; Larese Filon et al., 2015; Monteiro-Riviere, 2014; NIOSH, 2007; Oberdörster et al., 2005b; Roco, 2005; SCCP, 2007) partly because many factors that influence absorption were not studied and the opinions of these investigators were generalized based on limited studies with only a few types of NP. Many of these studies were conducted on very large particles that were not of nanosize (<100 nm). Also, these discrepancies may be related to differences in NP composition, surface chemistry, vehicles or solvents, techniques and methods of exposure, and analytical analysis, laboratory conditions, and duration of the experiment. The respiratory route of exposure has been studied extensively (Donaldson et al., 2006; Geiser et al., 2003; Geys et al., 2006; Lam et al., 2004; Larese Filon et al., 2015; Limbach et al., 2007; Magrez et al., 2006; Monteiro-Riviere, 2014; Muller et al., 2005; Nel et al., 2006; Oberdörster et al., 2005a; Rotoli et al., 2008; Shimada et al., 2006; Shvedova et al., 2005) (see Chapter 9), but skin exposure has been neglected being considered less permeable with a lower perception of risk. For a more complete discussion on the aspects of skin penetration with NPs, see the reviews by Monteiro-Riviere and Baroli (2010) and Monteiro-Riviere (2014).

Nanomaterials have widespread use not only in biomedical applications, but also in occupational and consumer scenarios. Consumers or workers may have compromised skin with an altered barrier from conditions, such as atopic dermatitis, psoriasis, or eczema on their hands or other parts of their body, which could influence the rate of absorption or penetration of nanomaterials through skin. The transdermal flux of nanomaterials has obvious implications to toxicology, therapeutics, and drug delivery, but there is a need to know the depth and mechanism of NP penetration through the rate-limiting barrier of the stratum corneum.

Several studies have evaluated the hypothesis that NP may become lodged in the lipid matrix of the skin (Monteiro-Riviere et al., 2007; Zhang and Monteiro-Riviere, 2008; Zhang et al., 2008). The importance of NP size, shape, charge, surface properties, and vehicle, as well as animal species will determine penetration and should be considered when designing studies to assess whether or not NP penetration occurs. There is limited information on the in vivo dermal penetration of nanomaterials in humans; however, there are several published studies in various in vitro model systems and on excised animal and human skins.

Barrier Modifications

The effects of solvents and vehicles on skin absorption are well-known. The barrier properties may be altered either by mechanical action, ultraviolet radiation, or by chemical agents, after occupational or accidental spillage, or topical creams, such as sunscreens, cosmetics, insect repellents, or drugs. Many solvents can cause a range of structural alterations to the intercellular lipid bilayers of the stratum corneum by increasing the fluidization, and delipidization of the lipid, thereby changing the polarity, and phase separation causing vesicles within the lipid layers. Damage to desmosomes may create fissures within the lipid bilayer and damage to the fibrillar keratin of the stratum corneum may create vacuoles. All of these solvent actions will influence the rate of absorption for chemicals (Monteiro-Riviere et al., 2001; Riviere and Brooks, 2005, 2011; Riviere and Monteiro-Riviere, 2010; Roberts et al., 2008) but with nanomaterials only a few studies have investigated these effects.

Mechanical Action

Mechanical stressors may affect how NP can traverse through the skin. In an occupational setting, repetitive motions, such as constant hand movements could accentuate biomechanical forces that may alter the structural organization of skin thereby leading to an increase in the permeability of the skin. The effects of flexing for 30 min on normal skin with FITC-conjugated dextran beads with a diameter of 0.5 μm showed penetration through the stratum corneum layer and into the viable epidermis (Tinkle et al., 2003). Studies in flow-through diffusion cells showed penetration of a very small fullerene amino acid-derivatized peptide NP of 3.5 nm topically applied to dermatomed porcine skin flexed at a 45-degree angle at a frequency of 20 flexes/min for 60 or 90 min (Rouse et al., 2008). After 8 and 24 h, TEM analysis revealed NP localization within the intercellular spaces of the stratum granulosum, suggesting that penetration of the derivatized fullerene occurs through intercellular lipid moieties (Monteiro-Riviere et al., 2007; Rouse et al., 2007). The effects of applied strain on quantum dot (QD) cellular uptake showed that application of physiological load conditions may increase the permeability of the cell membrane allowing for an increase in QD uptake (Rouse et al., 2008). However, QD applied to rat skin flexed for 60 min showed that larger NPs QD655-COOH (18 nm) and QD565-COOH (14 nm) did not penetrate at 8 and 24 h (Zhang et al., 2008).

QD penetration was assessed in intact, tape-stripped, and abraded skins which are methods used to mimic barrier perturbation. Studies were conducted with two different sizes of

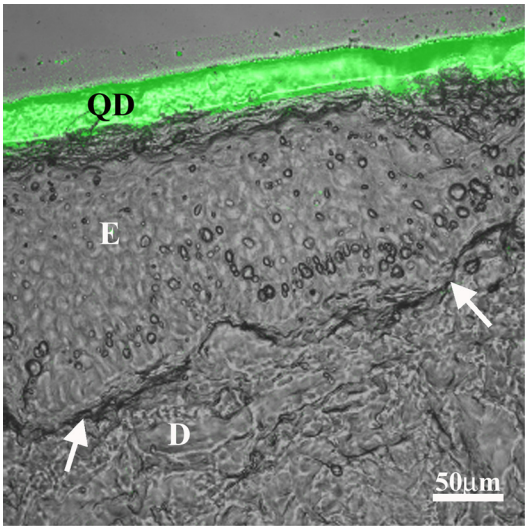


FIGURE 15.1 Laser confocal microscopy of tape-stripped human skin exposed to QD565-COOH for 8 h in flow-through diffusion cells. The *arrows* depict the interface of the epidermis *(E)* and dermis *(D)* of the skin. Quantum dot *(QD)* remained on the surface of the epidermis.

QD with carboxylic acid coatings on rat skin in flow-through diffusion cells for 8 and 24 h. Tape-stripped skin showed QD remained on the surface of the viable epidermis. In abraded skin, QD penetrated into the viable dermis at both 8 and 24 h, while intact skin depicted QD only in the stratum corneum (Zhang et al., 2008). QD565-COOH was topically applied to human skin that was tape-stripped 30 times to remove the stratum corneum in flow-through diffusion cells for 8 h and was detected by laser confocal microscopy on the surface of the viable epidermal layer of the skin (Monteiro-Riviere and Inman, 2008) (Fig. 15.1).

UVB-Damaged Skin

Studies with sunscreens containing zinc isotopes (^{68}Zn) topically applied in humans for 5 days have been conducted on sunburned damaged [ultraviolet B (UVB) radiation] skin and showed higher levels of zinc in the blood and urine of individuals receiving the nanosunscreen. However, whether ^{68}Zn was absorbed as ZnO particles or soluble Zn is unknown (Gulson et al., 2010). Other studies with sunscreens containing titanium dioxide (TiO_2) and zinc oxide (ZnO)NP in different water and oil formulations were studied on in vivo and in vitro UVB-damaged porcine skin and TiO_2 showed deep penetration into the stratum corneum (Fig. 15.2A) layers, while ZnO remained primarily on the surface (Fig. 15.2B) (see TiO_2 and ZnO section). However, with more sophisticated microscopic techniques, such as time-of-flight secondary ion mass spectrometry (TOF-SIMS), Ti was detected within the epidermis and superficial dermis and Zn was limited to the stratum corneum layer. This study showed TiO_2 and ZnO sunscreen formulations were slightly enhanced in UVB-damaged skin, but transdermal absorption was not detected (Monteiro-Riviere et al., 2011b).

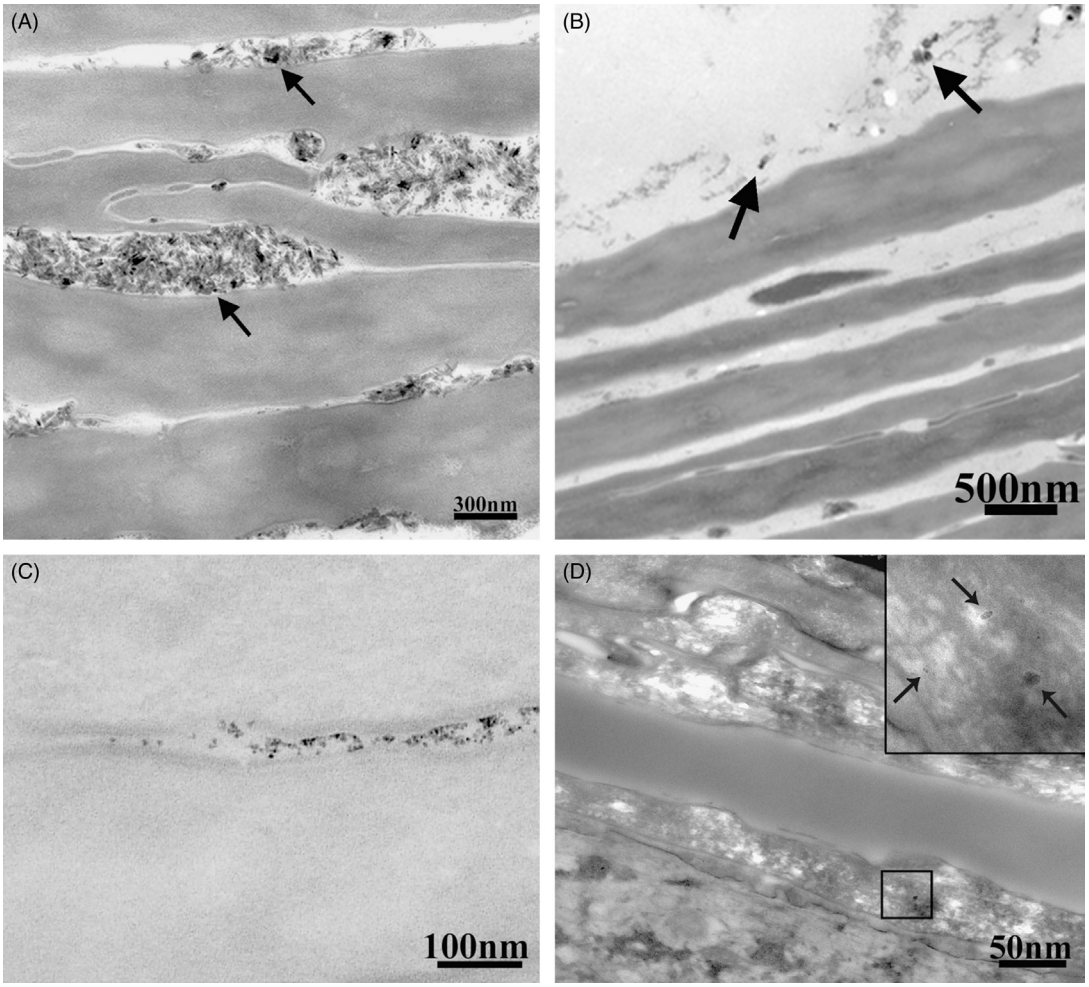


FIGURE 15.2 (A) Transmission electron micrograph (TEM) of TiO_2NP *(arrows)* within the intercellular lipid space of the stratum corneum layers of in vivo skin treated for 48 h with a sunscreen formulation. (B) TEM of ZnONP on the surface of stratum corneum of ultraviolet B (UVB)-exposed skin treated with ZnO sunscreen formulation for 48 h. (C) TEM showing the localization of QD621 within the intercellular lipid bilayers of the upper stratum corneum. (D) TEM of 13 nm AuNP *(arrows)* localized in the stratum corneum of human skin.

Solvent Effects

The impact of solvents on skin penetration was studied with pristine fullerenes (C_{60}) in toluene, cyclohexane, chloroform, and mineral oil in weanling pigs. The fullerene in the solvent was applied to the skin daily for 4 days to simulate occupational exposures. The dose sites were tape-stripped 26 times for quantitative analysis by simplified liquid–liquid extraction along with skin biopsies. The C_{60} dose in toluene, cyclohexane, or chloroform showed that the pristine fullerenes penetrated deeper into the stratum corneum layers, but more C_{60}

was found in the stratum corneum when dosed in chloroform. No C_{60} was detected in the skin with the mineral oil. Similar studies were conducted with in vitro flow-through diffusion cells which also showed that no fullerenes were present in the receptor fluid (Xia et al., 2010).

Terpenes are highly lipophilic aliphatic compounds in essential oils that are generally regarded as safe and have been found to change the stratum corneum lipid organization from orthorhombic to hexagonal (Dos Anjos and Alonso, 2008; Roberts et al., 2008; Sapra et al., 2008). Human skin was pretreated for 1 h with the terpenes (menthol, eucalyptol, limonene) or ethanol vehicle and dosed with different charged QDs of two different sizes in the in vitro flow-through diffusion cells for 8 h. Laser scanning confocal microscopy showed no penetration of QD into the viable layers of the skin, but detected QD in the upper stratum corneum layers. TEM showed slightly deeper QD penetration in the stratum corneum in the terpene-pretreated skin, but QDs were not detected in the perfusate. These studies showed that terpenes caused only a slight enhancement of QD penetration into the stratum corneum but no absorption occurred through skin (Monteiro-Riviere et al., 2011a).

Carbon-Based Nanomaterials

Carbon-based nanomaterial absorption through skin may not be similar to chemical absorption since these nanomaterials are engineered differently and are of various sizes, and therefore are not homogeneous like chemicals (Monteiro-Riviere and Inman, 2006). Carbon nanomaterials are one of the most important classes of NP because of their large variety of applications. They include: (1) carbon nanotubes, single-walled carbon nanotubes (SWCNT), and multiwalled carbon nanotubes (MWCNT), with diameters ranging from a few to hundreds of nanometers and length up to a few micrometers, and (2) fullerenes with a size less than 100 nm. Carbon nanotubes are quite large compared to carbon fullerene NP and will not penetrate through the skin. Therefore, most studies have used fullerenes due to their small size to study the penetration through skin. A study in which C_{60} with a fluorescently tagged nuclear localization signal (C_{60}-NLS-FITC) was topically applied at 4.4 μg and 1.1 μg/cm^2 to porcine skin in flow-through diffusion cells for 24 h showed penetration of the NP through all epidermal layers by confocal microscopy. TEM showed that the C_{60}-NLS-FITC localized within the intercellular bilipid layers of the stratum corneum cell layers (Rouse et al., 2007). The addition of 1% Pluronic F127 surfactant greatly enhanced the permeability for all treatments (Monteiro-Riviere et al., 2006).

Quantum Dots

Several investigators have studied the penetration of QDs in skin because they are easy to track by fluorescent methods due to their intense and photostable fluorescence and their commercial availability with different surface coatings. QDs of different shapes, sizes, and charges were studied. QD565 and QD655 contain a cadmium/selenium (CdSe) core with a zinc sulfide (ZnS) shell. QD565 are spherical with a diameter of 4.6 nm, while QD655 are ellipsoid with a size of 6 nm (minor axis) × 12 nm (major axis). The hydrodynamic diameters for QD565 are 35 nm for the polyethylene glycol (PEG), uncharged; 14 nm for the carboxylic acid (COOH), negatively charged; and 15 nm for the PEG-amine (NH_2), positively charged. The hydrodynamic diameters of QD655 were 45 nm (PEG), 18 nm (COOH), and 20 nm (NH_2)

(Ryman-Rasmussen et al., 2006). In comparison, QD621-PEG have a CdSe core and CdS shell coated with PEG polymer coils, and are nail shaped as seen by TEM, with the mean width of 5.78 ± 0.97 nm and length of 8.40 ± 1.9 nm and a hydrodynamic size of 39 ± 1 nm as evaluated by size-exclusion chromatography (Yu et al., 2007). Penetration of QD565 and QD655 with diverse physicochemical properties was studied in porcine flow-through diffusion cells at an occupationally relevant dose over a work day. QD565 with PEG, PEG-NH_2, or COOH coatings showed penetration into the stratum corneum and localization within the epidermal and dermal layers by 8 h. PEG- and PEG-NH_2-coated QD655 were localized within the epidermal layers by 8 h. The penetration of QD655-COOH was evident only at 24 h in the epidermal layers (Ryman-Rasmussen et al., 2006). Skin absorption of QD565 with three surface modifications was evaluated in human skin with in vitro flow-through and static diffusion cells at various pHs. Laser scanning confocal microscopy detected penetration into intact viable epidermis of skin for QD-PEG at pH 8.3, but not at pH 7.0. QD did not penetrate beyond the stratum corneum of intact skin but, after tape stripping 30 times to remove most of the stratum corneum, were found in the viable epidermal layers (Prow et al., 2012).

QD621 topically applied to dermatomed porcine skin in flow-through diffusion cells were located primarily in the normal stratum corneum, although a small amount of fluorescence was detected in the upper epidermal layers. Occasionally, QD621 were seen in the outer root sheath of the hair follicle (Zhang et al., 2008). By TEM, the QD were limited to the upper intercellular spaces of the stratum corneum of porcine skin (Fig. 15.2C).

Neutral and charged QD621 were infused into the arterial vascular supply of the isolated perfused skin flap model to mimic systemic distribution after exposure by any route. Biodistribution studies showed that QD621 can migrate out of the capillaries into the surrounding tissue (Lee et al., 2007). QD621 that were intradermally injected into SKH-1 hairless mice migrated from the injection site to regional lymph nodes through the lymphatic duct system and then to the liver and other organs (Gopee et al., 2007). Other investigators studied QD penetration in rat skin and found Cd within the liver and kidney after skin exposure (Tang et al., 2013). Though this experiment was conducted in rats, it raises the question if rat is a suitable model, and also the systemic toxicity of Cd release from QDs.

TiO_2 and ZnO

The global production of TiO_2 and ZnO for use in skincare products was 1000 tons in 2003 and 2004 (Borm et al., 2006). The Australian Therapeutic Goods Administration estimated that in 2005, 70% of all sunscreens containing TiO_2 and 30% containing ZnO were formulated with NP (Faunce et al., 2008). Sunscreen use is highly recommended to prevent skin cancer, sunburn, photoaging, and skin wrinkles but traditional sunblocks may cause irritation. Nanoscale titanium dioxide (TiO_2) and micronized ZnONPs less than 100 nm have been incorporated into sunscreens to provide an effective broad spectrum physical sunblocker and being transparent is more aesthetically acceptable to the consumers. Several in vivo and in vitro studies, including the European NANODERM project, have shown negligible or no penetration of intact skin with different formulations and concentrations of TiO_2NP under various experimental conditions (Bennat and Müller-Goymann, 2000; Cross et al., 2007; Dussert and Gooris, 1997; Gamer et al., 2006; Kertész et al., 2005; Kiss et al., 2008; Lademann et al., 1999; Mavon et al., 2007; Menzel et al., 2004; NANODERM, 2007; Pflücker et al., 2001; Sadrieh

et al., 2010; SCCNFP, 2000; Schulz et al., 2002; Tan et al., 1996). The EU Scientific Committee on Consumer Products in 2007 stated that a safety assessment of nanosized TiO_2 in cosmetics should also take into account abnormal skin conditions and the impact of mechanical effects on skin penetration (SCCP, 2007).

Some investigators have suggested that penetration can occur when the skin barrier is damaged. Humans exposed for 5 days outdoors to sunscreens containing ZnONP showed small increases in the Zn tracer in the blood and urine by very sensitive methods but it is not known whether ^{68}Zn was absorbed as ZnO or soluble Zn (Gulson et al., 2010). Recently, in vitro and in vivo studies were conducted with TiO_2NP and ZnONP to UVB-damaged porcine skin with four different sunscreen formulations (Monteiro-Riviere et al., 2011b). Flow-through diffusion cells were treated 24 h with four sunscreen formulations: 10% coated TiO_2 in o/w; 10% coated TiO_2 in w/o; 5% coated ZnO in o/w; and 5% uncoated ZnO in o/w. TiO_2 primary particle size was 10×50 nm with mean agglomerates of 200 nm; ZnO mean was 140 nm. Skin was evaluated by LM, SEM, TEM, and TOF-SIMS. UVB-exposed skin showed TiO_2NP down to 17 layers into the stratum corneum compared to normal skin (Fig. 15.2A), while ZnO remained on the surface. TOF-SIMS analysis showed TiO_2 and ZnO penetration. The perfusate analyzed by TEM/EDX or inductively coupled plasma mass spectrometry (ICP-MS) detected no Ti or Zn, indicating minimal transdermal absorption. The in vivo porcine skin was dosed for 24 h occluded with the same formulations and at 48 h (Monteiro-Riviere et al., 2011a). The TiO_2 in o/w formulation penetrated 13 layers deep into UVB-damaged stratum corneum, while only 7 layers deep in non-UVB treated skin; TiO_2 in w/o penetrated deeper in the UVB-damaged stratum corneum. Coated and uncoated ZnNP in o/w were localized to the upper 1–2 superficial stratum corneum in UVB-damaged and normal skin (Fig. 15.2B). Only using more sophisticated techniques like TOF-SIMS showed Ti within the epidermis and superficial dermis, and Zn limited to the stratum corneum and upper epidermis. In summary, UVB-damaged skin slightly enhanced TiO_2NP or ZnONP penetration in sunscreen formulations but no transdermal absorption was detected (Monteiro-Riviere et al., 2011b). In studies with human skin, TiO_2NP applied to in vitro human skin in Franz cells did not find penetration through the skin even by damaging the skin with a needle (Mauro et al., 2015a). A 90-day study with ZnONP in rats showed no toxic effects but Zn was detected in the internal organs most probably due to oral ingestion through licking the skin (Ryu et al., 2014). ZnONP in three formulations commonly used in commercially available sunscreens did not show penetration of ZnNP into the viable skin layers but showed an increase in the Zn ion fluorescence signal in the viable epidermis (Holmes et al., 2016). In other studies, ZnONP were investigated ex vivo and in vivo for only 2 h using human skin that was either intact or wounded by tape stripping or microneedled; these studies showed, by multiphoton tomography, that there was a significant difference in the intact and wounded skin even after several washes and significant amounts of ZnONP were retained at the punctured sites (Raphael et al., 2013).

There are many conflicting reports as to the penetration of TiO_2 and many of these studies cannot be compared for the variables within each study are so diverse. Hence, there are species anatomical differences in skin, hair follicle density, and regional differences in absorption, as well as differences in vehicles used, and the duration of most of these experiments is very short. For a more complete discussion on the aspects of skin penetration with NP see the reviews by Monteiro-Riviere and Baroli (2010), Monteiro-Riviere and Riviere (2009), and Larese Filon et al. (2015). Since all studies have found that ZnONP do not penetrate skin even

with sophisticated microscopic techniques, the European Scientific Committee on Consumer Products and the Australian Therapeutics Goods Administration have come to a consensus that sunscreens containing ZnONP will not have adverse effects on normal skin (Australian Therapeutics Goods Administration, 2013; SCCS, 2012).

Gold (Au)NPs

In vitro skin permeation of AuNP was first studied by Sonavane et al. (2008) in rat skin Franz static diffusion cells with 15, 102, and 19 nm. AuNP showed size-dependent permeation through rat skin and TEM revealed accumulation of small NP within the deep layer of skin but the larger particles were observed mainly in the epidermis and dermis. This is not surprising because the larger NPs were not of nanosize (<100 nm). Other authors confirmed that AuNP of 12.6 nm can permeate intact and damaged human skin in Franz static diffusion cells in a dose-dependent manner (Fig. 15.2D) (Larese Filon et al., 2011). Also, when greater amounts of Au were applied (3 times), there was a parallel increase in permeation (3 times). Evaluation of the Au into skin revealed that Au concentration decreased from the superficial to deeper skin layers and a significant amount was found in damaged skin compared to intact skin. The abraded skin had an increase in only the amount of Au that remained in the skin but not in the receptor compartment. This may be due to a strong interaction between NP and the skin cells and extracellular matrix that might hinder the permeation of particle migration, and the short 24-h period of time. When 4.9 nm AuNP in water was applied to in vitro human skin, no penetration was noted (Labouta and Schneider, 2013); however, when repeated with 6 nm AuNP in toluene, penetration occurred in the stratum corneum and epidermis.

Silver (Ag)NPs

AgNP absorption through intact skin is low (<1 ppm) since much of the free ion is precipitated as Ag sulfide in the superficial layers of the stratum corneum. An in vivo daily repetitive skin study for 14 days was conducted using 25 and 35 nm carbon-coated AgNP, as well as freshly synthesized or thoroughly washed 20, 50, and 80 nm AgNP topically applied to the backs of weanling pigs. AgNP penetration was observed only in the superficial layers of the stratum corneum by TEM and confirmed by EDS which detected the localization of Ag (Samberg et al., 2010). Skin penetration of AgNP of 25 nm was demonstrated in intact and damaged in vitro human skin in static diffusion cells (Larese Filon et al., 2009) but with low flux compared to the absorption rates for metal powders (Larese Filon et al., 2004). TEM confirmed the presence of AgNP in the stratum corneum and the outermost surface of the epidermis but not in the dermis. These results suggest that a small fraction of the NP were dissolved and diffused through the skin layers as elemental Ag.

Polymer-coated AgNP of 19 nm in synthetic sweat applied to fresh, cryopreserved, and glycerolized human skin in Franz diffusion cells for 24 h showed silver flux penetration was greater in the glycerolized skin (Bianco et al., 2014). Silver was also detected in the reticular dermis after topical application of human skin with an Acticoat containing 10–40 nm AgNP, but no penetration was seen with the larger 70 nm AgNP on porcine skin in vitro (Vogt et al., 2014).

Silver release from textiles containing nanosilver was studied in Franz diffusion cells with human skin and found Ag and AgCl aggregates up to 1 μm in both the epidermis and dermis (Bianco et al., 2015). The large size of these particles suggests that most of the aggregation occurred in the skin. The formation of these aggregates most likely slowed systemic absorption of Ag but they could form a reservoir that would prolong the release of Ag ions.

TOXICITY STUDIES

Irritation

There is little information whether NP on the surface of the skin can cause local irritation due to mechanical actions (Crosera et al., 2009). In particular, local irritation of carbon nanotubes have been reported on the conjunctiva of the eye of animals but no effect was noted on the skin (Kishore et al., 2009). Carbon fiber dermatitis has been reported in humans after exposure to carbon nanofibers (hyperkeratosis) (Eedy, 1996).

Sensitization

The clinical manifestation of skin sensitization is an allergic contact dermatitis, but the sensitization potential of nanomaterials is unknown. NPs that penetrate into the epidermis may cause an allergic reaction due to the presence of sensitization substances, such as metals (e.g., nickel, cobalt, chromium, and so on) that can induce an allergic contact dermatitis or asthma. To date, there is one case report that describes dermatitis and respiratory systems in a female exposed to nickel NPs (Journeay and Goldman, 2014). However, current data suggest that there is a potential for an increase in exposure because many nanomaterials/NPs contain a metal catalyst. Since NPs have a larger bioavailable surface area, it has been hypothesized that they can interact significantly with the immune system to induce an allergic type of response upon skin contact. Metals, such as cobalt, nickel, and chromium are common NPs that could trigger an allergic response. Presumably, no allergic response would occur with nonallergenic metal NPs, such as Au or Ag. On the other hand, it has been suggested that fullerenes may play a leading role in the inhibition of the in vitro and in vivo IgE-mediated allergic response, thus blocking histamine release. Fullerenes are also capable of inhibiting mast cells and basophilic allergic responses in vitro and anaphylaxis in vivo. This suggests these molecules have unrecognized antiallergic properties. This biological effect critically depends on the moieties that are added to the carbon cage and it has been suggested that they act on different Fc epsilon RI-specific molecules in mast cells and basophils. These fullerene derivatives were suggested to represent a new class of compounds that interfere with Fc epsilon RI signaling pathways to stabilize mast cells and basophils, and thus fullerene-based therapies may be a new approach for treating allergic diseases (Norton et al., 2010), and perhaps to control diseases largely influenced by these cells, including type I hypersensitivity, anaphylaxis, hay fever, asthma, arthritis, multiple sclerosis, and other autoimmune diseases. Obviously, a great deal of research is required to assess this hypothesis.

Carbon-Based Nanomaterials

A major concern in conducting toxicity studies with carbon nanomaterial is the nature of the controls (reference material of known toxicity) employed. Carbon black (CB), a nanoscale particulate, has often been recommended to serve as a negative (nontoxic) control. The use of CB has grown out of its previous role in serving as a control in inhalational toxicology studies. However, caution must be taken when utilizing CB because of its ability to adsorb colorimetric chemical markers used in biochemical assays, thus leading to unreliable findings (Monteiro-Riviere and Inman, 2006; Monteiro-Riviere et al., 2009). Therefore, multiple approaches including TEM, mRNA assay, and protein expression assays should be used to assess the toxicological effects of these NPs (Monteiro-Riviere and Inman, 2006). Ten different viability assays were used to assess the cytotoxicity in HEK with five different nanomaterials (SWCNT, C_{60}, CB, nC_{60}, and QD). Nanomaterials tested were carbon or noncarbon based and soluble or nonsoluble in aqueous media. Although these assays provide accurate viability data for small molecule cytotoxicity studies, they were less reliable when assessing nanomaterials or NPs (Monteiro-Riviere et al., 2009).

Due to the poor solubility of fullerenes, limited research has been conducted in biological systems. Functionalization of C_{60} with the addition of amino acids, hydroxyl groups, or carboxyl groups can increase the ability of the NP to interact with the biological environment (Colvin, 2003; Sayes et al., 2004; Yang and Barron, 2004). ^{14}C-labeled underivatized C_{60} exposure to immortalized HEK demonstrated no effect on proliferation and fibroblasts but showed that the radiolabel was taken up; it was unclear whether NP actually entered the cell or associated with the cell membrane (Scrivens et al., 1994). HEK exposed to nC_{60} ranging from 0.0000025 to 0.0005 mg/mL and $C_{60}(OH)_{24}$ ranging from 0.000005 to 0.001 mg/mL for 24 and 48 h showed a significant decrease in viability by 24 h. Interleukin (IL)-8 and IL-6 were significantly greater than controls but IL-10, IL-1β, and TNF-α were below detectable limits. The $C_{60}(OH)_{24}$ showed no toxicity at all concentrations. These results showed that derivatized $C_{60}(OH)_{24}$ is nontoxic, and nC_{60} is toxic at concentrations as low as 0.00025 mg/mL in HEK (Inman et al., 2006). HEK exposed to a functionalized C_{60}-based amino acid [bucky amino acid (Baa)] fullerene at various concentrations depicted a statistically significant decrease in viability for concentrations of 0.4 and 0.04 mg/mL and a significant increase in IL-8 for the 0.04 mg/mL dose by 8, 12, and 24 h. IL-6 and IL-1β were greater at 24 and 48 h, but no significant TNF-α or IL-10 expression was noted. TEM depicted this Baa in vacuoles of HEK (Rouse et al., 2006). HEK exposed to 0.4–0.001 mg/mL of C_{60}-NLS-FITC for 24 and 48 h showed a decrease in viability that was statistically significant at 0.4 mg/mL at both 24 and 48 h. Cytokines IL-8 and IL-6 showed a statistically significant increase at 0.2 and 0.4 mg/mL. TEM of the C_{60}-NLS-FITC showed localization in vacuoles in close proximity to the nucleus (Fig. 15.3A) (Monteiro-Riviere et al., 2006). The endocytic pathways of a water-soluble fullerene-substituted phenylalanine (Baa) poly-lysine derivative [Baa-Lys(FITC)-$(Lys)_8$-OH] with a positive surface charge and an FITC label was studied in HEK. The cellular uptake pathway was mediated by caveolae/lipid rafts and cytoskeletal components and the inhibitor for the scavenger receptor completely blocked the uptake (Zhang et al., 2009). Functionalized fullerenes with hydroxyl groups can increase the solubility and interactions with cells. In vitro toxicity studies with three hydroxylated fullerenes at several concentrations were conducted in HEK and showed no cytotoxicity or inflammation up to 8.55 μg/mL (Saathoff et al., 2011).

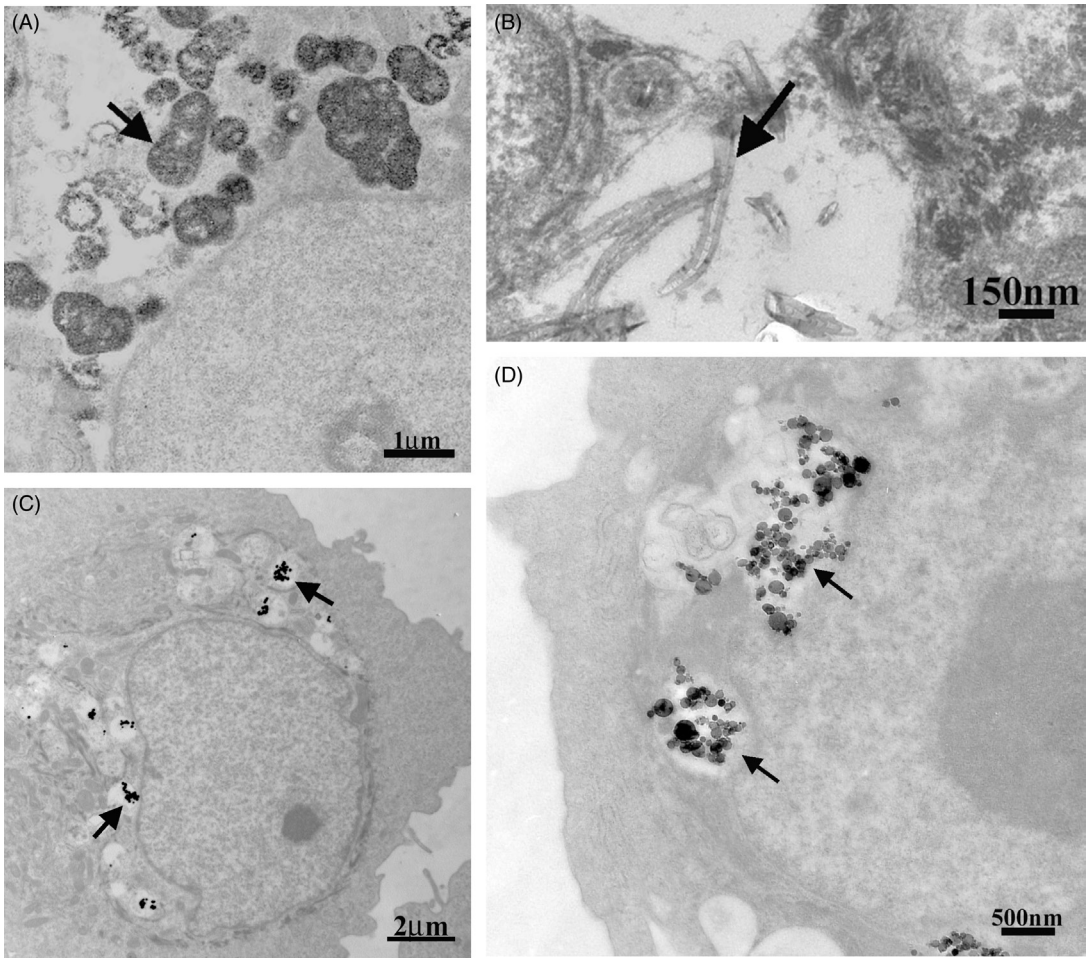


FIGURE 15.3 (A) TEM of the bioaccumulation of C_{60}-NLS-FITC NP localized in the cytoplasm but in close proximity to the nucleus of the HEK. (B) TEM depicting multiwalled carbon nanotubes (MWCNT) *(arrow)* within an intracellular cytoplasmic vacuole of an HEK. (C) TEM of 20 nm AgNP *(arrows)* within the intracytoplasmic vacuoles of an HEK. (D) TEM of 80 nm AlNP localized within the intracytoplasmic vacuoles of an HEK.

In addition, hydroxylated fullerenes were intravenously injected in rats and assessed at 8, 24, and 48 h showed no clinical chemistry changes and full histopathology on 29 target tissues did not show any adverse effects attributed to the fullerenes (Monteiro-Riviere et al., 2012)

C_{60} Fullerenes

Buckyballs or fullerenes are molecular structures made up of 60 or more carbon atoms. Fullerenes applied topically to mouse skin over 72 h had no effect on DNA synthesis. However, repeated application of fullerenes to mouse skin after initiation with dimethlybenzanthracene

(DMBA) for 24 weeks showed promotion with 12-0-tetradecanoylphorbol-13-acetate (TPA) that resulted in benign skin tumors (Nelson et al., 1993). C_{60}-NLS-FITC was topically applied at 4.4 μg and 1.1 μg/cm^2 to porcine skin in flow-through diffusion cells for 24 h with and without 1% Pluronic F127 surfactant or NLS-FITC alone showed penetration of the NP through all epidermal layers by confocal microscopy. TEM depicted C_{60}-NLS-FITC localized within the intercellular bilipid layers of the stratum corneum. The addition of a surfactant greatly enhanced the permeability for all treatments. These findings suggest that small substituted fullerenes can penetrate through intact skin and can elicit an inflammatory response (Monteiro-Riviere et al., 2006).

SWCNTs

Immortalized nontumorigenic human epidermal (HaCaT) cells exposed to unrefined SWCNT showed the formation of free radicals and a decrease in cell viability upon exposure for 18 h; however, inflammatory markers of irritation or inflammation were not evaluated (Shvedova et al., 2003). HEK dosed with 6-aminohexanoic acid-derivatized single-walled carbon nanotubes (AHA-SWCNT) at various concentrations exhibited a significant decrease in viability ($P < 0.05$) from 0.00005 to 0.05 mg/mL after 24 h. Biomarkers of inflammation, such as IL-6, increased in HEK treated with 0.05 mg/mL from 1 to 48 h, while IL-8 showed a significant increase at 24 and 48 h. Expression of other cytokines, such as TNF-α, IL-10, and IL-1β was not significant. TEM showed that AHA-SWCNT localized within intracytoplasmic vacuoles (Zhang et al., 2007). Studies with 1% Pluronic F127 surfactant used to disperse the AHA-SWCNT aggregates in the cell culture medium caused less toxicity (Zhang et al., 2007). Another study showed that SWCNT can activate NF-κB in a dose-dependent manner in HEK (Manna et al., 2005). Granulomas similar to those induced by foreign body reaction were seen 1 week after carbon nanotubes resembling stacked hats were implanted in the subcutaneous tissue of rats. Yokoyama et al. (2005) studied so-called hat-stacked carbon nanofibers and found no severe inflammatory responses after 4 weeks following subcutaneous implantation in rats.

MWCNTs

HEK exposed to unfunctionalized MWCNT at 0.1, 0.2, and 0.4 mg/mL concentrations for 1, 2, 4, 8, 12, 24, and 48 h showed a dose-dependent decrease in viability and a dose-dependent increase in the proinflammatory mediator IL-8. MWCNT were localized within cytoplasmic vacuoles (59% at 24 h and 84% at 48 h) (Fig. 15.3B) (Monteiro-Riviere et al., 2005b). When Pluronic F127 surfactant was added to the media it dispersed the MWCNT and reduced aggregation and was nontoxic to the HEK. MWCNT alone without surfactants were equally toxic and caused an increase in IL-8 expression. However, MWCNT with surfactants caused a decrease in IL-8 suppression than when exposed to medium alone. This suggests that surfactant–nanotube interactions alter the surface properties, thus modulating their interaction with viable cells similar to derivatized particles have altered cytotoxicity (Monteiro-Riviere et al., 2005a). Studies with lectin inhibitors that normally inhibit melanosome transfer pathways were studied to determine whether they play a role in reducing the cellular uptake of MWCNT. The lectin cocktails decreased MWCNT at the cell surface and reduced cellular

uptake (Zhang and Monteiro-Riviere, 2010). MWCNT have been shown to alter protein expression associated with metabolism, cell signaling, stress, and expression of cytoskeletal elements and vesicular trafficking (Witzmann and Monteiro-Riviere, 2006, 2007).

Quantum Dots

The cytotoxicity and proinflammatory potential of QD with different surface coatings were studied to determine if they cause differential cytotoxic effects on HEK and cytokine release. Toxicity was observed for PEG-amine and QD-COOH at 48 h with slight toxicity with PEG alone. The QD-COOH caused an increase in IL-1β, IL-6, and IL-8. Therefore, this study showed that the surface coatings of QD can play a role in cytotoxicity and immunotoxicity (Ryman-Rasmussen et al., 2007a). Endocytic pathways of untargeted QD were studied in HEK with three different surface coatings to determine the time-course effects of surface coating, temperature, culture medium or inhibitors of the cell cycle, and endocytosis. The time-course studies showed an increase in cellular interactions with time and media supplements, but decreasing the temperature decreased the magnitude of interactions of all three surface coatings. The biochemical mediators able to inhibit endocytosis were influenced by these variables (Ryman-Rasmussen et al., 2007b). Cytotoxicity of QD621 showed a dose-dependent decrease in viability from 0.3125 to 10 nM that was statistically significant at 1.25–10 nM compared to the controls. The cytotoxic and inflammatory responses of IL-8 and IL-6 were dose and time dependent. TEM verified that QD621 also were localized within the cells and along the periphery of the cell membrane after 24 h (Zhang et al., 2008).

There is a lack of understanding on the mechanism of QD cellular uptake and toxicity. Studies investigated the mechanism of uptake with QD655 with a carboxylic acid surface coating and showed that QD uptake was by lipid rafts and not clathrin or caveolae in HEK. However, QD were first internalized into early endosomes and then transferred to late endosomes or lysosomes. In addition, using 24 endocytic interfering agents showed that the QD endocytic pathways are regulated primarily by the G-protein–coupled receptor–associated pathway and low density lipoprotein receptor/scavenger receptor. There was minimal toxicity with the 20 nM dose in HEK at 48 h but not at 24 h. These findings were one of the first studies that provided insight into the specific mechanism of QD NP cellular uptake and that the surface coating, size, and charge of QD NP are important parameters in determining how NP cellular uptake occurs in mammalian cells (Zhang and Monteiro-Riviere, 2009).

Silver (Ag)NPs

Incorporation of AgNP is commonly used in the professional and personal healthcare fields and in consumer products, such as surgical and food handling tools, clothing, cosmetics, contact lens cases, catheters, and even children's toys. Skin exposure to AgNP is particularly high due to their increasing inclusion into textiles, burn creams, jewelry, acupuncture needles, and catheters. In fact, according to the database of the Nanotechnology Project of the Woodrow Wilson International Center for Scholars, AgNPs are the most commonly used material in all nanotechnology consumer products (Woodrow Wilson International Center for Scholars, 2011). Release of Ag ions from wound dressings can be triggered by wound fluids and tissue exudates. The Ag content of dressings varies from <0.1 mg/cm^2 to more

than 1 mg/cm^2 with the amount of Ag ions released ranging from <1 to >70 ppm. Several studies have evaluated the toxicity of Ag from AgNP-containing wound dressings and have shown elevated serum enzyme levels (including liver enzymes) as well as argyria (Wijnhoven et al., 2009). Argyria is a benign skin condition characterized by the bluish-graying of the skin that occurs through the preferential Ag deposition in the basal lamina of skin.

Ag in its bulk metallic form is inert and exerts no biocidal action but ionizes in the presence of water or tissue fluids to release antimicrobial Ag ions. The speciation of Ag does not play a large part in its toxicity, unlike other heavy metals. The toxicity of Ag^{3+} or Ag^{2+} is unlikely due to the transient nature of these forms of Ag, particularly in an aqueous environment. Furthermore, there is no discernable difference between Ag ions released from NPs from those generated by other sources, such as electrolysis or dissolution of ions from Ag salts. However, the rate of Ag ion production, as well as the distribution of Ag in tissue, may differ substantially between AgNP and other sources of silver ions due to the ability of AgNP to release ions into specific tissues, cell membranes, or cells. Additionally, once NPs are located within a site, they have the ability for protracted release of Ag ions, thus increasing the potential toxic impact. Furthermore, agglomeration of AgNP may change the rate of Ag ion offloading from their surface.

The cytotoxicity and inflammatory potential of AgNP were evaluated in HEK following 24 h exposure to 25 and 35 nm carbon-coated AgNP, as well as to either freshly synthesized or thoroughly washed 20, 50, and 80 nm AgNP at concentrations ranging from 0.00054 to 1.7 ppm (Samberg et al., 2010). Although cytotoxicity was only observed with freshly synthesized AgNP, small agglomerates of each of the AgNP were observed within the intracytoplasmic vacuoles of HEK (Fig. 15.3C). These same AgNP of different sizes, surface conditions, and synthesis methods were evaluated in *Escherichia coli*, Ag-resistant *E. coli*, *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), and *Salmonella* spp. This study yielded a range of minimal inhibitory concentrations, and was based on whether the AgNP solutions were contaminated by particle synthesis reducing agents. While freshly synthesized AgNPs were toxic to all bacterial strains at concentrations between 3.0 and 8.0 ppm, the washed AgNPs and the carbon-coated AgNPs were toxic to all bacterial strains at concentrations between 64.0 and 1024.0 ppm (Samberg et al., 2011). HEK and fibroblasts treated with AgNP dissolved out of commercially bought Ag-based wound dressings showed cytotoxicity at 15 μg/mL (Burd et al., 2007). Interactions of AgNP ranging from 7 to 20 nm in size were studied in human skin carcinoma cells and human fibrosarcoma cells and showed the onset of apoptosis at concentrations of 0.78 and 1.56 μg/mL, respectively (Arora et al., 2008).

Other Metals

Studies with 6.9 nm maghemite NP coated with tetramethylammonium hydroxide (TMAOH) and dispersed in a TMAOH aqueous solution, and iron NP (51%, 4.9 nm by TEM) coated with sodium bis(2-ethylexyl) sulfosuccinate (AOT) and dispersed in an AOT-rich aqueous solution were applied to in vitro human skin for 24 h. NPs were located at the stratum corneum–stratum granulosum interface, in hair follicles, and in the viable epidermis by TEM and EDS-SEM. NPs were not found in the receptor fluid by ICP-OES (Baroli et al., 2007). Aluminum (Al) is one of the most ubiquitous elements in our environment and is covered by an oxide film that generally inhibits corrosion. Al is found in explosives,

artillery surface coatings, and on rocket fuels. The cytotoxicity of AlNP of 30 nm did not cause mortality in immortalized mouse keratinocytes (Rooney et al., 2004). HEK exposed to 50 and 80 nm of AlNP in various concentrations showed no toxicity with four different viability assays (Monteiro-Riviere et al., 2010). AlNPs were also evaluated at different times (0 and 24 h) and at different temperatures (25 and 37°C) in both water and cell culture medium to determine what factors influenced cellular uptake in HEK. AlNPs were observed in HEK (Fig. 15.3D) in all conditions. AlNP agglomerated in deionized water and increased in size in the suspension at both 25 and 37°C. The hydrodynamic size of the NP was much greater in the cell culture medium, probably due to an increase in agglomeration that would reduce the effective dose of the NP to the HEK (Monteiro-Riviere et al., 2010). Others have suggested that Al found in antiperspirants may be linked to breast cancer (Exley et al., 2007). When 80 nm cobalt (Co)NPs were applied to in vitro Franz diffusion cells with intact and damaged human skin, Co flux was detected in the receiving solution after 24 h (Larese Filon et al., 2013). Statistically significant differences were found with Co flux permeation but only with damaged in vitro human skin (Mauro et al., 2015b). Higher permeation profiles were shown with 77 nm nickel nanoparticles (NiNPs) in synthetic sweat in needle-damaged skin compared to intact skin (Crosera et al., 2016). Rhodium (Rh)NP of 5.3 nm did not permeate through intact human skin but small amounts of Rh did permeate through damaged skin while platinum (Pt)NP of 5.8 nm were found only in damaged skin (Mauro et al., 2015a). Metal permeation or any chemical or NP penetration through in vitro human skin in diffusion cells is highly variable because investigators use different methods, receiving solutions, protocols, and duration of the experiment. It is difficult to compare these studies because human in vitro skin is not always fresh, but frozen and has undergone a surgical scrub that has removed the upper layers of the stratum corneum thereby damaging the barrier and making the skin more permeable.

TAKE-HOME MESSAGES

- The available literature has shown minimal penetration of NPs into the skin.
- NP physicochemical properties are important determinants to modulate skin penetration.
- Long-term in vivo studies under different conditions are needed to assess the true potential of NP penetration.
- No firm conclusion on possible adverse effects of NP on skin is possible on the basis of available data. Once again, long-term studies are needed.

References

Arora, S., Jain, J., Rajwade, J.M., Paknikar, K.M., 2008. Cellular responses induced by silver nanoparticles: in vitro studies. Toxicol. Lett. 179, 93–100.

Australian Therapeutics Goods Administration, 2013. Office of Scientific Evaluation Literature Review on the Safety of Titanium Dioxide and Zinc Oxide Nanoparticles in Sunscreens. R13/588508.

Baroli, B., Ennas, M.G., Loffredo, F., Isola, M., Pinna, R., Lopez-Quintela, A., 2007. Penetration of metallic nanoparticles in human full-thickness skin. J. Invest. Dermatol. 127, 1701–1712.

Bennat, C., Müller-Goymann, C.C., 2000. Skin penetration and stabilization of formulations containing microfine titanium dioxide as physical UV filter. Int. J. Cosmet. Sci. 22, 271–283.

Bianco, C., Adami, G., Crosera, M., Larese, F., Casarin, S., Castagnoli, C., Stella, M., Maina, G., 2014. Silver percutaneous absorption after exposure to silver nanoparticles: a comparison study of three human skin graft samples used for clinical applications. Burns 40 (7), 1390–1396.

Bianco, C., Kezic, S., Crosera, M., Svetlic˘ic´, V., Šegota, S., Maina, G., Romano, C., Larese, F., Adami, G., 2015. In vitro percutaneous penetration and characterization of silver from silver-containing textiles. Int. J. Nanomedicine 10, 1899–1908.

Borm, P.J.A., Robbins, D., Haubold, S., et al., 2006. The potential risks of nanomaterials: a review carried out for ECETOC. Part. Fibre Toxicol. 3, 11.

Bronaugh, R.L., 2008. Skin penetration of nanoparticles. In: Proceedings of PPP2008, Perspectives in Percutaneous Penetration, La Grande-Motte, March 25–29th, 2008, p. 1.

Burd, A., Kwok, C.H., Hung, S.C., et al., 2007. A comparative study of the cytotoxicity of silver-based dressings in monolayer cell, tissue explant, and animal models. Wound Repair Regen. 15, 94–104.

Chen, X., Schluesener, H.J., 2008. Nanosilver: a nanoproduct in medical application. Toxicol. Lett. 176, 1–12.

Colvin, V.L., 2003. The potential environmental impact of engineered nanomaterials. Nat. Biotechnol. 21, 1166–1170.

Cork, M.J., Robinson, D.A., Vasilopoulos, A., et al., 2006. New perspectives on epidermal barrier dysfunction in atopic dermatitis: gene–environment interactions. J. Allergy Clin. Immunol. 118, 3–21.

Crosera, M., Adami, G., Mauro, M., Bovenzi, M., Baracchini, E., Larese Filon, F., 2016. In vitro dermal penetration of nickel nanoparticles. Chemosphere 145, 301–306.

Crosera, M., Bovenzi, M., Maina, G., et al., 2009. Nanoparticle dermal absorption and toxicity: a review of the literature. Int. Arch. Occup. Environ. Health 82, 1043–1055.

Cross, S.E., Innes, B., Roberts, M., Tsuzuki, T., Robertson, T.A., McCormick, P., 2007. Human skin penetration of sunscreen nanoparticles: in-vitro assessment of a novel micronized zinc oxide formulation. Skin Pharmacol. Physiol. 20, 148–154.

Donaldson, K., Aitken, R., Tran, L., Stone, V., Duffin, R., Forrest, G., Alexander, A., 2006. Carbon nanotubes: a review of their properties in relation to pulmonary toxicology and workplace safety. Toxicol. Sci. 92, 5–22.

Dos Anjos, J.L.V., Alonso, A., 2008. Terpenes increase the partitioning and molecular dynamics of an amphipathic spin label in stratum corneum membranes. Int. J. Pharm. 350, 103–112.

Dussert, A.S., Gooris, E., 1997. Characterization of the mineral content of a physical sunscreen emulsion and its distribution onto human stratum corneum. Int. J. Cosmet. Sci. 19, 119–129.

Eedy, D.J., 1996. Carbon-fibre-induced airborne irritant contact dermatitis. Contact Dermatitis 35, 362–363.

Elias, P.M., 2010. Defensive functions of the stratum corneum: intergrative aspects. Monteiro-Riviere, N.A. (Ed.), Toxicology of the Skin—Target Organ Series, vol. 29, Informa Healthcare, New York, NY, pp. 19–28.

Elias, P.M., 1983. Epidermal lipids, barrier function, and desquamation. J. Invest. Dermatol. 80, 44s–49s.

Elsner, P., 1994. Irritant dermatitis in the workplace. Dermatol. Clin. 12, 461–467.

EPA, 2007. Nanotechnology White Paper. Prepared for the US Environmental Protection Agency by Members of the Nanotechnology Workgroup, a Group of EPA's Science Policy Council. US Environmental Protection Agency, Washington, DC.

Exley, C., Charles, L.M., Barr, L., Martin, C., Polwart, A., Darbre, P.D., 2007. Aluminium in human breast tissue. J. Inorg. Biochem. 101, 1344–1346.

Faunce, T., Murray, K., Nasu, H., Bowman, D., 2008. Sunscreen safety: the precautionary principle, the Australian Therapeutic Goods Administration and nanoparticles in sunscreens. Nanoethics 2, 231–240.

Filon, F.L., Boeninger, M., Maina, G., Adami, G., Spinelli, P., Damian, A., 2006. Skin absorption of inorganic lead and the effects of skin cleansers. J. Occup. Environ. Med. 48, 692–699.

Fiserova-Bergerova, V., Pierce, J.T., Droz, P.O., 1990. Dermal absorption potential of industrial chemicals: criteria for skin notation. Am. J. Ind. Med. 17, 617–635.

Fluhr, J.W., Dickel, H., Kuss, O., Weyher, I., Diepgen, T.L., Berardesca, E.I., 2002. Impact of anatomical location on barrier recovery, surface pH and stratum corneum hydration after acute barrier disruption. Br. J. Dermatol. 146, 770–776.

Gamer, A.O., Leibold, E., van Ravenzwaay, B., 2006. The in vitro absorption of microfine zinc oxide and titanium dioxide through porcine skin. Toxicol. In Vitro 20, 301–307.

Geiser, M., Schurch, S., Gehr, P., 2003. Influence of surface chemistry and topography of particles on their immersion into the lung's surface-lining layer. J. Appl. Physiol. 94, 1793–1801.

Geys, J., Coenegrachts, L., Vercammen, J., Engelborghs, Y., Nemmar, A., Nemery, B., Hoet, P.H., 2006. In vitro study of the pulmonary translocation of nanoparticles: a preliminary study. Toxicol. Lett. 160, 218–226.

Gopee, N.V., Roberts, D.W., Webb, P., et al., 2007. Migration of intradermally injected quantum dots to sentinel organs in mice. Toxicol. Sci. 98, 249–257.
Gulson, B., McCall, M., Korsch, M., et al., 2010. Small amounts of zinc from zinc oxide particles in sunscreens applied outdoors are absorbed through human skin. Toxicol. Sci. 118, 140–149.
Gwinn, M.R., Vallyathan, V., 2006. Nanoparticles: health effects—pros and cons. Environ. Health Perspect. 114, 1818–1825.
Holbrook, K., Odland, G., 1974. Regional differences in the thickness (cell layers) of the human stratum corneum: an ultrastructural analysis. J. Invest. Dermatol. 62, 415–422.
Holmes, A.M., Song, Z., Moghimi, H.R., Roberts, M.S., 2016. Relative penetration of zinc oxide and zinc ions into human skin after application of different zinc oxide formulations. ACS Nano 10, 1810–1819.
Inman, A.O., Sayes, C.M., Colvin, V.L., Monteiro-Riviere, N.A., 2006. Nano-C_{60} and derivatized C_{60} toxicity in human epidermal keratinocytes. Toxicol. Sci. 90 (S-1), 167.
Journeay, W.S., Goldman, R.H., 2014. Occupational handling of nickel nanoparticles: a case report. Am. J. Ind. Med. 57, 1073–1076.
Kertész, Z.S., Szikszai, Z., Gontier, E., et al., 2005. Nuclear microprobe study of TiO_2-penetration in the epidermis of human skin xenografts. Nucl. Instr. Meth. Phys. Res. B 231, 280–285.
Kielhorn, J., Melching-Kollmuß, S., Mangelsdorf, I., 2006. World Health Organization (WHO), Environmental Health Criteria 235: Dermal Absorption.
Kishore, A.S., Surekha, P., Murthy, P.B., 2009. Assessment of the dermal and ocular irritation potential of multi-walled carbon nanotubes by using in vitro and in vivo methods. Toxicol. Lett. 191, 268–274.
Kiss, B., Biró, T., Czifra, G., et al., 2008. Investigation of micronizided titanium dioxide penetration in human skin xenografts and its effect on cellular functions of human skin-derived cells. Exp. Dermatol. 17, 659–667.
Labouta, H.I., Schneider, M., 2013. Interaction of inorganic nanoparticles with the skin barrier: current status and critical review. Nanomedicine 9 (1), 39–54.
Lademann, J., Weigmann, H.J., Rickmeyer, C., et al., 1999. Penetration of titanium dioxide in sunscreen formulation into the horny layer and the follicular orifice. Skin Pharmacol. Appl. Skin Physiol. 12, 247–256.
Lam, C.W., James, J.T., McCluskey, R., Hunter, R.L., 2004. Pulmonary toxicity of single-wall carbon nanotubes in mice 7 and 90 days after intratracheal instillation. Toxicol. Sci. 77, 126–134.
Landmann, L., 1986. Epidermal permeability barrier: transformation of lamellar granule-disks into intercellular sheets by a membrane-fusion process, a freeze-fracture study. J. Invest. Dermatol. 87, 202–209.
Lansdown, A.B., Taylor, A., 1997. Zinc and titanium oxides: promising UV-absorbers but what influence do they have on the intact skin? Int. J. Cosmet. Sci. 19, 167–172.
Larese Filon, F., Adami, G., Venier, M., et al., 2004. In vitro percutaneous absorption of cobalt. Int. Arch. Environ. Health 77, 85–89.
Larese Filon, F., Crosera, M., Adami, G., Bovenzi, M., Rossi, F., Maina, G., 2011. Human skin penetration of gold nanoparticles through intact and damaged skin. Nanotoxicology 5, 493–501.
Larese Filon, F., Crosera, M., Timeus, E., Adami, G., Bovenzi, M., Ponti, J., Maina, G., 2013. Human skin penetration of cobalt nanoparticles through intact and damaged skin. Toxicol. In Vitro 27 (1), 121–127.
Larese Filon, F., D'Agostin, F., Bovenzi, M., et al., 2009. Human skin penetration of silver nanoparticles through intact and damaged skin. Toxicology 255, 33–37.
Larese Filon, F., Mauro, M., Adami, G., Bovenzi, M., Crosera, M., 2015. Nanoparticles skin absorption: new aspects for a safety profile evaluation. Regul. Toxicol. Pharmacol. 72, 310–322.
Lee, H.A., Imran, M., Monteiro-Riviere, N.A., Colvin, V.L., Yu, W.W., Riviere, J.E., 2007. Biodistribution of quantum dot nanoparticles in perfused skin: evidence of coating dependency and periodicity in arterial extraction. Nano Lett. 9, 2865–2870.
Limbach, L.K., Wick, P., Manser, P., Grass, R.N., Bruinink, A., Stark, W.J., 2007. Exposure of engineered nanoparticles to human lung epithelial cells: influence of chemical composition and catalytic activity on oxidative stress. Environ. Sci. Technol. 41, 4158–4163.
Madison, K.C., Swartzendruber, D.C., Wertz, P.W., 1987. Presence of intact intercellular lipid lamellae in the upper layers of the stratum corneum. J. Invest. Dermatol. 88, 714–718.
Magrez, A., Kasas, S., Salicio, V., et al., 2006. Cellular toxicity of carbon-based nanomaterials. Nano Lett. 6, 1121–1125.
Manna, S.K., Sarkar, S., Barr, J., et al., 2005. Single-walled carbon nanotube induces oxidative stress and activates nuclear transcription factor-κB in human keratinocytes. Nano Lett. 5, 1676–1684.

Mauro, M., Crosera, M., Bianco, C., Adami, G., Montini, T., Fornasiero, P., Jaganjac, M., Bovenzi, M., Larese Filon, F., 2015a. Permeation of platinum and rhodium nanoparticles through intact and damaged human skin. J. Nanopart. Res. 17, 253.

Mauro, M., Crosera, M., Pelin, M., Florio, C., Bellomo, F., Adami, G., Apostoli, P., DePalma, G., Bovenzi, M., Campanini, M., Filon, F., 2015b. Cobalt oxide nanopartilces: behavior towards intact and impaired human skin and keratinocytes toxicity. Int. J. Environ. Res. Public Health 12 (7), 8263–8280.

Mavon, A., Miquel, C., Lejeune, O., Payre, B., Moretto, P., 2007. In vitro percutaneous absorption and in vivo stratum corneum distribution of an organic and mineral sunscreen. Skin Pharmacol. Physiol. 20, 10–20.

Menon, G.K., Feinfold, K.R., Elias, P.M., 1992. The lamellar secretory response to barrier disruption. J. Invest. Dermatol. 98, 279–289.

Menten, D.A., 1976a. A minimum surface mechanism to account for the organization of cells into columns in the mammalian epidermis. Am. J. Anat. 145, 1–22.

Menten, D.A., 1976b. Liquid film model of tetrakaidecahedral packing to account for the establishment of epidermal cell columns. J. Invest. Dermatol. 66, 283–291.

Menzel, F., Reinert, T., Vogt, J., Butz, T., 2004. Investigations of percutaneous uptake of ultrafine TiO_2 particles at the high energy ion nanoprobe LIPSION. Nucl. Instr. Meth. Phys. Res. B 219–220, 82–86.

Monteiro-Riviere, N.A., 1991. Comparative anatomy, physiology, and biochemistry of mammalian skin. In: Hobson, D.W. (Ed.), Dermal and Ocular Toxicology Fundamentals and Methods. CRC Press, Boca Raton, FL, pp. 3–71.

Monteiro-Riviere, N.A., 2006. The integument. In: Eurell, J., Frappier, B. (Eds.), Dellmann's Textbook of Veterinary Histology. sixth ed. Blackwell Publishing, Ames, IA, pp. 320–349.

Monteiro-Riviere, N.A., 2008. Anatomical factors that affect barrier function. In: Zhai, H., Wilhelm, K.P., Maibach, H.I. (Eds.), Dermatotoxicology. seventh ed. CRC Press, New York, NY, pp. 39–50.

Monteiro-Riviere, N.A., 2010. Structure and function of skin. Monteiro-Riviere, N.A. (Ed.), Toxicology of the Skin—Target Organ Series, vol. 29, Informa Healthcare, New York, NY, pp. 1–18.

Monteiro-Riviere, N.A., 2014. Safety implications of nanomaterial exposure to skin. In: Monteiro-Riviere, N.A., Tran, C.L. (Eds.), Nanotoxicology: Progress Toward Nanomedicine. second ed. CRC Press, Boca Raton, FL, pp. 247–272, (Chapter 13).

Monteiro-Riviere, N.A., Baroli, B., 2010. Nanomaterial penetration. Monteiro-Riviere, N.A. (Ed.), Toxicology of the Skin—Target Organ Series, vol. 29, Informa Healthcare, New York, NY, pp. 333–346.

Monteiro-Riviere, N.A., Bristol, D.G., Manning, T.O., Riviere, J.E., 1990. Interspecies and interregional analysis of the comparative histological thickness and laser Doppler blood flow measurements at five cutaneous sites in nine species. J. Invest. Dermatol. 95, 582–586.

Monteiro-Riviere, N.A., Inman, A.O., 2006. Challenges for assessing carbon nanomaterial toxicity to the skin. Carbon 44, 1070–1078.

Monteiro-Riviere, N.A., Inman, A.O., 2008. Evaluation of quantum dot nanoparticle penetration in human skin. Toxicologist 102 (Suppl. 1), 1029.

Monteiro-Riviere, N.A., Inman, A.O., Erdmann, D., Xia, X., Riviere, J.E., 2011a. Terpene effects on penetration of nanoparticles in human skin. Toxicologist 120 (Suppl. 2), 2154.

Monteiro-Riviere, N.A., Inman, A.O., Mak, V., Wertz, P., Riviere, J.E., 2001. Effect of selective lipid extraction from different body regions on epidermal barrier function. Pharm. Res. 18, 992–998.

Monteiro-Riviere, N.A., Inman, A.O., Ryman-Rasmussen, J.P., 2007. Dermal effects of nanomaterials. In: Monteiro-Riviere, N.A., Tran, C.L. (Eds.), Nanotoxicology: Characterization, Dosing, and Health Effects. Informa Healthcare, New York, NY, pp. 317–338.

Monteiro-Riviere, N.A., Inman, A.O., Wang, Y.Y., Nemanich, R.J., 2005a. Surfactant effects on carbon nanotube interactions with human epidermal keratinocytes. Nanomedicine 1, 293–299.

Monteiro-Riviere, N.A., Inman, A.O., Zhang, L.W., 2009. Limitations and relative utility of screening assays to assess engineered nanoparticle toxicity in a human cell line. Toxicol. Appl. Pharmacol. 234, 222–235.

Monteiro-Riviere, N.A., Linder, K.E., Inman, A.O., Saathoff, J.G., Xia, X.R., Riviere, J.E., 2012. Lack of hydroxylated fullerene toxicity after intravenous administration to female Sprague-Dawley rats. J. Toxicol. Environ. Health A 75 (7), 367–373.

Monteiro-Riviere, N.A., Nemanich, R.J., Inman, A.O., Wang, Y.Y., Riviere, J.E., 2005b. Multi-walled carbon nanotube interactions with human epidermal keratinocytes. Toxicol. Lett. 155, 377–384.

Monteiro-Riviere, N.A., Oldenburg, S.J., Inman, A.O., 2010. Interactions of aluminum nanoparticles in human epidermal keratinocytes. J. Appl. Toxicol. 30, 276–285.

Monteiro-Riviere, N.A., Riviere, J.E., 2009. Interaction of nanomaterials with skin: aspects of absorption and biodistribution. Nanotoxicology 3, 188–193.
Monteiro-Riviere, N.A., Wiench, K., Landsiedel, R., Schulte, S., Inman, A.O., Riviere, J.E., 2011b. Safety evaluation of sunscreen formulations containing titanium dioxide and zinc oxide nanoparticles in UVB sunburned skin: an in vitro and in vivo study. Toxicol. Sci. 123, 264–280.
Monteiro-Riviere, N.A., Yang, J., Inman, A.O., Ryman-Rasmussen, J., Barron, A.R., Riviere, J.E., 2006. Skin penetration of fullerene substituted amino acids and their interactions with human epidermal keratinocytes. Toxicol. Sci. 90 (Suppl. 1), 168.
Muller, J., Huaux, F., Moreau, N., et al., 2005. Respiratory toxicity of multi-wall carbon nanotubes. Toxicol. Appl. Pharmacol. 207, 221–231.
NANODERM, 2007. Quality of Skin as a Barrier to Ultra-Fine Particles. Final Report. Project Number: QLK4-CT-2002-02678. Available from: www.uni-leipzig.de/~nanoderm/
Nel, A., Xia, T., Mädler, L., Li, N., 2006. Toxic potential of materials at the nanolevel. Science 311, 622–627.
Nelson, M.A., Frederick, E.D., Bowden, G.T., Hooser, S.B., Fernando, Q., Carter, D.E., 1993. Effects of acute and subchronic exposure of topically applied fullerene extracts on the mouse skin. Toxicol. Ind. Health 9, 623–630.
Nielsen, J.B., Grandjean, P., 2004. Criteria for skin notation in different countries. Am. J. Ind. Med. 45, 275–280.
Nielsen, J.B., Nielsen, F., Sørensen, J.A., 2007. Defense against dermal exposures is only skin deep: significantly increased penetration through slightly damaged skin. Arch. Dermatol. Res. 299, 423–431.
NIOSH (National Institute for Occupational Safety and Health), 2007. Progress Toward Safe Nanotechnology in the Workplace—A Report from the NIOSH Nanotechnology Research Center, DHHS (NIOSH). Publication No. 2007-123. Available from: www.cdc.gov/niosh
Norton, S.K., Dellinger, Z., Zhou, Z., et al., 2010. A new class of human mast cell and peripheral blood basophil stabilizers that differentially control allergic mediator release. Clin. Trans. Sci. 3, 158–169.
Oberdörster, G., Maynard, A., Donaldson, K., ILSI Research Foundation/Risk Science Institute Nanomaterial Toxicity Screening Working Group, et al., 2005a. Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy. Part. Fibre Toxicol. 2, 8.
Oberdörster, G., Oberdörster, E., Oberdörster, J., 2005b. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environ. Health Perspect. 113, 823–839.
Pflücker, F., Wendel, V., Hohenberg, H., et al., 2001. The human stratum corneum layer: an effective barrier against dermal uptake of different forms of topically applied micronized titanium dioxide. Skin Pharmacol. Appl. Skin Physiol. 14 (S1), 92–97.
Prow, T.W., Monteiro-Riviere, N.A., Inman, A.O., et al., 2012. Quantum dot penetration into viable human skin. Nanotoxicology 6, 173–185.
Raphael, A.P., Sundh, D., Grice, J.E., Roberts, M.S., Soyer, H.P., Prow, T.W., 2013. Zinc oxide nanoparticle removal from wounded human skin. Nanomedicine 8 (11), 1751–1761.
Riviere, J.E., 1994. Absorption and distribution. In: Hodgson, E., Levi, P.R. (Eds.), Introduction to Biochemical Toxicology. second ed. Appleton & Lange Publishers, Norwalk, CT, pp. 11–49.
Riviere, J.E., Brooks, J.D., 2005. Predicting skin permeability from complex chemical mixtures. Toxicol. Appl. Pharmacol. 208, 99–110.
Riviere, J.E., Brooks, J.D., 2011. Predicting skin permeability from complicated chemical mixtures: dependency of quantitative structure permeability relationships (QPSR) on biology of skin models used. Toxicol. Sci. 119, 224–232.
Riviere, J.E., Monteiro-Riviere, N.A., 2010. Toxicokinetics: dermal exposure and absorption of chemicals and nanomaterials. McQueen, C. (Ed.), Comprehensive Toxicology, vol. 1, second ed. Elsevier, New York, NY, pp. 111–122.
Roberts, M.S., Gierden, A., Riviere, J.E., Monteiro-Riviere, N.A., 2008. Solvents and vehicle effects on the skin. In: Roberts, M.S., Walters, K.A. (Eds.), Dermal Absorption and Toxicity Assessment. second ed. Informa Healthcare, New York, NY, pp. 433–447.
Roco, M.C., 2005. Environmentally responsible development of nanotechnology. Environ. Sci. Technol. 39, 106A–112A.
Rooney, A.D., Jones, R.L., Mattie, D.R., Schlager, J.J., Hussain, S.M., 2004. In Vitro Toxicity of Nanoparticles in Mouse Keratinocytes and Endothelial Cells. United States Air Force Research Laboratory, AFRL-HE-WP-TR-2005-0091.
Rotoli, B.M., Bussolati, O., Bianchi, M.G., et al., 2008. Non-functionalized multi-walled carbon nanotubes alter the paracellular permeability of human airway epithelial cells. Toxicol. Lett. 178, 95–102.

Rouse, J.G., Haslauer, C.M., Loboa, E.G., Monteiro-Riviere, N.A., 2008. Cyclic tensile strain increases interactions between human epidermal keratinocytes and quantum dot nanoparticles. Toxicol. In Vitro 22, 491–497.
Rouse, J.G., Yang, J., Barron, A.R., Monteiro-Riviere, N.A., 2006. Fullerene based amino acid nanoparticle interactions with human epidermal keratinocytes. Toxicol. In Vitro 20, 1313–1320.
Rouse, J.G., Yang, J., Ryman-Rasmussen, J.P., Barron, A.R., Monteiro-Riviere, N.A., 2007. Effects of mechanical flexion on the penetration of fullerene amino acid-derivatized peptide nanoparticles through skin. Nano Lett. 7, 155–160.
Ryman-Rasmussen, J.P., Riviere, J.E., Monteiro-Riviere, N.A., 2006. Penetration of intact skin by quantum dots with diverse physicochemical properties. Toxicol. Sci. 91, 159–165.
Ryman-Rasmussen, J.P., Riviere, J.E., Monteiro-Riviere, N.A., 2007a. Surface coatings determine cytotoxicity and irritation potential of quantum dot nanoparticles in epidermal keratinocytes. J. Invest. Dermatol. 127, 143–153.
Ryman-Rasmussen, J.P., Riviere, J.E., Monteiro-Riviere, N.A., 2007b. Variables influencing interactions of untargeted quantum dot nanoparticles with skin cells and identification of biochemical modulators. Nano Lett. 7, 1344–1348.
Ryu, H.J., Seo, M.Y., Jung, S.K., Maeng, E.H., Lee, S.Y., Jang, D.H., Lee, T.J., Jo, K.Y., Kim, Y.R., Cho, K.B., Kim, M.K., Lee, B.J., Son, S.W., 2014. Zinc oxide nanoparticles: a 90-day repeated-dose dermal toxicity study in rats. Int. J. Nanomedicine 9 (Suppl. 2), 137–144.
Saathoff, J.G., Inman, A.O., Xia, X.R., Riviere, J.E., Monteiro-Riviere, N.A., 2011. In vitro toxicity assessment of three hydroxylated fullerenes in human skin cells. Toxicol. In Vitro 25, 2105–2112.
Sadrieh, N., Wokovich, A.M., Gopee, N.V., et al., 2010. Lack of significant dermal penetration of titanium dioxide from sunscreen formulations containing nano- and submicron-size TiO_2 particles. Toxicol. Sci. 115, 156–166.
Samberg, M.E., Oldenburg, S.J., Monteiro-Riviere, N.A., 2010. Evaluation of silver nanoparticle toxicity in skin in vivo and keratinocytes in vitro. Environ. Health Perspect. 118, 407–413.
Samberg, M.E., Orndorff, P.E., Monteiro-Riviere, N.A., 2011. Antibacterial efficacy of silver nanoparticles of different sizes, surface conditions and synthesis methods. Nanotoxicology 5, 244–253.
Sapra, B., Jain, S., Tiwary, A.K., 2008. Percutaneous permeation enhancement by terpenes: mechanistic view. AAPS J. 10, 120–132.
Sartorelli, P., Ahlers, H.W., Alanko, K., et al., 2007. How to improve skin notation. Position paper from a workshop. Regul. Toxicol. Pharmacol. 49, 301–307.
Sayes, C.M., Fortner, J.D., Guo, W., et al., 2004. The differential cytotoxicity of water-soluble fullerenes. Nano Lett. 4, 1881–1887.
SCCNFP, 2000. Opinion of the Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers Concerning Titanium Dioxide. European Commission, Brussels, Belgium, Colipa No. S 75.
SCCP (Scientific Committee on Consumer Products), 2007. Preliminary Opinion on Safety of Nanomaterials in Cosmetic Products. European Commission, Brussels, Belgiumhttp://ec.europa.eu/health/archive/ph_risk/committees/04_sccp/docs/sccp_o_123.pdf.
SCCS, 2012. Scientific Committee on Consumer Safety Opinion on Zinc Oxide, SCCS/1489/12.
Scheuplein, R.J., 1967. Mechanisms of percutaneous absorption. II. Transient diffusion and the relative importance of various routes of skin penetration. J. Invest. Dermatol. 48, 79–88.
Schulz, J., Hohenberg, H., Pflücker, F., et al., 2002. Distribution of sunscreens on skin. Adv. Drug Deliv. Rev. 54 (S1), S157–S163.
Scrivens, W.A., Tour, J.M., Creek, K.E., Pirisi, L., 1994. Synthesis of ^{14}C-labeled C_{60}, its suspension in water, and its uptake by human keratinocytes. J. Am. Chem. Soc. 116, 4517–4518.
Shimada, A., Kawamura, N., Okajima, M., Kaewamatawong, T., Inoue, H., Morita, T., 2006. Translocation pathway of the intratracheally instilled ultrafine particles from the lung into the blood circulation in the mouse. Toxicol. Pathol. 34, 949–957.
Shvedova, A.A., Castranova, V., Kisin, E., et al., 2003. Exposure to carbon nanotube material: assessment of cytotoxicity using human keratinocytes cells. J. Toxicol. Environ. Health 66, 1909–1926.
Shvedova, A.A., Kisin, E.R., Mercer, R., et al., 2005. Unusual inflammatory and fibrogenic pulmonary responses to single-walled carbon nanotubes in mice. Am. J. Physiol. Lung Cell Mol. Physiol. 289, L698–L708.
Sonavane, G., Tomoda, K., Sano, A., Ohshima, H., Terada, H., Makino, K., 2008. In vitro permeation of gold nanoparticles through rat skin and rat intestine: effect of particle size. Colloids Surf. B 65, 1–10.
Swartzendruber, D.C., Wertz, P.W., Kitko, D.J., Madison, K.C., Downing, D.T., 1989. Molecular models of the intercellular lipid lamellae in mammalian stratum corneum. J. Invest. Dermatol. 92, 251–257.
Swartzendruber, D.C., Wertz, P.W., Madison, K.C., Downing, D.T., 1987. Evidence that the corneocyte has a chemically bound lipid envelope. J. Invest. Dermatol. 88, 709–713.

Tan, M.H., Commens, C.A., Burnett, L., Snitch, P.J., 1996. A pilot sudy on the percutaneous absorption of microfine titanium dioxide from sunscreens. Australas. J. Dermatol. 37, 185–187.
Tang, L., Zhang, C., Song, G., Jin, X., Xu, Z., 2013. In vivo skin penetration and metabolic path of quantum dots. Sci. Chin. Life Sci. 56 (2), 181–188.
Tinkle, S.S., Antonini, J.M., Rich, B.A., et al., 2003. Skin as a route of exposure and sensitization in chronic beryllium disease. Environ. Health Perspect. 111, 1202–1208.
Vermula, P.K., Anderson, R.R., Karp, J.M., 2011. Nanoparticles reduce nickel allergy by capturing metal ions. Nat. Nanotechnol. 5, 291–295.
Vogt, A., Rancan, F., Ahlberg, S., Nazemi, B., Choe, C.S., Darvin, M.E., Hadam, S., Blume-Peytavi, U., Loza, K., Diendorf, J., Epple, M., Graf, C., Ruhl, E., Meinke, M.C., Lademann, J., 2014. Interaction of dermatologically relevant nanoparticles with skin cells and skin. Nanotechnology 5, 2363–2373.
Wijnhoven, S.W.P., Peijnenburg, W.J.G.M., Herberts, C.A., et al., 2009. Nano-silver: a review of available data and knowledge gaps in human and environmental risk assessment. Nanotoxicology 3, 109–138.
Witzmann, F.A., Monteiro-Riviere, N.A., 2006. Multi-walled carbon nanotube exposure alters protein expression in human keratinocytes. Nanomedicine 2, 158–168.
Witzmann, F.A., Monteiro-Riviere, N.A., 2007. Effect of carbon nanotube exposure on keratinocyte protein expression. In: Monteiro-Riviere, N.A., Tran, C.L. (Eds.), Nanotoxicology: Characterization, Dosing, and Health Effects. Informa Healthcare, New York, NY, pp. 197–224.
Woodrow Wilson International Center for Scholars, 2011. A Nanotechnology Consumer Products Inventory. Available from: www.nanotechproject.org/consumerproducts
Xia, X.R., Monteiro-Riviere, N.A., Riviere, J.E., 2010. Skin penetration and kinetics of pristine fullerenes (C_{60}) topically exposed in industrial organic solvents. Toxicol. Appl. Pharmacol. 242, 29–37.
Yang, J., Barron, A.R., 2004. A new route to fullerene substituted phenylalanine derivatives. Chem. Commun. 24, 2884–2885.
Yokoyama, A., Sato, Y., Nodasaka, Y., et al., 2005. Biological behavior of hat-stacked carbon nanofibers in the subcutaneous tissue in rats. Nano Lett. 5, 157–161.
Yu, W.W., Chang, E., Falkner, J.C., et al., 2007. Forming biocompatible and nonaggregated nanocrystals in water using amphiphilic polymers. J. Am. Chem. Soc. 129, 2871–2879.
Zhang, L.W., Monteiro-Riviere, N.A., 2008. Assessment of quantum dot penetration into intact, tape stripped, abraded and flexed rat skin. Skin Pharmacol. Physiol. 21, 166–180.
Zhang, L.W., Monteiro-Riviere, N.A., 2009. Mechanisms of quantum dot nanoparticle cellular uptake. Toxicol. Sci. 110 (1), 138–155.
Zhang, L.W., Monteiro-Riviere, N.A., 2010. Lectins modulate multi-walled carbon nanotubes cellular uptake in human epidermal keratinocytes. Toxicol. In Vitro 24, 546–551.
Zhang, L.W., Yang, J., Barron, A.R., Monteiro-Riviere, N.A., 2009. Endocytic mechanisms and toxicity of a functionalized fullerene in human cells. Toxicol. Lett. 191, 149–157.
Zhang, L.W., Yu, W.W., Colvin, V.L., Monteiro-Riviere, N.A., 2008. Biological interactions of quantum dot nanoparticles in skin and in human epidermal keratinocytes. Toxicol. Appl. Pharmacol. 228, 200–211.
Zhang, L.W., Zeng, L., Barron, A.R., Monteiro-Riviere, N.A., 2007. Biological interactions of functionalized single-wall carbon nanotubes in human epidermal keratinocytes. Int. J. Toxicol. 26, 103–113.

CHAPTER

16

Gastrointestinal System

*Mark A. Jepson**, *Hans Bouwmeester***,†

***University of Bristol, Bristol, United Kingdom; **RIKILT—Wageningen UR, Wageningen, The Netherlands; †Wageningen University, Wageningen, The Netherlands**

OUTLINE

Introduction 381

Conditions and Barriers in the Gastrointestinal Tract 383

Uptake of Nanomaterials in the Gastrointestinal Tract 384

In Vitro Models to Study Uptake 385

Potential Toxicity of Nanomaterials in the Gastrointestinal Tract 387

Conclusions 389

Take-Home Messages 390

Acknowledgments 390

References 390

INTRODUCTION

The rapid expansion in nanotechnology increases the likelihood of exposure to nanomaterials and as a consequence increases the need to assess the potential health impact of such exposure. The oral route of exposure is nowadays recognized as an important exposure route, simply because of the wide range of emerging applications in food, such as the enhancement of texture, color, flavor, and food packaging safety (Bouwmeester et al., 2014; Sozer and Kokini, 2009). In the past decade, considerable progress has been made in our understanding of the mechanisms underlying the toxicity of nanomaterials, and the impact, behavior, and interaction of nanomaterials in the gastrointestinal tract (GIT).

A great diversity of materials is used in emerging food applications, examples include silver and zinc oxide nanoparticles, and food additives like titanium dioxide (TiO_2) and silicon dioxide (SiO_2). TiO_2, as titanium white, and SiO_2 are produced at high production volumes (Piccinno et al., 2012), and are authorized ingredients in food, consumer, and medicinal

Adverse Effects of Engineered Nanomaterials. http://dx.doi.org/10.1016/B978-0-12-809199-9.00016-1
Copyright © 2017 Elsevier Inc. All rights reserved.

products (EU Official Journal of the European Union, 2008a,b; European Commission, 1994; U.S. Food and Drug Administration, 2002). Both materials, produced mainly by flame processes or precipitation methods, consist mainly (SiO_2) or partly (TiO_2) of primary particles in the nanosize range (i.e., <100 nm) that may be aggregated and agglomerated to form larger structures (Peters et al., 2012, 2014; Sepeur et al., 2008; Weir et al., 2012).

Nanomaterials incorporated in food, toothpaste, cosmetics, and sunscreen also carry a clear potential of inadvertent ingestion for a large proportion of the population. Furthermore, the more widely studied inhalation route of nanomaterials will also result in secondary exposure of GIT following clearance from the respiratory tract. Only a few studies estimated the daily exposure to nanomaterials via food (van Kesteren et al., 2015; Weir et al., 2012). So we can conclude that the extent of exposure to nanomaterials is still poorly quantified and there is a need to understand the potential impact these materials may have within the GIT itself and the extent to which they may be translocated from GIT to the circulation and peripheral organs (primarily the liver and spleen), where they have the potential to exert toxic effects. One area that has been studied more extensively is the uptake of "nanocarriers" developed as vehicles for oral drug and vaccine delivery, which depend on their ability to interact with, and in some cases to be transported by, GIT epithelia. While data on the uptake of such particulate delivery devices identifies potential routes for uptake of other nanomaterials, it also highlights the complexity of particle interactions in the GIT which currently prevents accurate prediction of the behavior of individual nanomaterials.

In this chapter, we will discuss the different routes for uptake and transport of nanomaterials that exist in the GIT and review available literature characterizing these routes and their relevance for uptake of nanomaterials into the body. Much of the available data on nanomaterial uptake and transport from both in vivo and in vitro studies has come more from the pharmaceutical research field where development and characterization of particulate delivery vehicles for drugs and/or vaccines has been a hot topic for many years. Several in vivo studies have been performed to assess the gastrointestinal uptake and distribution of nanomaterials (Johnston et al., 2010; Lefebvre et al., 2015; Schleh et al., 2012), and larger particulates (Brayden et al., 2005; Jepson et al., 1993a,b, 1996). It is important to understand the potential routes for uptake and the factors that may limit or promote such uptake to estimate the risk to the organism of nanomaterials transport from the GIT lumen to peripheral tissues.

The chapter will also outline what is known about the toxicity of nanomaterials in the gut, and highlight where information is lacking. It has been hypothesized that particulates such as TiO_2 might play a role in the pathogenesis of inflammatory bowel disease (Butler et al., 2007; Lomer et al., 2002) and some experimental evidence supports this hypothesis (Nogueira et al., 2012), but direct evidence of a causal link between nanomaterials and gut pathology is lacking. For mechanistic studies in vitro models can be used and we will highlight the most frequently used approaches. Classical approaches are based on Caco-2 cells, a human adenocarcinoma cell-line that, when grown on permeable supports, differentiates to form a polarized epithelial layer with well-developed microvilli and tight junctions that closely models the epithelium of small intestine and is widely used to study drug absorption among other aspects of gut function (Artursson and Borchardt, 1997), but more advanced models are also used nowadays including coculture and 3D GIT models (Lefebvre et al., 2015). Detailed discussion of the potential toxicity mechanisms of nanomaterials in GIT cells is beyond the scope of this chapter and to a large extent these mirror the mechanisms described in other cell

types. However, it should also be kept in mind that cell differentiation into fully polarized epithelia with microvilli that more closely resemble in vivo GIT lining may reduce NM uptake (Cartiera et al., 2009; Hansen et al., 2009) and some studies have suggested that inflammatory responses to nanomaterials may be less prominent in GIT compared with other cells (Powell et al., 2000, 2010). The specialized morphology and function of differentiated GIT epithelial cells also needs to be carefully considered in the context of nanotoxicity assessment because effects of nanomaterials on epithelia may severely compromise GIT function in ways that would not be apparent from traditional toxicity assays.

CONDITIONS AND BARRIERS IN THE GASTROINTESTINAL TRACT

Ingested nanomaterials encounter conditions within the GIT that are very different from those encountered when contacting the organism via other routes and it is not yet clear how this may affect their potential toxicity (Bellmann et al., 2015). For example, the low pH of the stomach will critically affect nanomaterial properties (McCracken et al., 2013; Mwilu et al., 2013; Peters et al., 2012; Rogers et al., 2012; Walczak et al., 2013) and the likelihood that they dissociate to yield products with differing toxicity profiles. The extreme pH shifts encountered during gut transit and the presence of exogenous molecules coingested with nanomaterials and the proteins, mucus, and bile acids secreted within the gut may also affect the physicochemical and aggregation properties of nanomaterials (Lichtenstein et al., 2015; Walczak et al., 2015b). In comparison with some other exposure routes, such as in the alveoli of the lungs, residence time within GIT may be much shorter due to normal gut transit, reducing cellular exposure to ingested nanomaterials unless they are mucoadhesive or bioadhesive.

As we have seen, there are outstanding questions concerning the quantity and status of nanomaterials arriving in the gut. It is also important to consider the cellular composition of the GIT epithelium and additional barriers that limit nanomaterial exposure. The bulk of the epithelial lining in small intestine is made up of enterocytes, columnar epithelial cells with well-developed microvillus brush-border, and sealed by tight junctions that act as a barrier to passage of most materials. Interspersed between the enterocytes are the goblet cells which, by secreting mucus into the gut lumen, provide an additional barrier to the diffusion of particulates toward the epithelium (Behrens et al., 2002). The way particulate material interacts with secreted mucus depends to a large extent on particle size and surface properties, smaller particles penetrating better and positively charged particles being mucoadhesive (Behrens et al., 2002).

The cell surface glycocalyx, which is composed of glycosylated proteins within the membrane, constitutes a size-selective barrier to the close approach of particulates to the apical membrane of enterocytes. The glycocalyx is thinner in the small intestine compared with the large intestine. Though this makes the small intestine a prominent place for nanoparticle uptake, the glycocalyx layer also represents a hindrance allowing selective passage of materials. The glycocalyx can entrap nanoparticles (and thus reduce their translocation) because it poses a physical barrier due to its thickness, density, negative charge, and constant renewal (Cone, 2009; Crater and Carrier, 2010; Szentkuti and Lorenz, 1995). The glycocalyx has been shown to reduce interaction of even nanosized material with receptors on host cells

(Frey et al., 1996; Mantis et al., 2000). In addition, the glycocalyx has a protective function for biorelevant fluids present in the lumen of the gut.

A minor part of the GIT lining is composed of specialized epithelium that plays a prominent role in particle uptake. It has been recognized, since the early 1970s that Peyer's patches and analogous gut-associated lymphoid tissues (GALT) in the large intestine are able to accumulate particulate material from the gut lumen, including some in the nanoparticle size-range, such as India ink and ferritin (Bockman and Cooper, 1973). This phenomenon is a consequence of morphological and functional specialization of the epithelium overlying the lymphoid follicles (the follicle-associated epithelium or FAE), including the presence of specialized antigen-sampling M cells (Bockman and Cooper, 1973; Owen and Jones, 1974), which exhibit a remarkable capacity to transport a range of material, from proteins to bacteria, and deliver them to the underlying lymphoid cells.

UPTAKE OF NANOMATERIALS IN THE GASTROINTESTINAL TRACT

Uptake of nanomaterials in the GIT is potentially a major exposure route with important implications for their toxicity in peripheral organs as well as locally within the GIT. Many particle uptake studies have focused on Peyer's patches due to the presence of M cells, which are known to transport a broad range of particulate material including polystyrene (Foster et al., 1998; Jepson et al., 1993a; Pappo and Ermak, 1989), polylactide coglycolide (Desai et al., 1996; Garinot et al., 2007; Jepson et al., 1993b) and chitosan (Kadiyala et al., 2010) particles, viruses (Fotopoulos et al., 2002; Ouzilou et al., 2002), and bacteria (Autenrieth and Firsching, 1996; Clark et al., 1994, 1996, 1998; Sansonetti and Phalipon, 1999). It is important to avoid overestimating the role of M cells in bulk particle uptake as several lines of evidence suggest that significant uptake of particulate material over a broad size range occurs in non-lymphoid tissues, which comprises the vast majority of the GIT. For example, a range of particulate carriers including poly(D,L-lactide) and chitosan particles were not selectively taken up by rodent Peyer's patches compared to villi (Behrens et al., 2002; McClean et al., 1998). Similarly, while uptake of polystyrene particles in the GIT of orally dosed rats was greater into lymphoid tissues, a significant proportion was also absorbed by apparently normal villi (Jani et al., 1990; Hillery et al., 1994), with this uptake increasing as size decreased from micron to 100 nm and later suggesting that nanosized particles may access additional uptake routes to those available to larger particles. These data indicate that the lower efficiency of nanomaterials uptake in villus epithelium might be offset by its vastly greater surface area compared with the specialized FAE.

Although these studies have not precisely located sites of uptake at the cellular level, it is frequently assumed that uptake is transcellular due to the apparent integrity of the villus epithelium. Furthermore, experiments with cultured GIT epithelial cells have also demonstrated their capacity to internalize nanomaterials (Gaiser et al., 2009; Koeneman et al., 2010) as discussed later. While uptake of larger particulates can only be achieved through phagocytosis or macropinocytosis, which are not considered to be major uptake in fully differentiated GIT epithelia, except for the specialized antigen-sampling M cells, particles in the nanosize range may access additional uptake routes, such as clathrin- and caveoli-dependent endocytosis

which operate in polarized epithelia, although brush-border development may also limit these forms of endocytosis (Hansen et al., 2009).

One potential route for uptake of particles of various sizes is the phenomenon of "persorption," which was described in the 1960s by Volkheimer who described GIT translocation of particles up to several microns in diameter (e.g., yeast and starch granules) following oral dosing of experimental animals and humans (Volkheimer, 1977). Although this phenomenon is not generally considered to be a very significant route of transport, limited uptake of luminal material including nanomaterials is an inevitable consequence of the infrequent breaches in the epithelial lining occurring even in healthy GIT due to sloughing of aged enterocytes and localized damage. The concept that normal cell turnover may play a role in particle absorption was revived by the demonstration of nano-gold uptake through dying enterocytes (Hillyer and Albrecht, 2001).

The tight junctions between GIT epithelial cells are required for normal barrier and absorptive functions due in part to their blockage of the diffusion of molecules and ions between cells. This is, nevertheless, a highly regulated barrier and under some conditions significant tight junction leakage occurs, leading to paracellular transport of molecules and of particulate material including bacteria (Berg, 1999). There is some experimental evidence that nanomaterials of 1–2 nm might access the paracellular transport route, even in healthy GIT (Schleh et al., 2012). In addition, polycationic molecules can trigger selective and reversible opening of tight junctions (McEwan et al., 1993), a property that is being investigated for its potential to increase drug absorption from particulate delivery vehicles (Makhlof et al., 2011). It has also been reported that polymer-coated gold nanoparticles of 5–20 nm diameter and varying surface charge, can transiently open tight junctions of polarized Caco-2 cell layers, a property which may contribute to their own transport and that of bystander molecules (Lin et al., 2011). Similarly, nanosized insulin-complexes based on amine-modified comb-like polyesters reversibly decrease transepithelial electrical resistance (TEER) and enhance uptake into Caco-2 cell layers (Simon et al., 2007). In general, it seems unlikely that paracellular transport will be a widespread route for nanomaterial penetration of the healthy GIT wall apart from those nanomaterials that are small enough, or have the necessary surface properties to mediate tight junction opening. However, it is also important to bear in mind that this strict limitation on paracellular transport may not apply to all individuals since bacterial translocation can be enhanced by conditions, such as trauma, inflammation, stroke, and chronic alcohol use (Berg, 1992, 1999) and this leakage will most likely be mirrored by increased uptake of other material. Support for this suggestion arises from the findings that alcohol treatment and irradiation enhance penetration of 2 μm polystyrene particles by causing tight junction leakage in cultured Caco-2 cells (Moyes et al., 2008, 2010).

In Vitro Models to Study Uptake

There is limited information available from in vivo studies to address questions regarding the mechanisms of particle uptake in GIT. Mechanistic studies are more readily performed in cell culture models which are amenable to experimental manipulation. Various in vitro models have been developed for both these mechanistic studies, and screening for the uptake likelihood of nanomaterials (Braakhuis et al., 2015; Lefebvre et al., 2015). The most relevant in vitro studies of nanomaterials transport and toxicity have largely focused on Caco-2 cells,

a human adenocarcinoma cell-line widely used in pharmaceutical research (Artursson and Borchardt, 1997). Caco-2 cells are also becoming a popular cell model in the broader field of nanotoxicology, since they can provide a physiologically relevant in vitro model for initial testing, with consequent limitation of the need to utilize animal models. Unfortunately, many studies on uptake of nanomaterials and their effects on Caco-2 cells have focused only on nonpolarized cells grown on solid supports, which are of more limited value because they do not represent the physiological properties of the GIT epithelium. For example, it is known that brush-border differentiation is incomplete in Caco-2 cells grown for short periods and that uptake mechanisms are downregulated with brush-border differentiation (Hansen et al., 2009).

A potential drawback of Caco-2 monolayers is the lack of a glycocalyx or mucus layer (its importance was discussed earlier), which can, however, be introduced by coculturing Caco-2 cells with HT29-MTX cells (human colon adenocarcinoma mucus-secreting cells) (Behrens et al., 2002; Mahler et al., 2009; Scaldaferri et al., 2012; Walczak et al., 2015a). Mucus has a protective function for biorelevant fluids present in the lumen of the gut, and direct exposure of Caco-2 cells (without a mucus layer) to lumen content stimulants reduces the barrier function of Caco-2 cells models (Ingels et al., 2002). This is probably due to the lack of mucus layer making the Caco-2 cells much more sensitive to direct exposure to the low pH and high osmolality of the buffers (Westerhout et al., 2014). The inclusion of a mucus layer is important for more recently developed approaches where models to simulate the conditions in the GIT have been integrated with epithelial in vitro models (see below).

To further increase the complex anatomy of the human gut epithelium, human intestine microfold (M) cells are introduced in epithelial monolayers. While M cells in total compose less than 1% of the small intestine epithelial cell layer, they are responsible for the uptake and translocation of relatively larger particles. Coculture models of caco-2 and M cells have been developed (Antunes et al., 2013; Bouwmeester et al., 2011; des Rieux et al., 2005, 2007; Gullberg et al., 2000; Kerneis et al., 1997; Martinez-Argudo et al., 2007; Walczak et al., 2015a).

Orally ingested nanoparticles are exposed to continuously changing conditions while transiting through the GIT, which influences their nature and characteristics (Bellmann et al., 2015). Modifications to in vitro models for the GIT have been developed to mimic the gastrointestinal environment as closely as possible, to generate physiologically relevant results. Human digestion models can first be simple and static, and mimic only gastric or small intestinal conditions, in which materials are incubated with simulated gastric fluids, simulated small intestinal fluids or buffers at static pH values (Minekus et al., 2014; Mwilu et al., 2013). More complex static models often include most of the relevant GI conditions, that is, the oral, gastric, small intestinal, and large intestinal conditions (Newsome, 2014; Oomen et al., 2003; Van de Wiele et al., 2007; Versantvoort et al., 2005). Recently, these models have been used to assess the fate of 60-nm silver nanoparticles, and nanometer-sized silica (synthetic amorphous silica) nanoparticles during digestion (Peters et al., 2012; Walczak et al., 2013).

Advances in the field of miniaturized and microfluidic devices have recently led to the development of so-called organ-on-a-chip models (Bhatia and Ingber, 2014; Huh et al., 2012; Moraes et al., 2012; van der Meer and van den Berg, 2012). These devices provide a high control on the cell microenvironment (e.g., physical and chemical parameters) together with dynamic culture conditions (Whitesides, 2006). A gut-on-a-chip model integrating peristaltic

motion associated and a two-channel flow representing the lumen and plasma has been used to grow Caco-2 cells. It has been shown that under these conditions Caco-2 cells produce mucus and develop microvilli (Kim and Ingber, 2013).

A diverse range of nanomaterials are transported across fully differentiated Caco-2 cells. For example, the polarized Caco-2 cell model has revealed transport of TiO_2, SiO_2, CeO_2, and silver nanoparticles, seemingly by a transcellular route (Bhattacharjee et al., 2013; des Rieux et al., 2005, 2007; Gaiser et al., 2009; Mahler et al., 2009; Natoli et al., 2012; Nkabinde et al., 2012; Walczak et al., 2015a,b). Furthermore, uptake of PLGA nanoparticles of average diameter 95 nm was detected in polarized Caco-2, although this was significantly lower than in other epithelial cells lines (Cartiera et al., 2009). Despite the availability of this model, there is still no clear consensus as to which specific uptake route is more important and it remains likely that these will depend on the specific properties of individual nanomaterial. For example, dendrimers, which are <10 nm NP with potential as drug carriers (Duncan and Izzo, 2005), were shown in one study to be transported across Caco-2 cell layers with negligible toxicity (El-Sayed et al., 2002), whereas another report suggested the paracellular transport with concomitant loss of transepithelial electrical resistance (TEER), which might indicate toxicity and that was reduced by PEGylation (Sweet et al., 2009).

POTENTIAL TOXICITY OF NANOMATERIALS IN THE GASTROINTESTINAL TRACT

Several recent reports and reviews have highlighted the lack of data on the potential toxicity of food-borne particulates and the need to prioritize research in this area (Bouwmeester et al., 2009; Card et al., 2011). The relative lack of solid data on nanomaterials toxicity in GIT is compounded by the complexity of the environment, shortcomings of in vitro models, and limited information about exposure levels.

As we have seen, a number of studies have recently investigated the extent to which nanomaterials ingestion results in uptake from the gut lumen, and the sites of this uptake. Some studies have provided evidence of toxicity of metal NP (e.g., copper, silver, and gold) at relatively high doses in mice or rats (Chen et al., 2006; Kim et al., 2010; Loeschner et al., 2011; Park et al., 2010; van der Zande et al., 2012, 2014; Zhang et al., 2010). The highly acidic environment encountered in the stomach has been proposed to play a major role in the toxicity of copper and silver nanoparticles by triggering release of ions (Loeschner et al., 2011; Meng et al., 2007; van der Zande et al., 2012). These and other studies highlight the potential influence of the pH shifts encountered during GIT transit in both stability and dispersal/aggregation of nanomaterials, potentially influencing local toxicity as well as their ability to penetrate the GIT barriers (Kadiyala et al., 2010; Wang et al., 2008). The possibility that gastric fluid potentiates nanomaterials toxicity has also been highlighted by in vitro data suggesting low pH-mediated release of cadmium ions from CdSe quantum dots exacerbates toxicity of these NP in GIT cells (Wang et al., 2008).

While most studies to date have focused on biodistribution of nanomaterials and systemic effects in animals to the administered nanomaterials, it is also important to consider local effects on GIT pathophysiology, and the potential for indirect and chronic effects on GIT health. One under-explored area of relevance here is the potential effect of nanomaterials on the

normal bacterial microflora which is now known to play key roles in GIT development and regulation of inflammation (Pietroiusti et al., 2015; Reiff and Kelly, 2010). For example, concerns have been raised about the potential impact that the known antibacterial effects of silver NP may have on the microflora, and its potential impact on GIT function. The extent to which any such effects of nanomaterials influence GIT health is currently unclear.

Since dysregulation of inflammation is a common cause of GIT disease, the well-documented inflammatory responses induced by nanomaterials in airways, for example, give rise to concern that nanomaterials might trigger inflammatory bowel disease. This possibility received further attention due to the identification of pigmented cells in human Peyer's patches which result from macrophages accumulating mineral material, such as TiO_2 absorbed from the gut lumen by Peyer's patch FAE and the presence of similar material in inflammatory aggregates of Crohn's disease patients (Shepherd et al., 1987). The potential role of dietary particles including nanomaterials in inflammatory bowel disease has been further characterized and discussed elsewhere (Nogueira et al., 2012; Powell et al., 1996, 2000) and, although some subsequent studies have failed to identify a clear causal link with gut pathology, the possibility remains that dietary particulates may regulate inflammatory responses, perhaps in concert with bacterial antigens (Butler et al., 2007; Lomer et al., 2004). It is also important to bear in mind that inflammatory bowel disease is known to be multifactorial and that some individuals are predisposed to development of disease. It is therefore plausible that responses to nanomaterials might vary dramatically between individuals and the presence of other causative factors.

As with the characterization of nanomaterials uptake, most in vitro assessment of nanomaterials toxicity in GIT cells have focussed on the Caco-2 cell-line. For example, a range of food-related nanomaterials, including silver, TiO_2, SiO_2, and ZnO induced significant cytotoxicity and oxidative DNA damage in cultured Caco-2 cells (Bastian et al., 2009; Sahu et al., 2014; Song et al., 2014), but the significance of these findings to real GIT exposure remains uncertain due to limitation of these studies to nonpolarized cells, which poorly reflect the GIT epithelium, and the continuing uncertainty over realistic exposure levels. In contrast, other studies have pointed to a relative lack of toxicity of many particulates in Caco-2 cells, including delivery vehicles, TiO_2 and ferrous oxide nanoparticles, although ZnO NP exhibited significant toxicity in this cell model (Zhang et al., 2010). Other studies have shown pure cobalt and cobalt-doped tungsten carbide nanoparticle exert toxicity in nonpolarized Caco-2, and suggest that these cells are unusually sensitive to cobalt in nanoparticle form (Horev-Azaria et al., 2011; Semete et al., 2010). Fewer studies have investigated toxicity of nanomaterials in differentiated Caco-2 cells, but recent publications have reported a range of adverse responses to such cells exposed to silver nanoparticles (Böhmert et al., 2014, 2015; Lichtenstein et al., 2015; Oberemm et al., 2016). Other studies have shown that the responses of differentiated Caco-2 cells including the in vitro M cell model can be attributed mainly to silver ions (Bouwmeester et al., 2011).

Extrapolation of in vitro studies to the in vivo GIT must be approached with caution since true GIT may be less responsive as we have seen, but is also prone to specific damage mechanisms that cannot be modeled in vitro. Meaningful interpretation and comparison of the results obtained using different in vitro experiments and extrapolation to in vivo data require reliable characterization of the nanoparticles and their aggregates, as well as matrix-based influences on the nanoparticles. Therefore, appropriate analytical techniques should

be applied to determine the nanoparticle size distribution, composition, and concentration in the experimental samples. Analytical techniques to quantify and characterize nanomaterials in samples from in vitro studies and other biological specimens have been reviewed before (Bouwmeester et al., 2009; Braakhuis et al., 2015; Peters et al., 2012; Szakal et al., 2014).

CONCLUSIONS

Widespread exposure to nanomaterials via ingestion is an inevitable consequence of the expanding use of nanomaterials in food and other consumer products and is a cause for concern. However, the extent of ingestion and the potential risks this exposure imposes remain poorly defined. The studies discussed in this chapter go some way toward defining uptake mechanisms and suggest potential toxic mechanisms that warrant further investigation. Only a very limited number of risk assessments of nanomaterials to which humans are exposed orally have been performed (Dekkers et al., 2011; van Kesteren et al., 2015; Wijnhoven et al., 2009).

It is clear that multiple routes exist for nanomaterials to cross the GIT epithelial barrier and to be disseminated throughout the body, raising the possibility that ingested nanomaterials may have adverse effects in peripheral organs (to be discussed in other chapters). Significant uptake of some nanomaterials has been demonstrated in animal models following oral administration but these studies have largely focused on relatively high doses. Animal studies and cell culture models have highlighted the potential high capacity uptake route provided by the specialized M cells and also demonstrated that these specialized cells, which are massively outnumbered by other enterocytes, are not the only portal for nanomaterials penetration of the GIT wall. It is not yet clear to what extent uptake routes differ between nanomaterials types and the extent to which additional barriers (e.g., mucus and glycocalyx) and the changing GIT environment affect influence nanomaterials uptake. The prevailing data do, nevertheless, suggest that there is some commonality but also distinct differences in the behavior of nanomaterials in GIT.

In general low GI translocation rates have been observed for nanomaterials, in which detailed characterization of the nanomaterials has shown to be crucial (Loeschner et al., 2011; van der Zande et al., 2014). In addition, the low translocation rates bring significant analytical challenges. The absence of acute in vitro translocation should always be interpreted in relation to the sensitivity of the detection technique (both in terms of concentration and nanoparticle size). The introduction of in vitro models that allow chronic exposures are promising in that a chronic exposure better reflects the real-life human exposure. Data on biokinetics and kinetic modeling of nanomaterials is imperative to extrapolate from in vitro to in vivo. The overall resultant of absorption, distribution, metabolism, and excretion (ADME), that is, internal exposure, will determine target tissue doses and will be critical for the ultimate systemic adverse health effects (Geraets et al., 2014).

It is clear that at high doses some nanomaterials, for example, metal and metal oxide NP, and quantum dots, can exhibit severe toxicity in animal and cell culture models. In addition to cytotoxic effects, additional, more subtle effects have been identified with appropriate GIT culture models that might affect GIT function. However, it is not yet clear to what extent nanomaterials will have adverse effects at more realistic doses and the extent to which

some of the in vitro data reflects the in vivo situation. As with other areas of nanotoxicology, attention must be given to the stability of nanomaterials, with release of toxic ions potentially exacerbating toxicity of some nanomaterials and it is noteworthy that some recent studies have demonstrated the potential for the acidic gastric environment to increase nanomaterial toxicity. No clear evidence exists yet for a role of nanomaterials in inflammatory bowel disease or other immune-related pathologies. Nevertheless, the delicate balance of immune regulation in the GIT, and the key role played by commensal bacteria in this balance are additional causes of concern since the known effects of some nanomaterials suggests they have potential to contribute to disease.

Together, the studies carried out to date on nanomaterials suggest that GIT epithelia may be prone to damage that is not always readily modeled in vitro, at least without the relatively laborious and expensive use of fully differentiated GIT cell models. However, the relative paucity of data regarding toxicity mechanisms and considerable uncertainty regarding realistic exposure levels and behavior of nanomaterials in the complex GIT environment make it impossible to predict the likely health effects of nanomaterials.

TAKE-HOME MESSAGES

- Major questions remain about the potential for nanomaterials to have adverse effects in the GIT or elsewhere, via GIT uptake.
- Studies performed to date have identified several potential uptake and toxicity mechanisms, although further research is required to fully characterize these effects.
- Several simple as well as more advanced in vitro models have been developed to study uptake and effects of nanomaterials in the GIT.
- A thorough characterization of the nanomaterials used in these studies is pivotal, and several analytical chemistry approaches are now becoming available.
- To obtain more insight into the mechanisms behind nanoparticle uptake and translocation, in vitro models should be further developed to become closer to physiological conditions. Such in vitro models should contain the GIT microbiome to incorporate nanomaterial, microbiome, and GIT (immune) responses.

Acknowledgments

We would like to acknowledge many colleagues past and present who have contributed to our studies on gastrointestinal particle uptake and nanotoxicology. Funding for our research in this area has been provided by BBSRC, MRC, NERC, Wellcome Trust, ZonMW, Dutch Ministry of Economic Affairs, The Netherlands Food and Product Safety Authority, and by the European Commission through FP7- MARINA.

References

Antunes, F., Andrade, F., Araujo, F., Ferreira, D., Sarmento, B., 2013. Establishment of a triple co-culture in vitro cell models to study intestinal absorption of peptide drugs. Eur. J. Pharm. Biopharm. 83, 427–435.

Artursson, P., Borchardt, R.T., 1997. Intestinal drug absorption and metabolism in cell cultures: Caco-2 and beyond. Pharm. Res. 14, 1655–1658.

Autenrieth, I.B., Firsching, R., 1996. Penetration of M cells and destruction of Peyer's patches by Yersinia enterocolitica: an ultrastructural and histological study. J. Med. Microbiol. 44, 285–294.

Bastian, S., Busch, W., Kuhnel, D., Springer, A., Meissner, T., Holke, R., Scholz, S., Iwe, M., Pompe, W., Gelinsky, M., Potthoff, A., Richter, V., Ikonomidou, C., Schirmer, K., 2009. Toxicity of tungsten carbide and cobalt-doped tungsten carbide nanoparticles in mammalian cells in vitro. Environ. Health Perspect. 117, 530–536.

Behrens, I., Pena, A.I., Alonso, M.J., Kissel, T., 2002. Comparative uptake studies of bioadhesive and non-bioadhesive nanoparticles in human intestinal cell lines and rats: the effect of mucus on particle adsorption and transport. Pharm. Res. 19, 1185–1193.

Bellmann, S., Carlander, D., Fasano, A., Momcilovic, D., Scimeca, J.A., Waldman, W.J., Gombau, L., Tsytsikova, L., Canady, R., Pereira, D.I.A., Lefebvre, D.E., 2015. Mammalian gastrointestinal tract parameters modulating the integrity, surface properties, and absorption of food-relevant nanomaterials. Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol. 7, 609–622.

Berg, R.D., 1992. Bacterial translocation from the gastrointestinal tract. J. Med. 23, 217–244.

Berg, R.D., 1999. Bacterial translocation from the gastrointestinal tract. Adv. Exp. Med. Biol. 473, 11–30.

Bhatia, S.N., Ingber, D.E., 2014. Microfluidic organs-on-chips. Nat. Biotechnol. 32, 760–772.

Bhattacharjee, S., Ershov, D., Gucht, J., Alink, G.M., Rietjens, I.M., Zuilhof, H., Marcelis, A.T., 2013. Surface charge-specific cytotoxicity and cellular uptake of tri-block copolymer nanoparticles. Nanotoxicology 7, 71–84.

Bockman, D.E., Cooper, M.D., 1973. Pinocytosis by epithelium associated with lymphoid follicles in the bursa of Fabricius, appendix, and Peyer's patches. An electron microscopic study. Am. J. Anat. 136, 455–477.

Böhmert, L., Girod, M., Hansen, U., Maul, R., Knappe, P., Niemann, B., Weidner, S.M., Thünemann, A.F., Lampen, A., 2014. Analytically monitored digestion of silver nanoparticles and their toxicity on human intestinal cells. Nanotoxicology 8, 631–642.

Böhmert, L., Niemann, B., Lichtenstein, D., Juling, S., Lampen, A., 2015. Molecular mechanism of silver nanoparticles in human intestinal cells. Nanotoxicology 9, 852–860.

Bouwmeester, H., Dekkers, S., Noordam, M.Y., Hagens, W.I., Bulder, A.S., de Heer, C., ten Voorde, S.E.C.G., Wijnhoven, S.W.P., Marvin, H.J.P., Sips, A.J.A.M., 2009. Review of health safety aspects of nanotechnologies in food production. Regul. Toxicol. Pharmacol. 53, 52–62.

Bouwmeester, H., Poortman, J., Peters, R.J., Wijma, E., Kramer, E., Makama, S., Puspitaninganindita, K., Marvin, H.J., Peijnenburg, A.A., Hendriksen, P.J., 2011. Characterization of translocation of silver nanoparticles and effects on whole-genome gene expression using an in vitro intestinal epithelium coculture model. ACS Nano 5, 4091–4103.

Bouwmeester, H., Brandhoff, P., Marvin, H.J., Weigel, S., Peters, R.J., 2014. State of the safety assessment and current use of nanomaterials in food and food production. Trends Food Sci. Technol. 40, 200–210.

Braakhuis, H.M., Kloet, S.K., Kezic, S., Kuper, F., Park, M.V.D.Z., Bellmann, S., van der Zande, M., Le Gac, S., Krystek, P., Peters, R.J.B., Rietjens, I.M.C.M., Bouwmeester, H., 2015. Progress and future of in vitro models to study translocation of nanoparticles. Arch Toxicol 89 (9), 1469–1495.

Brayden, D.J., Jepson, M.A., Baird, A.W., 2005. Keynote review: intestinal Peyer's patch M cells and oral vaccine targeting. Drug Discov. Today 10, 1145–1157.

Butler, M., Boyle, J.J., Powell, J.J., Playford, R.J., Ghosh, S., 2007. Dietary microparticles implicated in Crohn's disease can impair macrophage phagocytic activity and act as adjuvants in the presence of bacterial stimuli. Inflamm. Res. 56, 353–361.

Card, J.W., Jonaitis, T.S., Tafazoli, S., Magnuson, B.A., 2011. An appraisal of the published literature on the safety and toxicity of food-related nanomaterials. Crit. Rev. Toxicol. 41, 22–49.

Cartiera, M.S., Johnson, K.M., Rajendran, V., Caplan, M.J., Saltzman, W.M., 2009. The uptake and intracellular fate of PLGA nanoparticles in epithelial cells. Biomaterials 30, 2790–2798.

Chen, Z., Meng, H., Xing, G., Chen, C., Zhao, Y., Jia, G., Wang, T., Yuan, H., Ye, C., Zhao, F., Chai, Z., Zhu, C., Fang, X., Ma, B., Wan, L., 2006. Acute toxicological effects of copper nanoparticles in vivo. Toxicol. Lett. 163, 109–120.

Clark, M.A., Jepson, M.A., Simmons, N.L., Hirst, B.H., 1994. Preferential interaction of Salmonella typhimurium with mouse Peyer's patch M cells. Res. Microbiol. 145, 543–552.

Clark, M.A., Reed, K.A., Lodge, J., Stephen, J., Hirst, B.H., Jepson, M.A., 1996. Invasion of murine intestinal M cells by Salmonella typhimurium inv mutants severely deficient for invasion of cultured cells. Infect. Immun. 64, 4363–4368.

Clark, M.A., Hirst, B.H., Jepson, M.A., 1998. M-cell surface beta1 integrin expression and invasin-mediated targeting of Yersinia pseudotuberculosis to mouse Peyer's patch M cells. Infect. Immun. 66, 1237–1243.

Cone, R.A., 2009. Barrier properties of mucus. Adv. Drug Deliv. Rev. 61, 75–85.

Crater, J.S., Carrier, R.L., 2010. Barrier properties of gastrointestinal mucus to nanoparticle transport. Macromol. Biosci. 10, 1473–1483.

Dekkers, S., Krystek, P., Peters, R., Lankveld, D., Bokkers, B., van Hoeven-Arentzen, P., Bouwmeester, H., Oomen, A.G., 2011. Presence and risks of nanosilica in food products. Nanotoxicology 5, 393–405.
des Rieux, A., Ragnarsson, E.G., Gullberg, E., Preat, V., Schneider, Y.J., Artursson, P., 2005. Transport of nanoparticles across an in vitro model of the human intestinal follicle associated epithelium. Eur. J. Pharm. Sci. 25, 455–465.
des Rieux, A., Fievez, V., Theate, I., Mast, J., Preat, V., Schneider, Y.J., 2007. An improved in vitro model of human intestinal follicle-associated epithelium to study nanoparticle transport by M cells. Eur. J. Pharm. Sci. 30, 380–391.
Desai, M.P., Labhasetwar, V., Amidon, G.L., Levy, R.J., 1996. Gastrointestinal uptake of biodegradable microparticles: effect of particle size. Pharm. Res. 13, 1838–1845.
Duncan, R., Izzo, L., 2005. Dendrimer biocompatibility and toxicity. Adv. Drug Deliv. Rev. 57, 2215–2237.
El-Sayed, M., Ginski, M., Rhodes, C., Ghandehari, H., 2002. Transepithelial transport of poly(amidoamine) dendrimers across Caco-2 cell monolayers. J. Control. Release 81, 355–365.
EU Official Journal of the European Union, 2008a. Commision Directive 2008/84/Ec of 27 August 2008 Laying Down Specific Purity Criteria on Food Additive Other Than Colours and Sweeteners.
EU Official Journal of the European Union, 2008b. Regulation (Ec) No. 1333/2008 of the European Parliament and of the Council of 16 December 2008 on Food Additives.
European Commission, 1994. European Parliament and Council Directive 94/36/EC of 30 June 1994 on colours in foodstuffs. Off. J. Eur. Communities L237, 13–29.
Foster, N., Clark, M.A., Jepson, M.A., Hirst, B.H., 1998. Ulex europaeus 1 lectin targets microspheres to mouse Peyer's patch M-cells in vivo. Vaccine 16, 536–541.
Fotopoulos, G., Harari, A., Michetti, P., Trono, D., Pantaleo, G., Kraehenbuhl, J.P., 2002. Transepithelial transport of HIV-1 by M cells is receptor-mediated. Proc. Natl. Acad. Sci. USA 99, 9410–9414.
Frey, A., Giannasca, K.T., Weltzin, R., Giannasca, P.J., Reggio, H., Lencer, W.I., Neutra, M.R., 1996. Role of the glycocalyx in regulating access of microparticles to apical plasma membranes of intestinal epithelial cells: implications for microbial attachment and oral vaccine targeting. J. Exp. Med. 184, 1045–1059.
Gaiser, B.K., Fernandes, T.F., Jepson, M., Lead, J.R., Tyler, C.R., Stone, V., 2009. Assessing exposure, uptake and toxicity of silver and cerium dioxide nanoparticles from contaminated environments. Environ. Health 8 (Suppl. 1), S2.
Garinot, M., Fievez, V., Pourcelle, V., Stoffelbach, F., des Rieux, A., Plapied, L., Theate, I., Freichels, H., Jerome, C., Marchand-Brynaert, J., Schneider, Y.J., Preat, V., 2007. PEGylated PLGA-based nanoparticles targeting M cells for oral vaccination. J. Control. Release 120, 195–204.
Geraets, L., Oomen, A.G., Krystek, P., Jacobsen, N.R., Wallin, H., Laurentie, M., Verharen, H.W., Brandon, E.F., de Jong, W.H., 2014. Tissue distribution and elimination after oral and intravenous administration of different titanium dioxide nanoparticles in rats. Part. Fibre Toxicol. 11, 30.
Gullberg, E., Leonard, M., Karlsson, J., Hopkins, A.M., Brayden, D., Baird, A.W., Artursson, P., 2000. Expression of specific markers and particle transport in a new human intestinal M-cell model. Biochem. Biophys. Res. Commun. 279, 808–813.
Hansen, G.H., Rasmussen, K., Niels-Christiansen, L.L., Danielsen, E.M., 2009. Endocytic trafficking from the small intestinal brush border probed with FM dye. Am. J. Physiol. Gastrointest. Liver Physiol. 297, G708–G715.
Hillery, A.M., Jani, P.U., Florence, A.T., 1994. Comparative, quantitative study of lymphoid and non-lymphoid uptake of 60 nm polystyrene particles. J. Drug Target 2, 151–156.
Hillyer, J.F., Albrecht, R.M., 2001. Gastrointestinal persorption and tissue distribution of differently sized colloidal gold nanoparticles. J. Pharm. Sci. 90, 1927–1936.
Horev-Azaria, L., Kirkpatrick, C.J., Korenstein, R., Marche, P.N., Maimon, O., Ponti, J., Romano, R., Rossi, F., Golla-Schindler, U., Sommer, D., Uboldi, C., Unger, R.E., Villiers, C., 2011. Predictive toxicology of cobalt nanoparticles and ions: comparative in-vitro study of different cellular models using methods of knowledge discovery from data. Toxicol. Sci. 122, 489–501.
Huh, D., Torisawa, Y.S., Hamilton, G.A., Kim, H.J., Ingber, D.E., 2012. Microengineered physiological biomimicry: organs-on-chips. Lab. Chip 12 (12), 2156–2164.
Ingels, F., Deferme, S., Destexhe, E., Oth, M., Van den Mooter, G., Augustijns, P., 2002. Simulated intestinal fluid as transport medium in the Caco-2 cell culture model. Int. J. Pharm. 232 (1–2), 183–192.
Jani, P., Halbert, G.W., Langridge, J., Florence, A.T., 1990. Nanoparticle uptake by the rat gastrointestinal mucosa: quantitation and particle size dependency. J. Pharm. Pharmacol. 42, 821–826.
Jepson, M.A., Simmons, N.L., Savidge, T.C., James, P.S., Hirst, B.H., 1993a. Selective binding and transcytosis of latex microspheres by rabbit intestinal M cells. Cell Tissue Res. 271, 399–405.

Jepson, M.A., Simmons, N.L., O'Hagan, D.T., Hirst, B.H., 1993b. Comparison of poly(DL-lactide-*co*-glycolide) and polystyrene microsphere targeting to intestinal M cells. J. Drug Target 1, 245–249.
Jepson, M.A., Clark, M.A., Foster, N., Mason, C.M., Bennett, M.K., Simmons, N.L., Hirst, B.H., 1996. Targeting to intestinal M cells. J. Anat. 189, 507–516.
Johnston, B.D., Scown, T.M., Moger, J., Cumberland, S.A., Baalousha, M., Linge, K., van Aerle, R., Jarvis, K., Lead, J.R., Tyler, C.R., 2010. Bioavailability of nanoscale metal oxides TiO(2), CeO(2), and ZnO to fish. Environ. Sci. Technol. 44, 1144–1151.
Kadiyala, I., Loo, Y., Roy, K., Rice, J., Leong, K.W., 2010. Transport of chitosan-DNA nanoparticles in human intestinal M-cell model versus normal intestinal enterocytes. Eur. J. Pharm. Sci. 39, 103–109.
Kerneis, S., Bogdanova, A., Kraehenbuhl, J.P., Pringault, E., 1997. Conversion by Peyer's patch lymphocytes of human enterocytes into M cells that transport bacteria. Science 277, 949–952.
Kim, H.J., Ingber, D.E., 2013. Gut-on-a-Chip microenvironment induces human intestinal cells to undergo villus differenetiation. Integr. Biol. 5, 1130–1140.
Kim, Y.S., Song, M.Y., Park, J.D., Song, K.S., Ryu, H.R., Chung, Y.H., Chang, H.K., Lee, J.H., Oh, K.H., Kelman, B.J., Hwang, I.K., Yu, I.J., 2010. Subchronic oral toxicity of silver nanoparticles. Part. Fibre Toxicol. 7, 20.
Koeneman, B.A., Zhang, Y., Westerhoff, P., Chen, Y., Crittenden, J.C., Capco, D.G., 2010. Toxicity and cellular responses of intestinal cells exposed to titanium dioxide. Cell Biol. Toxicol. 26, 225–238.
Lefebvre, D.E., Venema, K., Gombau, L., Valerio, Jr., L.G., Raju, J., Bondy, G.S., Bouwmeester, H., Singh, R.P., Clippinger, A.J., Collnot, E.M., Mehta, R., Stone, V., 2015. Utility of models of the gastrointestinal tract for assessment of the digestion and absorption of engineered nanomaterials released from food matrices. Nanotoxicology 9, 523–542.
Lichtenstein, D., Ebmeyer, J., Knappe, P., Juling, S., Böhmert, L., Selve, S., Niemann, B., Braeuning, A., Thünemann, A.F., Lampen, A., 2015. Impact of food components during in vitro digestion of silver nanoparticles on cellular uptake and cytotoxicity in intestinal cells. Biol. Chem. 396, 1255–1264.
Lin, I.C., Liang, M., Liu, T.Y., Ziora, Z.M., Monteiro, M.J., Toth, I., 2011. Interaction of densely polymer-coated gold nanoparticles with epithelial Caco-2 monolayers. Biomacromolecules 12, 1339–1348.
Loeschner, K., Hadrup, N., Qvortrup, K., Larsen, A., Gao, X., Vogel, U., Mortensen, A., Lam, H.R., Larsen, E.H., 2011. Distribution of silver in rats following 28 days of repeated oral exposure to silver nanoparticles or silver acetate. Part. Fibre Toxicol. 8, 18.
Lomer, M.C., Thompson, R.P., Powell, J.J., 2002. Fine and ultrafine particles of the diet: influence on the mucosal immune response and association with Crohn's disease. Proc. Nutr. Soc. 61, 123–130.
Lomer, M.C., Hutchinson, C., Volkert, S., Greenfield, S.M., Catterall, A., Thompson, R.P., Powell, J.J., 2004. Dietary sources of inorganic microparticles and their intake in healthy subjects and patients with Crohn's disease. Br. J. Nutr. 92, 947–955.
Mahler, G.J., Shuler, M.L., Glahn, R.P., 2009. Characterization of Caco-2 and HT29-MTX cocultures in an in vitro digestion/cell culture model used to predict iron bioavailability. J. Nutr. Biochem. 20, 494–502.
Makhlof, A., Werle, M., Tozuka, Y., Takeuchi, H., 2011. A mucoadhesive nanoparticulate system for the simultaneous delivery of macromolecules and permeation enhancers to the intestinal mucosa. J. Control. Release 149, 81–88.
Mantis, N.J., Frey, A., Neutra, M.R., 2000. Accessibility of glycolipid and oligosaccharide epitopes on rabbit villus and follicle-associated epithelium. Am. J. Physiol. Gastrointest. Liver Physiol. 278, G915–G923.
Martinez-Argudo, I., Sands, C., Jepson, M.A., 2007. Translocation of enteropathogenic Escherichia coli across an in vitro M cell model is regulated by its type III secretion system. Cell Microbiol. 9, 1538–1546.
McClean, S., Prosser, E., Meehan, E., O'Malley, D., Clarke, N., Ramtoola, Z., Brayden, D., 1998. Binding and uptake of biodegradable poly-DL-lactide micro- and nanoparticles in intestinal epithelia. Eur. J. Pharm. Sci. 6, 153–163.
McCracken, C., Zane, A., Knight, D.A., Dutta, P.K., Waldman, W.J., 2013. Minimal intestinal epithelial cell toxicity in response to short- and long-term food-relevant inorganic nanoparticle exposure. Chem. Res. Toxicol. 26 (10), 1514–1525.
McEwan, G.T., Jepson, M.A., Hirst, B.H., Simmons, N.L., 1993. Polycation-induced enhancement of epithelial paracellular permeability is independent of tight junctional characteristics. Biochim. Biophys. Acta 1148, 51–60.
Meng, H., Chen, Z., Xing, G., Yuan, H., Chen, C., Zhao, F., Zhang, C., Zhao, Y., 2007. Ultrahigh reactivity provokes nanotoxicity: explanation of oral toxicity of nano-copper particles. Toxicol. Lett. 175, 102–110.

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carriere, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., Marze, S., McClements, D.J., Menard, O., Recio, I., Santos, C.N., Singh, R.P., Vegarud, G.E., Wickham, M.S., Weitschies, W., Brodkorb, A., 2014. A standardised static in vitro digestion method suitable for food—an international consensus. Food Funct. 5, 1113–1124.

Moraes, C., Mehta, G., Lesher-Perez, S.C., Takayama, S., 2012. Organs-on-a-chip: a focus on compartmentalized microdevices. Ann. Biomed. Eng. 40 (6), 1211–1227.

Moyes, S.M., Killick, E.M., Morris, J.F., Kadhim, M.A., Hill, M.A., Carr, K.E., 2008. Changes produced by external radiation in parameters influencing intestinal permeability and microparticle uptake in vitro. Int. J. Radiat. Biol. 84, 467–486.

Moyes, S.M., Morris, J.F., Carr, K.E., 2010. Culture conditions and treatments affect Caco-2 characteristics and particle uptake. Int. J. Pharm. 387, 7–18.

Mwilu, S.K., El Badawy, A.M., Bradham, K., Nelson, C., Thomas, D., Scheckel, K.G., Tolaymat, T., Ma, L., Rogers, K.R., 2013. Changes in silver nanoparticles exposed to human synthetic stomach fluid: effects of particle size and surface chemistry. Sci. Total Environ. 447, 90–98.

Natoli, M., Leoni, B.D., D'Agnano, I., Zucco, F., Felsani, A., 2012. Good Caco-2 cell culture practices. Toxicol. In Vitro 26, 1243–1246.

Newsome, R., 2014. 2013 IFT International Food Nanoscience Conference. Proc. Compr. Rev. Food Sci. Food Saf 13, 190–228.

Nkabinde, L.A., Shoba-Zikhali, L.N., Semete-Makokotlela, B., Kalombo, L., Swai, H.S., Hayeshi, R., Naicker, B., Hillie, T.K., Hamman, J.H., 2012. Permeation of PLGA nanoparticles across different in vitro models. Curr. Drug Deliv. 9, 617–627.

Nogueira, C.M., de Azevedo, W.M., Dagli, M.L., Toma, S.H., Leite, A.Z., Lordello, M.L., Nishitokukado, I., Ortiz-Agostinho, C.L., Duarte, M.I., Ferreira, M.A., Sipahi, A.M., 2012. Titanium dioxide induced inflammation in the small intestine. World J. Gastroenterol. 18, 4729–4735.

Oberemm, A., Hansen, U., Böhmert, L., Meckert, C., Braeuning, A., Thünemann, A.F., Lampen, A., 2016. Proteomic responses of human intestinal Caco-2 cells exposed to silver nanoparticles and ionic silver. J. Appl. Toxicol. 36, 404–413.

Oomen, A.G., Rompelberg, C.J., Bruil, M.A., Dobbe, C.J., Pereboom, D.P., Sips, A.J., 2003. Development of an in vitro digestion model for estimating the bioaccessibility of soil contaminants. Arch. Environ. Contam. Toxicol. 44 (3), 281–287.

Ouzilou, L., Caliot, E., Pelletier, I., Prevost, M.C., Pringault, E., Colbere-Garapin, F., 2002. Poliovirus transcytosis through M-like cells. J. Gen. Virol. 83, 2177–2182.

Owen, R.L., Jones, A.L., 1974. Epithelial cell specialization within human Peyer's patches: an ultrastructural study of intestinal lymphoid follicles. Gastroenterology 66, 189–203.

Pappo, J., Ermak, T.H., 1989. Uptake and translocation of fluorescent latex particles by rabbit Peyer's patch follicle epithelium: a quantitative model for M cell uptake. Clin. Exp. Immunol. 76, 144–148.

Park, E.J., Bae, E., Yi, J., Kim, Y., Choi, K., Lee, S.H., Yoon, J., Lee, B.C., Park, K., 2010. Repeated-dose toxicity and inflammatory responses in mice by oral administration of silver nanoparticles. Environ. Toxicol. Pharmacol. 30, 162–168.

Peters, R., Kramer, E., Oomen, A.G., Rivera, Z.E., Oegema, G., Tromp, P.C., Fokkink, R., Rietveld, A., Marvin, H.J., Weigel, S., Peijnenburg, A.A., Bouwmeester, H., 2012. Presence of nano-sized silica during in vitro digestion of foods containing silica as a food additive. ACS Nano 6 (3), 2441–2451.

Peters, R.J.B., van Bemmel, G., Herrera-Rivera, Z., Helsper, H.P.F.G., Marvin, H.J.P., Weigel, S., Tromp, P.C., Oomen, A.G., Rietveld, A., Bouwmeester, H., 2014. Characterization of titanium dioxide nanoparticles in food products: analytical methods to define nanoparticles. J. Agric. Food Chem. 62 (27), 6285–6293.

Piccinno, F., Gottschalk, F., Seeger, S., Nowack, B., 2012. Industrial production quantities and uses of ten engineered nanomaterials in Europe and the world. J. Nanopart. Res. 14, 1109.

Pietroiusti, A., Magrini, A., Campagnolo, L., 2015. New frontiers in nanotoxicology: gut microbiota/microbiome-mediated effects of engineered nanomaterials. Toxicol. Appl. Pharmacol. 299, 90–95.

Powell, J.J., Ainley, C.C., Harvey, R.S., Mason, I.M., Kendall, M.D., Sankey, E.A., Dhillon, A.P., Thompson, R.P., 1996. Characterisation of inorganic microparticles in pigment cells of human gut associated lymphoid tissue. Gut 38, 390–395.

Powell, J.J., Harvey, R.S., Ashwood, P., Wolstencroft, R., Gershwin, M.E., Thompson, R.P., 2000. Immune potentiation of ultrafine dietary particles in normal subjects and patients with inflammatory bowel disease. J. Autoimmun. 14, 99–105.

Powell, J.J., Faria, N., Thomas-McKay, E., Pele, L.C., 2010. Origin and fate of dietary nanoparticles and microparticles in the gastrointestinal tract. J. Autoimmun. 34, J226–J233.

Reiff, C., Kelly, D., 2010. Inflammatory bowel disease, gut bacteria and probiotic therapy. Int. J. Med. Microbiol. 300, 25–33.

Rogers, K.R., Bradham, K., Tolaymat, T., Thomas, D.J., Hartmann, T., Ma, L.Z., Williams, A., 2012. Alterations in physical state of silver nanoparticles exposed to synthetic human stomach fluid. Sci. Total Environ. 420, 334–339.

Sahu, S.C., Roy, S., Zheng, J., Yourick, J.J., Sprando, R.L., 2014. Comparative genotoxicity of nanosilver in human liver HepG2 and colon Caco2 cells evaluated by fluorescent microscopy of cytochalasin B-blocked micronucleus formation. J. Appl. Toxicol. 34, 1200–1208.

Sansonetti, P.J., Phalipon, A., 1999. M cells as ports of entry for enteroinvasive pathogens: mechanisms of interaction, consequences for the disease process. Semin. Immunol. 11, 193–203.

Scaldaferri, F., Pizzoferrato, M., Gerardi, V., Lopetuso, L., Gasbarrini, A., 2012. The gut barrier: new acquisitions and therapeutic approaches. J. Clin. Gastroenterol. 46 (Suppl.), S12–S17.

Schleh, C., Semmler-Behnke, M., Lipka, J., Wenk, A., Hirn, S., Schäffler, M., Schmid, G.N., Simon, U., Kreyling, W.G., 2012. Size and surface charge of gold nanoparticles determine absorption across intestinal barriers and accumulation in secondary target organs after oral administration. Nanotoxicology 6, 36–46.

Semete, B., Booysen, L., Lemmer, Y., Kalombo, L., Katata, L., Verschoor, J., Swai, H.S., 2010. In vivo evaluation of the biodistribution and safety of PLGA nanoparticles as drug delivery systems. Nanomedicine 6, 662–671.

Sepeur, S., Laryea, N., Goedicke, S., Groβ, F., 2008. Nanotechnology: Technical Basics And Applications. Vincentz Network, Hannover.

Shepherd, N.A., Crocker, P.R., Smith, A.P., Levison, D.A., 1987. Exogenous pigment in Peyer's patches. Hum. Pathol. 18, 50–54.

Simon, M., Behrens, I., Dailey, L.A., Wittmar, M., Kissel, T., 2007. Nanosized insulin-complexes based on biodegradable amine-modified graft polyesters poly[vinyl-3-(diethylamino)-propylcarbamate-co-(vinyl acetate)-co-(vinyl alcohol)]-graft-poly(L-lactic acid): protection against enzymatic degradation, interaction with Caco-2 cell monolayers, peptide transport and cytotoxicity. Eur. J. Pharm. Biopharm. 66, 165–172.

Song, Y., Guan, R., Lyu, F., Kang, T., Wu, Y., Chen, X., 2014. In vitro cytotoxicity of silver nanoparticles and zinc oxide nanoparticles to human epithelial colorectal adenocarcinoma (Caco-2) cells. Mutat. Res. 769, 113–118.

Sozer, N., Kokini, J.L., 2009. Nanotechnology and its applications in the food sector. Trends Biotechnol. 27 (2), 82–89.

Sweet, D.M., Kolhatkar, R.B., Ray, A., Swaan, P., Ghandehari, H., 2009. Transepithelial transport of PEGylated anionic poly(amidoamine) dendrimers: implications for oral drug delivery. J. Control. Release 138, 78–85.

Szakal, C., Roberts, S.M., Westerhoff, P., Bartholomaeus, A., Buck, N., Illuminato, I., Canady, R., Rogers, M., 2014. Measurement of nanomaterials in foods: integrative consideration of challenges and future prospects. ACS Nano 8 (4), 3128–3135.

Szentkuti, L., Lorenz, K., 1995. The thickness of the mucus layer in different segments of the rat intestine. Histochem. J. 27, 466–472.

U.S. Food and Drug Administration, 2002. Code of Federal Regulations Title 21-Food and Drugs 21 CFR 73.2575, listing of color additives exempt from certification.

Van de Wiele, T.R., Oomen, A.G., Wragg, J., Cave, M., Minekus, M., Hack, A., Cornelis, C., Rompelberg, C.J., De Zwart, L.L., Klinck, B., Van Wijnen, J., Verstraete, W., Sips, A.J., 2007. Comparison of five in vitro digestion models to in vivo experimental results: lead bioaccessibility in the human gastrointestinal tract. J. Environ. Sci. Health A Tox. Hazard Subst. Environ. Eng 42, 1203–1211.

van der Meer, A.D., van den Berg, A., 2012. Organs-on-chips: breaking the in vitro impasse. Integr. Biol. 4, 461–470.

van der Zande, M., Vandebriel, R.J., Van Doren, E., Kramer, E., Herrera Rivera, Z., Serrano-Rojero, C.S., Gremmer, E.R., Mast, J., Peters, R.J.B., Hollman, P.C., Hendriksen, P.J., Marvin, H.J.P., Peijnenburg, A.A.C.M., Bouwmeester, H., 2012. Distribution, elimination, and toxicity of silver nanoparticles and silver ions in rats after 28-day oral exposure. ACS Nano 6, 7427–7442.

van der Zande, M., Vandebriel, R.J., Groot, M.J., Kramer, E., Herrera Rivera, Z.E., Rasmussen, K., Ossenkoppele, J.S., Tromp, P., Gremmer, E.R., Peters, R.J.B., Hendriksen, P.J., Marvin, H.J.P., Hoogenboom, R.L.A.P., Peijnenburg,

A.A.C.M., Bouwmeester, H., 2014. Sub-chronic toxicity study in rats orally exposed to nanostructured silica. Part. Fibre Toxicol. 11, 8.
van Kesteren, P.C.E., Cubadda, F., Bouwmeester, H., van Eijkeren, J.C.H., Dekkers, S., de Jong, W.H., Oomen, A.G., 2015. Novel insights into the risk assessment of the nanomaterial synthetic amorphous silica, additive E551, in food. Nanotoxicology 9, 442–452.
Versantvoort, C.H., Oomen, A.G., Van de Kamp, E., Rompelberg, C.J., Sips, A.J., 2005. Applicability of an in vitro digestion model in assessing the bioaccessibility of mycotoxins from food. Food Chem. Toxicol. 43, 31–40.
Volkheimer, G., 1977. Persorption of particles: physiology and pharmacology. Adv. Pharmacol. Chemother. 14, 163–187.
Walczak, A.P., Fokkink, R., Peters, R., Tromp, P., Herrera Rivera, Z.E., Rietjens, I.M., Bouwmeester, H., 2013. Behaviour of silver nanoparticles and silver ions in an in vitro human gastrointestinal digestion model. Nanotoxicology 7, 1198–1210.
Walczak, A.P., Kramer, E., Hendriksen, P.J., Tromp, P., Helsper, J.P., van der Zande, M., Rietjens, I.M., Bouwmeester, H., 2015a. Translocation of differently sized and charged polystyrene nanoparticles in in vitro intestinal cell models of increasing complexity. Nanotoxicology 9, 453–461.
Walczak, A.P., Kramer, E., Hendriksen, P.J., Helsingden, R., van der Zande, M., Rietjens, I.M., Bouwmeester, H., 2015b. In vitro gastrointestinal digestion increases the translocation of polystyrene nanoparticles in an in vitro intestinal co-culture model. Nanotoxicology 9, 886–894.
Wang, L., Nagesha, D.K., Selvarasah, S., Dokmeci, M.R., Carrier, R.L., 2008. Toxicity of CdSe nanoparticles in Caco-2 cell cultures. J. Nanobiotechnology 6, 11.
Weir, A., Westerhoff, P., Fabricius, L., Hristovski, K., Von Goetz, N., 2012. Titanium dioxide nanoparticles in food and personal care products. Environ. Sci. Technol. 46 (4), 2242–2250.
Westerhout, J., van de Steeg, E., Grossouw, D., Zeijdner, E.E., Krul, C.A., Verwei, M., Wortelboer, H.M., 2014. A new approach to predict human intestinal absorption using porcine intestinal tissue and biorelevant matrices. Eur. J. Pharm. Sci. 63, 167–177.
Whitesides, G.M., 2006. The origins and the future of microfluidics. Nature 442, 368–373.
Wijnhoven, S.W.P., Peijnenburg, W.J.G.M., Herberts, C.A., Hagens, W.I., Oomen, A.G., Heugens, E.H.W., Roszek, B., Bisschops, J., Gosens, I., Van De Meent, D., Dekkers, S., De Jong, W.H., Van Zijverden, M., Sips, A.J.A.M., Geertsma, R.E., 2009. Nano-silver—a review of available data and knowledge gaps in human and environmental risk assessment. Nanotoxicology 3, 109–138.
Zhang, X.D., Wu, H.Y., Wu, D., Wang, Y.Y., Chang, J.H., Zhai, Z.B., Meng, A.M., Liu, P.X., Zhang, L.A., Fan, F.Y., 2010. Toxicologic effects of gold nanoparticles in vivo by different administration routes. Int. J. Nanomedicine 5, 771–781.

CHAPTER

17

Reproduction and Development

*Luisa Campagnolo**, *Karin S. Hougaard*****

***University of Rome 'Tor Vergata', Rome, Italy; ****National Research Center for the Working Environment, Copenhagen, Denmark**

OUTLINE

Introduction 397

Fertility 398
- *Male Reproductive Function* 398
- *Female Fertility* 402

Nanoparticles and Developmental Toxicity 403
- *Metal-Based Nanoparticles* 404
- *Carbonaceous Particles* 408
- *Polystyrene Nanoparticles* 411

Discussion 412

Take-Home Messages 416

References 416

INTRODUCTION

The sensitivity of reproductive and developmental outcomes to toxic substances causes significant public concern. Unfortunately, there is at present a large discrepancy between the number of chemicals in commerce and the number that have been evaluated for reproductive toxicity (Lawson et al., 2003). For nanomaterials this aspect is even graver. Thus, toxicity of the bulk material might not be adequately assessed in the first place, and downsizing and engineering might convey physical and chemical qualities that differ from that of the corresponding bulk material. In 2015, the Nanotechnology Consumer Products Inventory (CPI) has been updated, and recognized that 1,600+ manufacturer-identified nanotechnology-based consumer products have been already introduced to the market (Vance et al., 2015). Exposure to nanomaterials might have detrimental consequences for fertility, pregnancy, and development of the offspring. Even if the field of nanotoxicology is rapidly expanding (Krug, 2014), the number of studies that have investigated the reproductive and developmental aspect of

Adverse Effects of Engineered Nanomaterials. http://dx.doi.org/10.1016/B978-0-12-809199-9.00017-3
Copyright © 2017 Elsevier Inc. All rights reserved.

nanomaterials is limited (Hougaard et al., 2015). At the same time, legislative measures aimed to control the exposure to engineered nanoparticles (ENPs) are currently lacking for pregnant women, as well as for women and men of childbearing age.

The present chapter aims to provide an overview on what is known so far on reproductive and developmental effects of the most commonly studied engineered nanomaterials, based on published investigations and reviews.

FERTILITY

Fertility includes the processes that are necessary for initiation of pregnancy. Production of the male and female germ cells (gametogenesis) is a highly regulated process that requires balance between proliferation, differentiation, and apoptosis. Also several less visible biological processes need to function correctly, for example, correct timing of hormonal activity or physiological parameters impacting the ability to fertilize, as well as libido, sexual behavior, and development of the fertilized ovum up to and including implantation (Lawson et al., 2003; Scientific Committee on Occupational Exposure Limits (SCOEL), 2009). Pollutants [among which nanoparticles (NPs) can be included] may adversely affect human reproductive health by directly influencing reproductive tissues or indirectly, by behaving as endocrine disrupters or interfering with the hormonal regulation of germ cell differentiation and maturation (Pryor et al., 2000; Lucas et al., 2009). Indeed, the ability of NPs to behave as endocrine disrupters has been recently recognized, although available information on this aspect of nanotoxicity is quite limited (Iavicoli et al., 2013) (and see Chapter 14). Perturbation of reproductive processes by chemicals and NPs might also lead to DNA mutations in somatic and in germ cells (Courbiere et al., 2013; Singer and Yauk, 2010). Studying the impact of nanotechnology on the reproductive health of both sexes is of major importance, since, for example, mutations in reproductive organs and cells represent not only a risk for the individual, but also for their progeny. In this respect, a different sensitivity to mutation-inducing agents is expected for exposures in adult males and females as the male process of spermiogenesis is continuous and takes place throughout the male fertile age. In comparison, female oogenesis is cyclic in nature and relies on the ovarian pool of oocytes that was established during fetal life.

Several reports demonstrate that ultrafine particles, the nonmanufactured counterparts to ENPs, can adversely affect male reproductive function (Ema et al., 2013; Yauk et al., 2008; Yoshida et al., 1999). Due to the often carbonaceous nature of ultrafine particles, for example, diesel exhaust particles, these are difficult to trace in biological tissues. In contrast, the chemical constitution of most ENPs allows such tracing, and over the last years, studies have been published on the distribution and the effect of ENP exposure to the reproductive organs in mammals.

Male Reproductive Function

The possibility that ENPs may interfere with male reproductive function has been addressed in vivo in several studies. One of the key questions is whether ENPs, independently of the route of administration, can reach the testes, and thus, potentially, act directly on the sperm producing tissue. It has to be considered that male germ cell maturation within the

seminiferous tubules occurs in a privileged environment, as the tight junctions between the somatic Sertoli cells forms the so-called blood-testis-barrier (BTB). Elucidating whether NPs eventually reach the lumen of the seminiferous tubules and thus the germinal epithelium by crossing the BTB is therefore of importance. One pioneering study on organ distribution of NPs reports that only gold particles of 10 and 250 nm accessed the testes 24 h after an intravenous injection whereas particles of 50 and 100 nm did not (de Jong et al., 2008). Other studies do, however, show that 50 nm iron-containing NPs and even 350 nm chitosan and 450 nm latex particles may access the testes (Choi et al., 2010; Klein et al., 2013; Kwon et al., 2008). However, in these studies crossing of the BTB was not observed, that is, particles were observed in the interstitial tissue outside the convoluted seminiferous tubules, or the location of particles was unclear (Choi et al., 2010; Klein et al., 2013; Kwon et al., 2008; Lan and Yang, 2012). Silica-coated magnetic particles of 50 nm administered intraperitoneally (25, 50, and 100 mg/kg) to mice for 4 weeks accumulated in the testis, without inducing histological changes of the organ at any dose level (Kim et al., 2006), suggesting that the mere presence of particles does not indicate toxicity. Indeed, in this study, localization of the nanoparticles appeared mainly interstitial and it is again not clear if nanoparticles accessed the BTB and reached the germ cell niche. Localization of nanoparticles in specific compartments (i.e., interstitial vs. intra tubular compartment) may influence toxicity. Recently, it has been demonstrated that 45 nm AgNPs, intravenously administered to rabbits (0.6 mg/kg), not only distributed to the testis, but also crossed the BTB; NPs were identified in the cytoplasm of Sertoli cell, in the nuclei of elongated spermatids and in ejaculated sperms (Castellini et al., 2014). A similar distribution has been observed in mice for 70 nm, but not for 300 nm, SiO_2 NPs after two consecutive intravenous injections of 0.8 mg per mouse, suggesting a size dependent ability of particles to cross the BTB (Morishita et al., 2012). Access to the testis and to the BTB may therefore depend on many factors. Chemical composition, size, and the method applied for analysis of particle distribution appear to be critical aspects to consider. It should be kept in mind that although the percentage of particles that enters an organ may be small (<1% of the dose), the particle number may be very high (Balasubramanian et al., 2010; Klein et al., 2013; Lan and Yang, 2012).

Adverse effects of NPs on spermatogenesis have been reported. Indeed, some studies demonstrate that carbon and metal nanoparticles can induce histological alterations and impair germ cell maturation, thus reducing the quality of sperms. For carbon black NPs, fertility parameters were investigated after intratracheal instillation of particles of different sizes (15, 56, and 95 nm) to sexually mature male mice (Fig. 17.1). The relatively high dose, 0.1 mg per mouse, was administered once weekly for 10 weeks. No changes in body or reproductive organ weights were observed, however, seminiferous tubules appeared vacuolated and the daily sperm production was decreased in all treated groups. Plasma levels of testosterone were increased, although only significantly so in the 14 and 56 nm particle groups. Interestingly, when males were exposed to the 14 nm particle but at particle numbers corresponding to the 56 nm particle group (weekly dose of 1.56 μg CB 14 nm per mouse) only daily sperm production was significantly decreased. The effect thus depended on particle mass rather than particle number (Yoshida et al., 2009a) (Fig. 17.2).

Repeated intravenous administration of multiwalled carbon nanotubes (MWCNT; 5 mg/kg every 3 days for 15 days to adult mice) altered architecture of the seminiferous tubules, and reduced thickness of the germ layer and number of Sertoli and spermatogonial cells, 15 days after the first dose. Increase in the testicular level of malondialdehyde indicated an

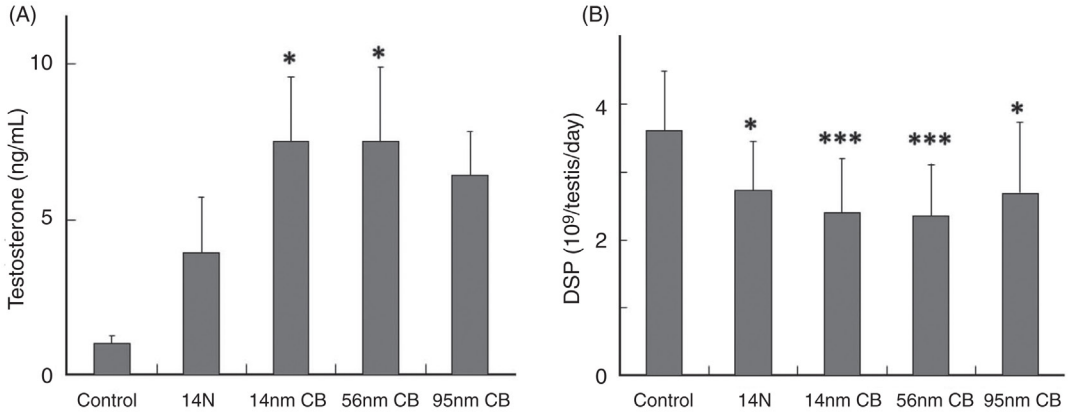


FIGURE 17.1 Effects on male reproductive parameters after exposure to carbon black particles of different sizes. Male mice were exposed to 0.1 mg carbon black per mouse by intratracheal instillation, once per week for 10 weeks. Particles were of three different sizes, that is, 14, 56, and 96 nm. Additional males were exposed to the 14 nm particle but at particle numbers corresponding to the 56 nm particle group (14 N; weekly dose of 1.56 μg CB 14 nm per mouse). Mean ± SD. *$p < 0.05$, *** $p < 0.001$. (A) Serum testosterone concentration, determined by ELISA (n = 15–16). (B) The efficiency of spermatogenesis, calculated as daily sperm production (DSP) (n = 15–16). *Source: Reproduced from Yoshida, S., Hiyoshi, K., Ichinose, T., Takano, H., Oshio, S., Sugawara, I. et al., 2009a. Effect of nanoparticles on the male reproductive system of mice. Int. J. Androl. 32, 337–342.*

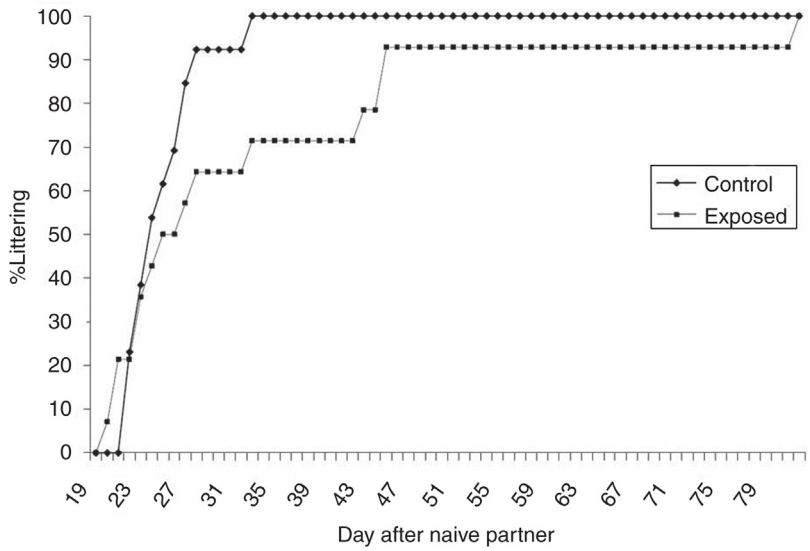


FIGURE 17.2 Time-to-first F2 litter. Littering curves for male offspring of sham and TiO_2-exposed pregnant mice (exposed by inhalation 1 h/day to 42 mg/m^3 aerosolized TiO_2-powder (1.7·106 particles/cm^3; peak-size: 97 nm) on gestation days 8–18. As adults, the male offspring were cross mated to mature, naïve female mice and time-to-first-delivery of F2 litter was recorded. Time to delivery of first F2 litter was delayed in exposed compared to control male offspring, however, nonsignificantly so (p = 0.12). Other studies report statistically significant effects on male reproductive measures after prenatal exposure to ENPs (Takeda et al., 2009; Yoshida et al., 2009b). This endpoint warrants further investigations in future studies. *Source: Originally published in Hougaard, K.S., Jackson, P., Jensen, K.A., Sloth, J.J., Loschner, K., Larsen, E.H. et al., 2010b. Effects of prenatal exposure to surface-coated nanosized titanium dioxide (UV-Titan). A study in mice. Part Fibre Toxicol. 7(16), 16.*

increased level of oxidative stress, which was returned to control levels at 60 and 90 days. Histological alterations were only sporadically observed at these later time points, suggesting an ability of the seminiferous epithelium to repair. No changes in plasma levels of sex hormones, DNA damage in sperm, total sperm concentration, motility, acrosome integrity, or mating behavior were observed. Effects did not depend on functionalization by carboxylation (negatively charged) or amination (positively charged). At all time-points, minimal changes were observed after only a single intravenous injection (Bai et al., 2010). Reversible damage to the testis has been also reported after five repeated intravenous administrations of 64 nm SiO_2 NPs to sexually mature mice over a period of 15 days. After 15 and 35 (but not 60) days from the first administration, DNA damage and decrease of sperm quality (decreased sperm concentration in epididymis and motility, increased rates of abnormal sperm) was observed (Xu et al., 2014). In the AgNP study described earlier, the localization of Ag NPs within the tubules was associated with reduced sperm motility and integrity of the acrosome and mitochondria, although no apparent morphological alteration of the testicular tissue was observed (Castellini et al., 2014).

Despite the data indicating toxicity of NPs to the testis, a few studies suggest that depending on their chemical composition, some NPs may have a beneficial, protective effect relative to external insults or ageing. Thus the administration of hydrated C_{60} fullerene in drinking water, rescued reproductive alterations observed in male rats with streptozotocin-induced diabetes (Bal et al., 2011). The fullerenes themselves did not induce changes in testicular values, apart from a small but significant increase in serum testosterone (Bal et al., 2011). Ameliorative effects on spermiogenesis in streptozotocin-induced diabetic rats have been also demonstrated for ZnO NP, after 30 days of oral administration of 10 mg/kg (Afifi et al., 2015). Similarly, pretreatment of male rats with fullerenol prevented increase in testicular levels of oxidative stress markers, normally induced by doxorubicin, probably through antioxidant capability or ability to bind free iron (Srdjenovic et al., 2010). Recently, cerium dioxide nanoparticles have been associated with improved quality of otherwise deteriorated male reproductive parameters in aged rats (Kobyliak et al., 2015).

One of the main mechanisms though which NPs may induce testicular damage appears to be oxidative stress. Indeed, findings in some studies indicate that particle exposure alters the oxidative environment in the testes by increasing the production of reactive oxygen species and decreasing the activity of antioxidant enzymes. Fan et al. (2006) compared the male reproductive effects in rats of nano- (10 nm) and microsized (1–5 μm) silicon dioxide particles, following inhalation exposure to 100 or 300 mg/m^3 for 2 h every second day for 65 days. Body weight decreased at both dose levels for the 10 nm particle, as did relative testes weight at 100 mg/m^3. Relative epididymal weight was increased at both dose levels—again only for the 10 nm particle. Exposure induced histopathological changes that were accompanied by reduced sperm counts and motility. In the testes, there were significant indications of oxidative stress in the 100 mg/m^3 group for the micrometre particles and at 300 mg/m^3 for both particle sizes. The authors concluded that the effects of nanoparticle exposure are more widespread than those observed after micrometre particle exposure (Fan et al., 2006) [discussed in (Lan and Yang, 2012)]. Oxidative stress has been established as one of the main causes of male infertility. Like any other cell, sperm cells face the oxygen-paradox: although low levels of ROS are required for sperm capacitation, excessive oxidative stress leads to impaired sperm functions compromising fertility (Aitken et al., 2012).

Adverse effects on reproductive functions have been reported in some in vitro studies, investigating the sensitivity of isolated testicular cell types to NPs. Several metal NPs interfered dose-dependently with cell metabolic activity and viability of a mouse spermatogonial stem cell line. The effects occurred independently of surface modifications, with silver NPs showing the highest toxicity (Braydich-Stolle et al., 2005). In the testes, the Leydig cells synthesize testosterone, which is critical for the male phenotype and spermatogenesis. In the mouse TM3 Leydig cell line, titanium dioxide (TiO_2, 25–70 nm) and carbon black (CB, 14 nm) NPs were both internalized by the cells, and TiO_2 particles, at a concentration of 10 and 100 μg/mL, transiently impaired cell proliferation and viability. The ability of 10 and 30 μg/mL of CB NPs to alter testosterone production was demonstrated by quantitative PCR of the steroidogenic acute regulatory (StAR) gene, as the encoded protein is important in the process of testosterone synthesis (Komatsu et al., 2008). Incubation of 9 nm gold NPs with a single donor sample of human sperm cells affected motility in 25% of the cells compared to 5% of the controls, and particles were shown to penetrate into sperm heads and tails (Wiwanitkit et al., 2009). In contrast, uptake by bovine sperm cells of Fe_3O_4 NPs, coated with poly-(vinyl alcohol) did not interfere with motility or ability to undergo the acrosome reaction, both fundamental processes for fertilization (Makhluf et al., 2008). More recently, Au and Ag NPs were demonstrated to be nonharmful to human spermatozoa up to a concentration of 500 μM, which would be unlikely to be reached in vivo (Moretti et al., 2013).

In conclusion, several studies indicate that exposure to some types of nanosized particles may interfere with male reproductive function, independently of the route of administration. When both biodistribution and toxicity have been assessed, it appears that presence of nanoparticles beyond the BTB is associated to alteration of fertility parameters (Castellini et al., 2014). However, further studies are needed to strictly correlate compartmental distribution of particles in the testis with reduced fertility. Chemical composition plays a role in the compartmental distribution of nanoparticles in the testis and their effect. Apart from a role for oxidative stress, little is known about the involved direct and indirect mechanisms driving toxicity (Lan and Yang, 2012). Furthermore, some particles may ameliorate rather than induce reproductive adverse effects.

Female Fertility

Reports on the biodistribution and action of NPs on the female reproductive process are scant. Differently from spermatozoa, the oocytes are not protected by a barrier similar to the BTB. They are, however, surrounded by a glycoproteic membrane, the zona pellucida, and a corona of somatic cells, the granulosa cells. Together, these form the cumulus-cell-oocyte-complex. The steroidogenic theca cells are more peripherally located. When intravenously administered at a dose levels of 35 and 66 μg to female mice early in gestation, 50 nm Ag NP were detected in the ovaries (Austin et al., 2012). Comparable dose levels (0.5 and 1 mg/kg) of smaller Ag NPs (20 nm) have been demonstrated to induce ovarian inflammation and apoptosis in the ovarian somatic cells (both granulosa and theca cells) of mice (Han et al., 2015). In one study, female mice were intratracheally instilled with 67 μg MWCNT per mouse on the day before cohabitation with a mature male. The delivery of the first litter was significantly delayed in exposed compared to sham instilled females. Litter sizes and birth weights compared across groups (Hougaard et al., 2013). Another study observed lowered pregnancy

rates after intratracheal instillation, once daily for 5 days, with 7.5 mg/kg of either Zinc oxide or composite carbon and Zinc oxide NPs during gestation. Control pregnancy rate was 100%, compared to 57.2% and 66.7% in the two treated groups (Zhang et al., 2008). Short term (5 days) intragastric administration of 1 and 2 mg/kg per day of food-grade TiO_2 NP (primary particle size <25 nm) increased the levels of titanium found in ovaries and induced apoptosis of ovarian granulosa cells and decreased testosterone in blood at the highest dose (Tassinari et al., 2014).

In vitro, granulosa cells from preovulatory rat follicles were incubated with 10 nm gold particles. Particles were internalized shortly after addition to media. Transmission electron microscopy showed particles to interact with mitochondria. Estrogen accumulation and secretion changed in a time dependent matter, initially increasing, but decreasing after 24 h (Stelzer and Hutz, 2009). Similarly, calcium phosphate NPs accumulated inside human granulosa cells; apoptosis increased, but hormonal production was unaltered (Liu et al., 2010). The ability of NPs to interfere with expansion of the cumulus-cell-oocyte-complex (a fundamental process for fertilization) and oocyte maturation has been demonstrated in vitro for Ag NPs, where particles were taken up by granulosa cells but not the oocyte (Tiedemann et al., 2014). A similar uptake has been demonstrated for 8 nm CeO^2 NPs (Courbiere et al., 2013). Apparently, the presence of the zona pellucida represented a selective barrier for the passage of NPs; when follicular cells and oocytes were treated in vitro with 100 μg/mL of CeO_2 NP, aggregates decorated the zona pellucida and were taken up by the oocyte only after the zona had been enzymatically removed. However, uptake by and toxicity to granulosa cells is in itself sufficient to perturb fertility.

Also for particles in ambient air, very little research has been conducted for female reproductive function. In a study by Veras et al. (2009), effects of ambient air pollution on female reproductive function was investigated in a two generation study in mice. Estrous cyclicity, ovarian follicle numbers, pregnancy, and couple-mediated outcomes were used as endpoints. The study showed that female exposure to air pollution prior to conception affected several functions and stages of reproduction (Veras et al., 2009).

Altogether, the available data may suggest a potential for adverse effects of nanoparticles on female reproductive function. However, the lack of information precludes any firm conclusions.

NANOPARTICLES AND DEVELOPMENTAL TOXICITY

Developmental toxicity embraces any effect interfering with normal development, before, as well as after birth. Embryotoxicity might manifest as reduced body weight in fetal life or at birth, growth, and developmental retardation, organ toxicity, structural defects, death, and abortion. Impaired postnatal development might appear as delayed physical development or as functional deficits, that is, developmental outcomes that do not have obvious structural correlates. They might appear before birth, but will in many cases not appear until after birth, as is the case impaired cognitive function or enhanced propensity to develop allergy. Generally, developmental effects might appear up to and including normal pubertal development (Scientific Committee on Occupational Exposure Limits (SCOEL), 2009; United States Environmental Protection Agency, 1991). Whereas effects induced by exposure to chemicals might

be reversible in the adult organism, a similar exposure of an organism undergoing development might lead to permanent effects (United States Environmental Protection Agency, 1991). Furthermore, the developing fetus is characterized by intense cellular division and differentiation, an environment that may represent a different milieu, than does an established tissue or even monocultures of dividing cells in vitro. The fetus also has relatively low levels of most xenobiotic-metabolizing and detoxifying enzymes (e.g., GST), which make tissues more sensitive to oxidative stress (Wells et al., 2009). In a model system for neuronal differentiation (PC-12 cells), silver NPs produced no or little oxidative stress in undifferentiated, dividing cells. This changed when the cells were induced to differentiate by addition of nerve growth factor. Then silver NPs were associated with robust oxidative stress and at the same time impairment of differentiation into the determined phenotype; silver NPs effects were different from those of silver ions (Powers et al., 2011). Furthermore, in mice, pregnancy has been shown to increase the inflammatory response to airway exposure to NPs (Fedulov et al., 2008; Lamoureux et al., 2010). For developmental toxicity, the placenta presents at least a potential barrier between the maternal blood and the fetal compartment (Pietroiusti et al., 2013). For some types of particles this barrier seems almost complete (Sadauskas et al., 2007) whereas for others some access to fetal tissues has been reported (Hougaard et al., 2011; Wick et al., 2010). Several factors may determine whether particles translocate across the placenta, and rodent studies using intravenous administration indicate that transplacental passage depends on particle characteristics (size, surface coating, charge), dose and duration of exposure, animal model, and timing in pregnancy (Buerki-Thurnherr et al., 2012; Pietroiusti et al., 2013), as well as the inflammatory condition of the exposed female (Tian et al., 2013). However, transplacental passage may not be necessary to have nanoparticle induced adverse effects on the embryo. The mere distribution of NPs to the placenta may suffice to induce alterations on fetal tissues, as it was recently demonstrated in vitro for cobalt-chromium NPs. Through an ATP-mediated intercellular signaling, NPs were able to induce DNA damage across an intact in vitro placental barrier, without particles actually crossing the barrier. Thus, the indirectly induced effects differed substantially from those induced in cells subjected to direct exposure to nanoparticles (Bhabra et al., 2009).

Metal-Based Nanoparticles

Titanium dioxide (TiO_2). Nanoparticulate titanium dioxide (TiO_2) is widely used in industrial products like lacquers and sunscreen lotions with "invisible" UV-filters and paints with photocatalytic-induced self-cleaning properties. It is employed as a food additive, under the name of E171, to give foods an opaque color. It is also frequently used as a generic model particle to illustrate effects of exposure to relatively inert nanoparticles. A single oral dose of 50 nm TiO_2 NPs (100 or 1000 mg/kg) to mouse dams during organogenesis has been demonstrated to significantly increase fetal abnormalities and death (Philbrook et al., 2011). When intravenously injected to pregnant mice females close to term, 70 nm TiO_2 NPs were able to cross the placenta barrier, reach the fetus, and induce in utero growth restriction (Yamashita et al., 2011). TiO_2 particles have been investigated for their effects on offspring male reproductive function, after exposure of pregnant mice to 0.1 mg per mouse 25–70 nm TiO_2 anatase particles by subcutaneous injection, at gestation days 3, 7, 10, and 14. Four days and 6 weeks after birth, aggregated particles of TiO_2 were identified in brain and testicular tissue from

male offspring. Particles were detected in spermatids, and Leydig and Sertoli cells alongside abnormal testicular morphology, and reduced number of Sertoli cells and daily sperm production. Sperm morphology was comparable between groups (Takeda et al., 2009). Whether developmental exposure influences also functional aspects of the nervous system was assessed by exposing lactating females to 25 nm anatase TiO_2 particles from postnatal day 2–21 (100 mg TiO_2/day), by gavage. Several parameters of synaptic plasticity were depressed in offspring hippocampal dentate gyrus. Effects were much less pronounced following exposure throughout gestation. The hippocampus is pivotal for higher order neural function, suggesting developmental exposure to TiO_2 might interfere with cognitive function in the offspring (Gao et al., 2011). This was specifically studied in offspring of pregnant mice exposed by inhalation for 1 h/day to 42 mg of rutile TiO_2 particles/m^3 on gestation days 8–18 (primary particle size: 21 nm, peak-size in exposure atmosphere: 97 nm). Exposure induced lung inflammation in the dams, but gestational and lactational parameters compared to that of controls. Gene expression patterns were however significantly altered in offspring liver a few days after birth, more so in females compared to males. As young adults, the F1 offspring underwent several neurobehavioral tests. Prenatally exposed offspring tended to avoid the central zone of the open field, and exposed female offspring displayed enhanced prepulse inhibition. Learning and memory were, however, unaffected in the Morris water maze, that among others depend on hippocampal function. This study also investigated functional reproductive implications by cross-mating the mature male and female F1 offspring to non-exposed mature mice. Time-to-delivery of the first F2 litter was somewhat (although nonsignificantly) delayed for exposed male, but not female offspring. Litter size, sex distribution, and birth weights of F2 litters were comparable to that of controls (Hougaard et al., 2010b). The daily sperm production was later assessed in males from the F1 as well as the F2 generation. DSP was not statistically significantly altered in either generation, although TiO_2 tended to reduce sperm counts in the F1 offspring, where also the time-to-delivery of the first litter was delayed (Kyjovska et al., 2013).

Offspring propensity to develop allergy was investigated after intranasal insufflation of pregnant mice with a single dose of 50 μg microsized TiO_2 at gestation day 14. When offspring were sensitized and exposed to the aerosolized model allergen of ovalbumin, neonates of exposed mothers developed a more pronounced asthmatic phenotype than did sham exposed control offspring (Fedulov et al., 2008).

Gold nanoparticles. Gold (Au) NPs have been already extensively applied in biology and medicine, and are also used in consumer products such as cosmetics. In vivo experiments have primarily been carried out with the zebra fish embryo as a model. A "random walk" of gold NPs in embryonic tissues has been proposed. When cleavage-stage zebra fish embryos were incubated for 120 h with stable, monodispersed (16.6 ± 0.9 nm), highly purified Au NPs (concentration range 0.025–1.2 nM), particles diffused through the chorionic pores and in the embryos via Brownian motion. A small number of dead or abnormal embryos was observed, with a no clear dose-response dependence, probably due to a random distribution of NPs within the embryonic tissues, that might lead to stochastic effects on embryonic development. Overall, the gold NPs showed low toxicity (Browning et al., 2009). Similarly, when mercaptobenzoic acid-coupled Au NPs of 25 and 40 nm in diameter were injected directly into zebra fish at the one-cell embryonic stage, particles were found in all cells and tissues of the larvae, which responded to mechanical stimuli, reflecting a normal development of

organs. Morphology appeared normal, and also gene expression was unaffected by the presence of particles (Wang et al., 2010). Altogether, gold NPs seems of low toxicity for embryonic development of the zebra fish in vivo, independent of size (30–100 nm) and tested surface modifications.

In mammals, placental crossing for gold NPs has been originally studied in an ex vivo model of human placental perfusion. In this model system, PEGylated Au NPs of 10 and 30 nm accumulated in the maternal layer of the human placenta, but no particles were detected in the fetal side of the organ (Myllynen et al., 2008). Contradictory results have been obtained in animal models in vivo. Some transplacental passage of 5 and 30 nm radiolabeled gold NPs have been demonstrated in one report in rats (Takahashi and Matsuoka, 1981), but placental impermeability to 2–40 nm particles, evaluated by autometallography, has been also reported in mice (Sadauskas et al., 2007). However, recently, the ability of Au NPs to cross the placental barrier as a function of gestational stage has been demonstrated in rodents. Intravenous administration of 15 nm Au NPs between 5.5 and 15.5 (0.9 mg/g body weight) reveals that these NPs are able to reach the placenta and extraembryonic tissues, but translocation beyond the placental barrier follows only if exposure occurs before gestational day 11.5. The type of surface modification is also a parameter influencing biodistribution, as fetal and extra-embryonic tissue accumulation of ferritin- and PEG-modified nanoparticles was considerably greater than that of citrate-capped nanoparticles. Interestingly, no sign of toxicity was observed on fetal and postnatal growth and fertility, even at the high dose of 7.2 mg/g body weight (Yang et al., 2012). Similar distribution have been observed for 20 and 50 nm Au NPs administered i.v. late in gestation (GD 16 and 17; 50 μg per mouse). Particles accumulated in maternal liver and placenta, but not in fetal organs, and did not induce toxicity. Increased presence of clathrin positive endocytic vesicle in syncytiotrophoblast and fetal endothelial cells argued for an increase in endocytic vesicles at the maternal–fetal barrier of mice treated with Au NPs (Rattanapinyopituk et al., 2014). The 20 nm transtrophoblastic channels have been recently demonstrated as the route through which small NPs may cross the placental barrier and reach fetal tissues. When 1.4, 18, and 80 nm radiolabeled Au NPs were administered to GD 18 pregnant rats, all three sizes were detected in the placenta, but only very low fractions of the two smaller particles were found in fetuses (Semmler-Behnke et al., 2014). Altogether, these data demonstrate a low but detectable transplacental crossing of Au NPs and, in accordance with results in nonmammalian models, indicate a low toxicity of Au NPs in pregnancy.

A low toxicity of Au NPs has been demonstrated also in vitro. Using a modified version of the embryonic stem cell test, gold NPs covered by hyaluronic acid were classified as weakly embryotoxic, while silane and gold coated cobalt ferrite NPs appeared nonembryotoxic (Di et al., 2010). When 11 nm Au NPs were injected into one blastomere of a 2 cell stage mouse embryo at a dose of 50 μg/mL, development to the blastocyst stage was not impaired as it was not affected the expression of genes involved in apoptosis or differentiation of the blastocyst inner cells mass (Taylor et al., 2014).

Silver nanoparticles. Silver (Ag) NPs have been already widely used in consumer products due to their antibacterial properties. Over the last 5 years, several studies have investigated the ability of Ag NPs, and released ions, to reach the placenta and/or induce adverse effects on embryonic development. Originally, the effect of Ag NP on mammalian development was studied in vitro. Incubation for 24 h of morula stage mouse embryos with silver NPs impaired

their ability to reach the blastocyst stage, increased the percentage of apoptotic cells and reduced the ability to undergo to in vitro outgrowth. In addition, when blastocysts, preincubated with 50 μM silver NPs, were transferred to the uterus of pseudo-pregnant females, implantation success was significantly decreased compared to nonexposed blastocysts. Also the percentage of implanted embryos that failed to develop normally was markedly higher in the treated group (Li et al., 2010). However, a relatively high dose of 50 μM (corresponding to 625 ng per embryo) was used in this study. When much lower doses (0.0005 ng per embryo) were directly injected into one blastomere of a 2-cell embryo, Ag NPs did not induce any effect on embryo development, while supplementation of the medium with silver ions (at a concentration of 50% of the Ag present in the injected blastomere) completely blocked development. Ag NPs injection had no effects on gene expression (Taylor et al., 2014). In vivo, intravenous administration of 35 and 66 μg per mouse of 50 and 10 nm Ag NPs during early mouse organogenesis resulted in silver accumulation in all organs, including embryos, where, however, accumulation was the lowest; only very mild embryotoxic effects were observed (Austin et al., 2012, 2015). In contrast, 100 and 1000 mg/kg orally administered 20 nm Ag NPs at a similar gestational stage were reported to significantly increase fetal mortality (Philbrook et al., 2011). However, repeated oral administrations (GD 6-19) of <10 nm NPs, although inducing oxidative stress in maternal liver, did not have any toxic effect on development (Yu et al., 2013). Particles of same size accumulated in the offspring of females orally administered with 250 mg/kg (Lee et al., 2012), indicating the possible transfer of Ag NPs from mother to fetus. These results also suggest that Ag NPs have low embryotoxicity, however too few in vivo studies are available. Further investigation on the effects of developmental exposure to Silver nanoparticles as well as of the relative contribution of particles and ions in tocicity is needed.

Zinc oxide nanoparticles. Not many studies have investigated the developmental toxicity of ZnO nanoparticles. Recently, one group studied the effect of repeated oral administration of positively and negatively charged 20 nm ZnO NPs (Hong et al., 2014a,b). The authors showed that after a daily exposure to 100, 200, or 400 mg/kg ZnO NPs (from GD 5 to GD 19), the content of Zn in fetal tissues was similar in control and treated groups; however, the positively charged NPs decreased fetal weight and increased fetal abnormalities at the highest dose. Apart from this, all parameters examined (e.g., implantations, resorptions and fetal deaths) were not affected by the treatment with either particle. In vitro studies using the embryonic stem cell test or EST (a validated assay developed for predicting embryotoxicity of soluble chemical compounds) have also shown low embryotoxic potential of ZnO NPs (Farcal et al., 2015).

Cadmium containing particles. Cadmium containing NPs have been considered promising for a wide range of industrial and medical applications. As an example, nanosized cadmium oxide (CdO) is used for the preparation of quantum dots (QD), which have been used as sensitive fluorescent probes for imaging and tumor targeting. However, Cd, which is a toxic heavy metal and a developmental toxicant (Thompson and Bannigan, 2008), is known to be released form QDs. Toxicity of Cd containing QDs (250 and 500 nmol/L) has been demonstrated after incubation with mouse preimplatation embryoes. QDs induced apoptosis of blastocysts and inhibited proliferation of the inner cell mass. A size and dose-dependent placental transfer of CD was demonstrated after intravenous administration of QDs. Toxicity was dependent on the amount of Cd contained in the QDs (range 50–125 μg), suggesting a

role for the released heavy metal in mediating the effect (Chu et al., 2010). Recently, chronic pulmonary exposure to 230 μg/m^3 of CdO NPs has been shown to decrease the incidence of pregnancy, and to affect in utero growth of the embryos and delay neonatal growth (Blum et al., 2012), as well as to induce renal dysfunction in mothers and offspring (Blum et al., 2015).

Carbonaceous Particles

Carbon-based NPs are a heterogeneous class of particles with very different structure whose common feature is a regular organization of carbon atoms.

Carbon nanotubes. Despite being considered among the most promising ENPs for several application (from industry to biomedicine), not much information on embryotoxic potential of single and multiwalled carbon nanotubes (CNTs) is available (Ema et al., 2015). When intravenously administered to pregnant mouse females soon after implantation, pristine and oxidized singlewalled CNTs (SWCNTs) have been demonstrated to induce early miscarriages, early and late resorptions and fetal abnormalities. Fetal alterations were observed at concentrations as low as 100 ng per mouse and were associated with extensive placental vascular lesions and increased production of reactive oxygen species (ROS) (Pietroiusti et al., 2011). Covalent fluorescent labelling allowed for identification of the distribution of short SWCNTs in placentas and embryonic membranes, but not in fetuses, indicating very low or no translocation of the particles across the placental barrier (Fig. 17.3). Distribution of particles in the placenta was per se able to induce fetal abnormalities. An interesting finding of this study is that repeated exposures at different stages of pregnancy, caused mild liver damage in dams and probably increased the teratogenic effects; however, given the low numerosity of the exposed population, additional studies are needed to confirm these results. Maternal organ distribution of SWCNT did not differ from organ distribution in nonpregnant females (Campagnolo et al., 2013b).

In one study, female mice were instilled with 67 μg multiwalled CNT (MWCNT) per mouse, either 4 times during gestation or once on the day before cohousing with a mature male. Litter sizes and birth weights compared across groups (Hougaard et al., 2013). Intravenous exposure of male mice to MWCNTs (5 mg/kg once every 3 days for 15 days) followed by pairing with naïve female mice, 15 or 60 days after the first injection, did not affect delivery, litter size, and offspring viability compared to pregnancies fathered by control mice. MWCNTs were therefore not associated with male mediated developmental toxicity (Bai et al., 2010). No toxicity was observed after 14 day-repeated oral administration of MWCNTs (dose range 8–1000 mg/kg/day) to pregnant rats. None of the developmental parameters examined (e.g., gestational index, embryo-fetal mortality, fetal and placental weights, sex ratio) were affected by the treatment (Lim et al., 2011). Interestingly, it has been demonstrated recently that gravidity/parity might play a role in nanoparticle mediated embryotoxicity, since, when exposed to the same dose, virgin mouse females were more prone to abortion than females that had already given birth once (Qi et al., 2014).

The effect of CNTs in vitro has been studied using mammalian embryonic stem cells (ES cells) and the EST. ES cells represent a good in vitro system to assess geno/embryotoxicity due to their high sensitivity to xenobiotics and DNA damaging agents. The EST predicted the results obtained in vivo demonstrated a high embryotoxic potential of oxidized SWCNTs

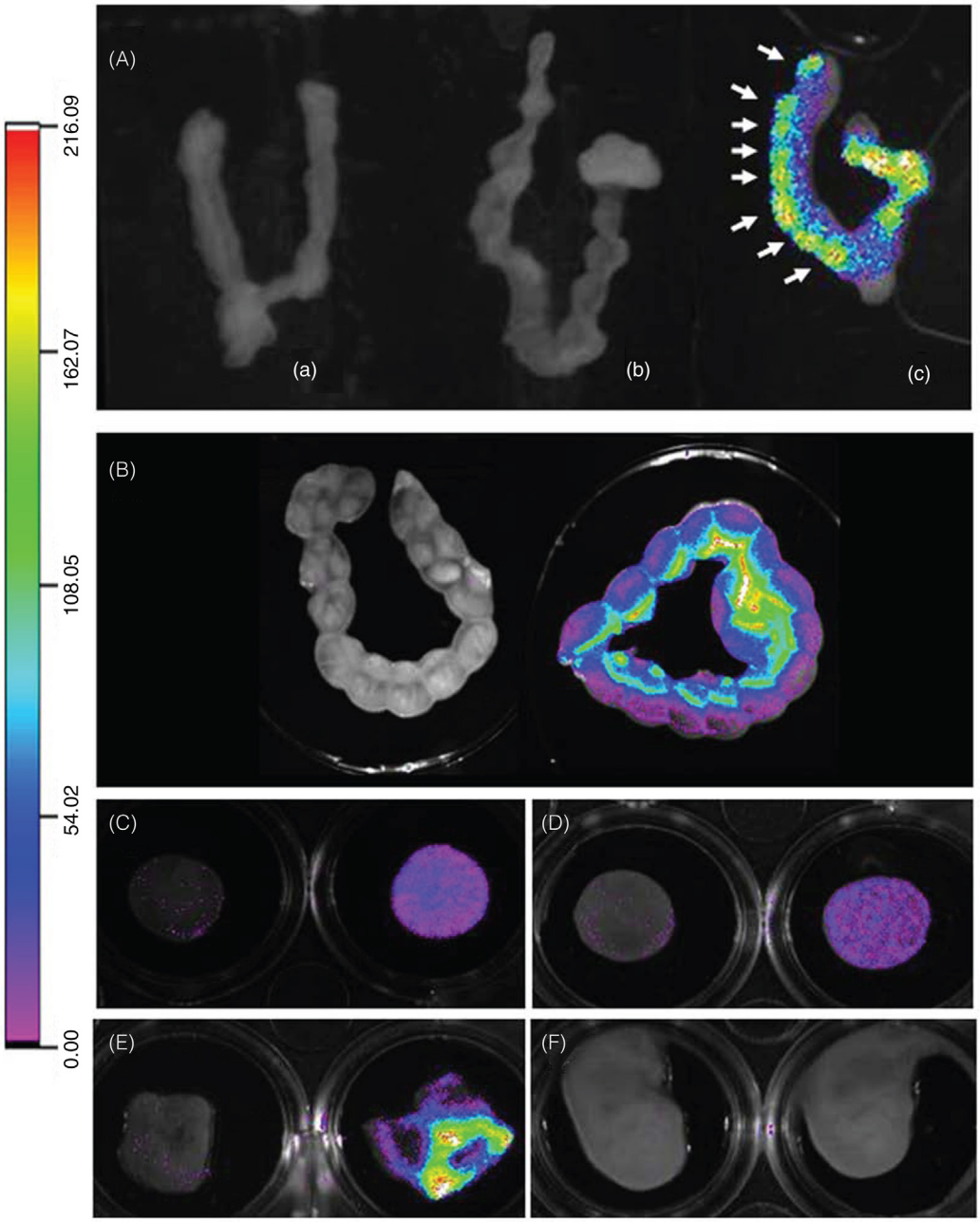


FIGURE 17.3 Analysis of the biodistribution of PEG-SWCNT-750 in the uterus, placenta, fetus, and fetal membranes. (A) At 6.5 dpc isolated uteri from dams exposed at 5.5 dpc to PEG-SWCNT-750 showed fluorescence in correspondence of the implanted embryos (c, *arrows*). No fluorescence was recorded in uteri collected from females exposed to either PBS or Seta750 (a and b, respectively). (B) fluorescence recorded in uteri collected from dams at 15.5 dpc that were either exposed to PBS (left) or PEG-SWCNT-750 (right) at day 14.5 of gestation. (C and D) 15.5 dpc isolated placentas from mice administered with PBS (left) or PEG-SWCNT-750 (right) with maternal (C) or fetal (D) side facing up. (E and F) 15.5 dpc fetal membranes (E) and fetuses (F) from PBS (left) or PEG-SWCNT-750 (right) treated dams. *Source: Reproduced from Campagnolo, L., Massimiani, M., Palmieri, G., Bernardini, R., Sacchetti, C., Bergamaschi, A. et al., 2013b. Biodistribution and toxicity of pegylated single wall carbon nanotubes in pregnant mice. Part Fibre. Toxicol. 10, 21.*

(Pietroiusti et al., 2011). Exposure of embryonic cells to MWCNTs reduced expression of stem cell markers after 2, 4, and 24 h, while the NPs entered the cells. In addition, the levels of p53 and phosphorylated p53 increased in a dose dependent manner within 2 h from the treatment, and markers of DNA damage were also induced.

*Fullerenes and fulleroles***.** Fullerenes are a class of NPs with a peculiar spherical cage structure usually composed of 60 carbon atoms. As already mentioned, this class of NPs has attracted much attention due to their significant antioxidants properties, since one molecule is able to neutralize 20 or more free radicals (Yadav and Kumar, 2008). Similarly to CNTs, not much information about the embryotoxic potential is available for fullerenes. A pioneering study investigated the embryotoxic effect of intraperitoneally injected poly(vinylpyrrolidone)-solubilized C_{60} fullerenes on mouse development When administered at high dosage (25–137 mg/kg) during early organogenesis (GD 10), fullerenes were found to distribute to the embryo, and to induce severe structural abnormalities and dead. Limitations to this study appear, as the doses used in the experiments were extremely high and only two mice per group were included (Tsuchiya et al., 1996). Most of the data on fullerenes and embryonic development have been obtained in aquatic species to investigate for their ecotoxicological potential on ground water pollution, and very few data on mammalian development are available. For example, fullerenes have been demonstrated to delay hatching, decreased heartbeat, and cause pericardial edema and ultimately death in zebra fish embryos at 0.2 and 1.5 mg/L, while even much higher concentrations of fullerols (hydroxylated C_{60} derivatives) did not elicit any toxicity (Usenko et al., 2007; Zhu et al., 2007). It is interesting to note, that differently from the protective antioxidant role demonstrated in the male germ line, fullerenes appear toxic with respect to embryonic development.

Carbon black. Carbon black (CB) is a common denomination for particles with a carbonaceous core, produced by incomplete combustion of heavy petroleum products and used as a black pigment. It has been used extensively as a model for diesel emission particles without the adhering hydrocarbon and metals (Ema et al., 2015). Effects of maternal pulmonary exposure to 14 nm carbon black on gestation, lactation, and DNA strand breaks were evaluated. Time mated mice were exposed by (1) inhalation of 42 mg/m^3 for 1 hour/day on gestation days 8–18, or (2) intratracheal instillations, on gestation days 7, 10, 15, and 18, with total doses of 11, 54, and 268 μg/animal. Lung inflammation was observed in exposed mothers at weaning. Neither inhalation nor instillation affected gestational and lactational measures, but inhalation increased levels of DNA strand breaks in the liver of mothers and offspring (Jackson et al., 2011). The instillation was also not associated with induction of mutations in the germ line of the femal offpring (Boisen et al., 2013). Exposure was furthermore associated with significantly and outspoken changes in gene expression in offspring liver a few days after birth, more so in female compared to male offspring (Jackson et al., 2012). When behavior was assessed in offspring of dams instilled at the highest dose level, habituation of activity in the open field was somewhat changed, but no significant effects was observed for the acoustic startle reaction (Jackson et al., 2013). Instillation of carbon black to pregnant at the highest dose level did not alter reproductive parameters in the F1 generation, but a decreased sperm production was observed in the F2 generation from the male (but not the female) germ line (Kyjovska et al., 2013). Pregnant mice were exposed to 14 nm carbon particles intratracheal instillation at day 7 and 14 of gestation.

Each dose was 0.2 mg. Again, no effects were observed for gestational parameters. At the ages of 5, 10, and 15 weeks, the testes from exposed offspring displayed significantly more seminiferous tubule damage and decreased daily sperm production compared to control offspring (Yoshida et al., 2009b). The potential involvement of prenatal exposure to respirable sized (microsized) carbon black in development of allergy was studied as described earlier for TiO_2, although the instillation dose was 5 times higher, that is, 250 μg CB. As for TiO_2, the exposed offspring developed a more pronounced asthmatic phenotype. A similar phenotype was observed after maternal exposure to 50 μg diesel exhaust particles (Fedulov et al., 2008). Allergic or inflammatory effects have been also demonstrated in male offspring after maternal intranasal instillation of 14 nm CB NPs (95 μg/kg body weight) on GD 9 and 15 (El-Sayed et al., 2015).

Polystyrene Nanoparticles

Among nanostructured polymeric materials, polystyrene has attracted much interest for its specific characteristics, such as stiffness, transparency, and easy processability. Polystyrene beads have been proposed for drug delivery, protein adsorption, and DNA transfection and are considered to be among the safest particles for experimental use. Bosman et al. (2005) demonstrated that mixed-size (between 40 and 120 nm) fluorescent polystyrene-based NPs were internalized by the 2-cell mouse embryo. Initially, particles were localized in the cytoplasm, but at later stages they translocated to the nuclei. Up to the blastocyst stage (the latest stage investigated), no impairment in the development was observed. Although a preferential localization of the NPs to the trophoblast cells was recorded, no significant difference in the ability of blastocysts to implant was evident between exposed and nonexposed groups (Bosman et al., 2005). Developmental toxicity of polystyrene NPs have also been investigated in postimplantation mouse embryos. Embryos were collected 7.5 days post coitum, that is, the beginning of mouse gastrulation, and injected via the extra-embryonic tissues with different sizes (20, 100, 200, and 500 nm) of amine- and carboxyl-functionalized fluorescent polystyrene NPs. After 12 hours, smaller carboxyl-functionalized NPs (20 nm) were distributed in all three germ layers of both the extra-embryonic tissues and the embryo proper, while bigger particles (>100 nm) distributed exclusively in the extra-embryonic compartment. In contrast, 200 nm amine-modified beads were able to access the embryonic tissues and induce growth retardation, thus indicating that surface chemistry, more that surface area, played a primary role for NP behavior and toxicity (Tian et al., 2009). In an in vitro study, mES cells were incubated with 0.4 or 12.2 million/mL of mixed-size (40–120 nm) fluorescently labeled particles for 6 days under conditions that would lead to differentiation of the cells. After one day, a slight reduction of cell viability was recorded. At 6 days, a nanoparticle influence on the type of differentiation was evident, since a shift from a squamous to a fibroblast and columnar phenotype was observed. Cytoskeletal reorganization was suggested to be responsible for such shift (Tran et al., 2007). Overall, data indicate that polystyrene NPs might interfere with proper differentiation of tissues. As the ability of 50–500 nm fluorescent polystyrene NPs to cross the blood-placental barrier has been demonstrated in the ex vivo model of placental transfusion (Wick et al., 2010), possible interference of polystyrene NPs with embryonic development needs to be properly assessed.

DISCUSSION

ENPs of various types are able to influence reproduction and development in several ways, as is evident from the earlier synthesis of published studies. Regarding reproductive function, experimental data show that ENPs may be found in the reproductive organs of experimental animals. Exposure to some ENPs has furthermore been observed to interfere adversely with important aspects of male reproductive function—also after exposure through the airways. Effects might be transient, as one study indicates that the seminiferous epithelium is able to repair damage induced by carbon nanotubes when followed up (Bai et al., 2010). Whether also female reproductive function is affected is a subject for study, but initial data indicate this may be the case (Hougaard et al., 2013). Effects of ENPs on central regulation of sex hormones that might indirectly interfere with spermatogenesis and oogenesis have not been reported so far, but it cannot be excluded that some particles may possess endocrine disrupting properties (Iavicoli et al., 2013). Interaction of ENPs with the hypothalamus-hypothesis-gonad axis might therefore be hypothesized (refer to Chapter 14).

Most ENPs have been shown to interfere with embryonic development, irrespective of size and chemical composition. Prenatal exposure to TiO_2 NPs influenced development of the offspring nervous system and the propensity to develop allergy and induced changes in gene expression in young offspring. Silver NPs appear to seriously impair embryonic development both in mammalian and nonmammalian. For carbonaceous nanoparticles, fullerenes induced structural and functional abnormalities in mouse and zebra fish embryos (Tsuchiya et al., 1996; Usenko et al., 2007). Inhalation of carbon black affected offspring DNA and immune function. When directly injected into zebra fish early embryos, carbon nanotubes induced acute and long-term effects, and in ES cells expression of genes associated with DNA (Cheng et al., 2009; Zhu et al., 2007). Gold NPs have been indicated as of low embryotoxic potential, as functionalized and nonfunctionalized particles only slightly affected zebra fish development (Bar-Ilan et al., 2009; Browning et al., 2009). The sensitivity of male reproductive organs and/or function to maternal particulate function has been investigated in mouse studies, for both TiO_2 and CB. It is of major concern that this organ system has showed sensitive over a range of particle sizes as well as after both airway and subcutaneous maternal exposure.

Information on developmental toxicity of ENPs has originally been obtained in the zebra fish. Although this model represents a valuable tool to screen for NP embryotoxicity and may give indications for risk assessment of NPs in humans, the lack of a placenta precludes exploitation for studying NP transfer from mother to fetus, a central aspect in environmental and occupational risk assessment of developmental toxicity. An in vitro test for developmental screening of NPs was recently proposed (Campagnolo et al., 2013a). Limitation of the test exists, as it does not include a simulated placental barrier. The ex vivo placental perfusion system might overcome this limitation, giving information of transplacental transfer of ENPs. Two reports have already exploited such system to study the mother-fetus transfer of gold and polystyrene NPs, showing that the ability of NPs to cross the blood-placental barrier is not size-dependent, as polystyrene NPs up to 240 nm are able to reach the fetal side of the organ, while much smaller gold NPs (30 nm) are confined to the maternal side (Myllynen et al., 2008; Wick et al., 2010). However, apoptosis occurring in the afterbirth placenta may result in permeability changes, which may in turn affect interpretation of data. In addition,

only term placentas are readily available for this approach, limiting studies to the putative effect to ENP exposure at the end of pregnancy.

The concept of engineering of nanomaterials is too recent for focused epidemiological studies to exist. However, the association between exposure to ambient air (and thus ultrafine) particles and adverse effects on especially development has been investigated. A systematic review of the association between air pollutants and birth outcomes based on 41 studies foundfine particulate matter (≤2.5 μm) to be associated with low birth weight, preterm birth, and being born small for gestational age (Backes et al., 2013; Dadvand et al., 2013; Shah and Balkhair, 2011). Overall, the evidence regarding particulate matter in ambient air on birth weight-related outcomes can be seen as strong (Hougaard et al., 2015). As such, the experimental studies on prenatal exposure to ENPs do not suggest fetotoxic potential of ENPs per se for maternal airway and oral exposure (Hougaard et al., 2010b; Hougaard et al., 2010a; Jackson et al., 2011; Warheit et al., 2015; Yoshida et al., 2009b). The basis for this conclusion is faint, however. Even if easily available, most mammalian studies on ENPs do not provide information on maternal weight gain, litter size, birth weights. In animals, rather many studies have investigated the effects of prenatal exposure to diesel exhaust and reported reduced body weight, altered immunological measures, increased frequency of DNA deletions, changes in sexual differentiation, and sperm production in the offspring [summarized in (Ema et al., 2013, 2015; Hougaard et al., 2008)]. Only few studies resuspended diesel exhaust particles, generally corroborating previous findings (Hemmingsen et al., 2009; Hougaard et al., 2008; Ritz et al., 2011). However, the relative roles of diesel exhaust particles, associated chemicals and exhaust gases in developmental toxicity remain to be clarified (Ema et al., 2015).

Mechanistically, exposure to ENPs may potentially affect reproduction and development through several modes of action. Toxicity can arise due to toxicological properties related to specific chemical constituents of the particles, as ENPs are often enriched with functional groups, or due to contaminants secondary to their production. As an example, embryotoxic effect of silver NPs has been proposed to be a consequence of silver ions released secondarily to in vivo nanoparticle destabilization (Bar-Ilan et al., 2009). The placenta acts as barrier for some but not all NPs, and particles have been identified in offspring tissues several weeks after birth [(Sadauskas et al., 2007; Takeda et al., 2009; Wick et al., 2010) and summarized in (Hougaard et al., 2011)]. Direct effects of particles on fetal development following maternal exposure are therefore possible, although the mere physical presence of particles does not necessarily interfere with development (Bar-Ilan et al., 2009; Park et al., 2009). However, many ENPs induce oxidative stress which poses a potential threat to germ cell, fetal and placental DNA integrity. Some NPs have been demonstrated to interact with proteins involved in DNA mismatch repair (Gupta et al., 2011) and altered DNA mismatch-repair machinery has been reported to cause germ line mutations (Tijsterman et al., 2002). Placental crossing of ENPs or even presence of particles in maternal blood might not be necessary in order for developmental toxicity to occur. Thus, lung exposure to particulates might induce airway inflammation with the resulting mediators representing potential adverse agents for reproductive and fetal development (Hougaard et al., 2010a,b). Interestingly, the lung inflammatory response to particles seems enhanced in pregnancy (Fedulov et al., 2008; Lamoureux et al., 2010). It is also of note that nanoparticles accumulated in maternal tissue may be transferred to the offspring during lactation (Melnik et al., 2013), potentially affecting postnatal development.

The published reports represent a great diversity of study designs, for example, with respect to choice of ENPs and surface modifications, model systems and animal species, dose levels, routes of exposure, and end points. This diversity makes it difficult to deduce general rules as to reproductive and developmental toxicity of ENPs. Thus, changes in hippocampal electrophysiological signals suggests that developmental exposure to particulate TiO_2 in rats interferes with brain development that would manifest as changes in cognitive function in offspring (Gao et al., 2011). However, when cognitive function was assessed in offspring of mothers inhaling TiO_2 during gestation, exposed offspring performed as well as control offspring (Hougaard et al., 2010b). This discrepancy might occur due to differences in exposure, with respect to both dose levels and route of exposure. Also, lactational exposure seemed to have a larger impact on hippocampal recordings than did gestational exposure. This could be due to a relatively increased transfer of the compound from mother to offspring during lactation or to increased sensitivity of the rat developing nervous system in lactation compared to the prenatal period. The latter is very important when translating these results to humans. Rodents are relatively delayed in CNS development at birth compared to humans, and at birth a rat compares to a human fetus approximately two trimesters into pregnancy (Morgane et al., 1992). Increased sensitivity during lactation in the rat would therefore indicate that the human fetus would display increased sensitivity during the last gestational trimester and shortly after birth.

Overall, available data on the effect of ENPs on male and female fertility as well as on developmental toxicity suggests that ENPs possess the potential to interfere with fertility and development. The diversity in applied study designs and investigated ENPs hampers deduction of general rules as to reproductive and developmental toxicity of ENPs. Physical-chemical properties influence reproduction and developmental toxicity of ENPs (Campagnolo et al., 2012). For ENPs in general, NP size or surface area is considered pivotal for the adverse response (Nel et al., 2006). However, findings in several experimental studies indicate that response does not depend strictly on size. Furthermore, surface chemistry seems important in some (Tian et al., 2009), but not other cases (Bai et al., 2010; Bar-Ilan et al., 2009). In other cases, the core material seems the primary determinator of toxicity, for example, for silver particles [e.g., (Bar-Ilan et al., 2009)]. In the same ex vivo model of placental transfer, 20–25 nm gold particles coated with polyethyleneglycol did not pass the placenta (Myllynen et al., 2008), where as much larger polystyrene beads passed with almost no hindrance (Wick et al., 2010). Finally, one study suggests that the route of exposure is crucial for developmental toxicity (Jackson et al., 2011). This will definitely be the case for reproductive and developmental toxicity suggested to occur due to particle induced lung inflammation (Hougaard et al., 2010a). At present, all nanomaterials developed for industrial or biomedical purposes should therefore, in principle, be tested for adverse effect on reproduction and development. Accidental exposures should be avoided, and conscious exposure should occur in controlled conditions, as data suggests several NPs to interfere with the reproductive apparatus even after airway exposure.

In protection against airborne exposure to chemicals in the workplace occupational exposure limits are pivotal. Awaiting the full implementation of the chemicals legislation, REACH (Chapter 7), limit values are in the EU set such that "exposure, even when repeated on a regular basis throughout a working life, will not lead to adverse effects on the health of exposed persons and/or their progeny at any time" (Scientific Committee on Occupational Exposure Limits (SCOEL), 2009). In principle, limit values therefore also protect the unborn child and

future generations. However, limit values are "predicted from the contemporary state of knowledge," which evidently is scarce for ENPs and for reproductive and developmental toxicity in general. Even if only limited data are available, "The absence of relevant data (on reproductive and developmental toxicity) will not normally be a factor in determining the size of an Uncertainty Factor" (Scientific Committee on Occupational Exposure Limits (SCOEL), 2009). Furthermore, nanomaterials are presently regulated similarly to the bulk material. Occupational exposure limits therefore provide little protection against potential reproductive and developmental toxicity of ENPs. Once pregnancy is a fact and "official," however, employers are obliged to assess risks and to remove, avoid, or reduce risks for pregnancy and the unborn child. These policies ignore that men and women might be exposed to reproductive toxicants while aiming to establish pregnancy, as well as the lag of time from conception to acknowledgement of pregnancy (European Agency for Safety and Health at Work, 2003). ENPs that are nonbiodegradable and resistant to excretion might present a special case, if accumulation in the body takes place during chronic exposure. This might translate into a slow release during pregnancy, even if exposure during pregnancy is avoided. Although speculative, it has been suggested for nervous tissue, that long term and low dose exposure might lead to chronic inflammation-like conditions (Simko and Mattsson, 2010), a condition that might prove detrimental for some reproductive processes. Ideally, in occupational settings women in the reproductive age and chronically exposed to NPs should be evaluated before initiation of pregnancy.

Increasing evidences have been accumulated indicating that some, but not all, ENPs may affect reproduction and development. The high number of variables characterizing the available data, for example, route of exposure, dose, size, make it difficult to extrapolate results. Future research ought to more systematically address reproductive and developmental toxicity of nanomaterials. A multidisciplinary approach, in which chemists, toxicologists, and embryologists/developmental biologist work together, should be preferred. An essential goal of research in reproductive and developmental toxicity of ENPs must be to (1) design studies that best represent human scenarios in terms of, for example, route of exposure, dosage, duration; (2) to identify most sensitive endpoints; (3) to deduce general rules as to regarding, for example, size and surface area, surface chemistry, core material, and route of exposure; (4) to include long term follow up studies to reveal subtle effects, which may manifest in the offspring only later in life.

Increased knowledge of potential mechanisms of toxicity is needed. It is especially important to clarify whether the mere presence of particles interferes with reproductive tissue and tissues undergoing development. Furthermore, there is a lack of in vivo studies investigating several dose levels and comparing nanosized particles with microsized particles.

One of the main topics that needs to be addressed when assessing the toxic potential of NPs is reproduction. Very little work has been done into particle effects on female reproductive capacity, and most of the information has been obtained in vitro. The two in vivo studies indicate that female fertility is susceptible to particle exposure. For the male reproductive system, concerns relate to the ability to bypass the BTB and accumulate within the seminiferous epithelium, and affect spermatogenesis, either directly or indirectly. It is also imperative to establish if the effects are permanent or can be reverted with time, and if toxicants affect germ line cells through inheritable mutations or subtle epigenetic modifications (e.g., DNA methylation) (Singer and Yauk, 2010).

Finally, important data could be provided without much effort, if reporting included information on common maternal and gestational measures (e.g., maternal weight gain, litter size, birth weights), and if study designs adhered to commonly accepted practices in reproductive and developmental toxicity, for example, by consulting guidelines before initiating experiments (Hougaard et al., 2015).

TAKE-HOME MESSAGES

- Over the last years, data have been accumulated suggesting that ENPs may adversely affect mammalian reproduction. While few data showing reversible detrimental effects of NPs on male reproductive function exist, very little is yet known about the female.
- The possibility that ENPs might adversely affect embryonic development appears real; however, the different chemical composition, the route of administration and the dose appear to play a major role in driving toxicity.
- Although the number of in vivo studies in mammalian species have greatly increased, the mechanisms driving reproductive and developmental toxicity of NPs are still poorly understood.

References

Afifi, M., Almaghrabi, O.A., Kadasa, N.M., 2015. Ameliorative effect of zinc oxide nanoparticles on antioxidants and sperm characteristics in streptozotocin-induced diabetic rat testes. Biomed. Res. Int. 2015, 153573.

Aitken, R.J., Jones, K.T., Robertson, S.A., 2012. Reactive oxygen species and sperm function—in sickness and in health. J. Androl. 33, 1096–1106.

Austin, C.A., Umbreit, T.H., Brown, K.M., Barber, D.S., Dair, B.J., Francke-Carroll, S., et al., 2012. Distribution of silver nanoparticles in pregnant mice and developing embryos. Nanotoxicology 6, 912–922.

Austin, C.A., Hinkley, G.K., Mishra, A.R., Zhang, Q., Umbreit, T.H., Betz, M.W., et al., 2015. Distribution and accumulation of 10 nm silver nanoparticles in maternal tissues and visceral yolk sac of pregnant mice, and a potential effect on embryo growth. Nanotoxicology 10, 1–8.

Backes, C.H., Nelin, T., Gorr, M.W., Wold, L.E., 2013. Early life exposure to air pollution: how bad is it? Toxicol. Lett. 216, 47–53.

Bai, Y., Zhang, Y., Zhang, J., Mu, Q., Zhang, W., Butch, E.R., et al., 2010. Repeated administrations of carbon nanotubes in male mice cause reversible testis damage without affecting fertility. Nat. Nanotechnol. 5, 683–689.

Bal, R., Turk, G., Tuzcu, M., Yilmaz, O., Ozercan, I., Kuloglu, T., et al., 2011. Protective effects of nanostructures of hydrated C(60) fullerene on reproductive function in streptozotocin-diabetic male rats. Toxicology 282, 69–81.

Balasubramanian, S.K., Jittiwat, J., Manikandan, J., Ong, C.N., Yu, L.E., Ong, W.Y., 2010. Biodistribution of gold nanoparticles and gene expression changes in the liver and spleen after intravenous administration in rats. Biomaterials 31, 2034–2042.

Bar-Ilan, O., Albrecht, R.M., Fako, V.E., Furgeson, D.Y., 2009. Toxicity assessments of multisized gold and silver nanoparticles in zebrafish embryos. Small 5, 1897–1910.

Bhabra, G., Sood, A., Fisher, B., Cartwright, L., Saunders, M., Evans, W.H., et al., 2009. Nanoparticles can cause DNA damage across a cellular barrier. Nat. Nanotechnol. 4, 876–883.

Blum, J.L., Xiong, J.Q., Hoffman, C., Zelikoff, J.T., 2012. Cadmium associated with inhaled cadmium oxide nanoparticles impacts fetal and neonatal development and growth. Toxicol. Sci. 26, 478–486.

Blum, J.L., Edwards, J.R., Prozialeck, W.C., Xiong, J.Q., Zelikoff, J.T., 2015. Effects of maternal exposure to cadmium oxide nanoparticles during pregnancy on maternal and offspring kidney injury markers using a murine model. J. Toxicol. Environ.Health A 78, 711–724.

Boisen, A.M., Shipley, T., Jackson, P., Wallin, H., Nellemann, C., Vogel, U., et al., 2013. In utero exposure to nanosized carbon black (Printex90) does not induce tandem repeat mutations in female murine germ cells. Reprod.Toxicol. 41, 45–48.

Bosman, S.J., Nieto, S.P., Patton, W.C., Jacobson, J.D., Corselli, J.U., Chan, P.J., 2005. Development of mammalian embryos exposed to mixed-size nanoparticles. Clin. Exp. Obstet. Gynecol. 32, 222–224.
Braydich-Stolle, L., Hussain, S., Schlager, J.J., Hofmann, M.C., 2005. In vitro cytotoxicity of nanoparticles in mammalian germline stem cells. Toxicol. Sci. 88, 412–419.
Browning, L.M., Lee, K.J., Huang, T., Nallathamby, P.D., Lowman, J.E., Xu, X.H., 2009. Random walk of single gold nanoparticles in zebrafish embryos leading to stochastic toxic effects on embryonic developments. Nanoscale 1, 138–152.
Buerki-Thurnherr, T., von, M.U., Wick, P., 2012. Knocking at the door of the unborn child: engineered nanoparticles at the human placental barrier. Swiss. Med. Wkly. 142, w13559.
Campagnolo, L., Massimiani, M., Magrini, A., Camaioni, A., Pietroiusti, A., 2012. Physico-chemical properties mediating reproductive and developmental toxicity of engineered nanomaterials. Curr. Med. Chem. 19 (26), 4488–4494.
Campagnolo, L., Fenoglio, I., Massimiani, M., Magrini, A., Pietroiusti, A., 2013a. Screening of nanoparticle embryotoxicity using embryonic stem cells. Methods Mol. Biol. 1058, 49–60.
Campagnolo, L., Massimiani, M., Palmieri, G., Bernardini, R., Sacchetti, C., Bergamaschi, A., et al., 2013b. Biodistribution and toxicity of pegylated single wall carbon nanotubes in pregnant mice. Part. Fibre. Toxicol. 10, 21.
Castellini, C., Ruggeri, S., Mattioli, S., Bernardini, G., Macchioni, L., Moretti, E., et al., 2014. Long-term effects of silver nanoparticles on reproductive activity of rabbit buck. Syst. Biol. Reprod. Med. 60, 143–150.
Cheng, J., Chan, C.M., Veca, L.M., Poon, W.L., Chan, P.K., Qu, L., et al., 2009. Acute and long-term effects after single loading of functionalized multi-walled carbon nanotubes into zebrafish (Danio rerio). Toxicol. Appl. Pharmacol. 235, 216–225.
Choi, M., Cho, M., Han, B.S., Hong, J., Jeong, J., Park, S., et al., 2010. Chitosan nanoparticles show rapid extrapulmonary tissue distribution and excretion with mild pulmonary inflammation to mice. Toxicol. Lett. 199, 144–152.
Chu, M., Wu, Q., Yang, H., Yuan, R., Hou, S., Yang, Y., et al., 2010. Transfer of quantum dots from pregnant mice to pups across the placental barrier. Small 6, 670–678.
Courbiere, B., Auffan, M., Rollais, R., Tassistro, V., Bonnefoy, A., Botta, A., et al., 2013. Ultrastructural interactions and genotoxicity assay of cerium dioxide nanoparticles on mouse oocytes. Int. J. Mol. Sci. 14, 21613–21628.
Dadvand, P., Parker, J., Bell, M.L., Bonzini, M., Brauer, M., Darrow, L.A., et al., 2013. Maternal exposure to particulate air pollution and term birth weight: a multi-country evaluation of effect and heterogeneity. Environ. Health Perspect. 121, 267–373.
de Jong, W.H., Hagens, W.I., Krystek, P., Burger, M.C., Sips, A.J., Geertsma, R.E., 2008. Particle size-dependent organ distribution of gold nanoparticles after intravenous administration. Biomaterials 29, 1912–1919.
Di, G.C., Lopez, D.R., De Lapuente, J., Mallafre, J.M., Suarez, M.B., 2010. Embryotoxicity of cobalt ferrite and gold nanoparticles: a first in vitro approach. Reprod. Toxicol. 30, 271–276.
El-Sayed, Y.S., Shimizu, R., Onoda, A., Takeda, K., Umezawa, M., 2015. Carbon black nanoparticle exposure during middle and late fetal development induces immune activation in male offspring mice. Toxicology 327, 53–61.
Ema, M., Naya, M., Horimoto, M., Kato, H., 2013. Developmental toxicity of diesel exhaust: a review of studies in experimental animals. Reprod. Toxicol. 42, 1–17.
Ema, M., Hougaard, K.S., Kishimoto, A., Honda, K., 2015. Reproductive and developmental toxicity of carbon-based nanomaterials: A literature review. Nanotoxicology, 1–22.
European Agency for Safety and Health at Work, 2003. Gender Issues in Safety and Health at Work. A Review. In: Kauppinen, K., Kumpulainen, R., Houtman, I., Copsey, S. (Eds.), Office for Official Publications of the European Communities, Luxemburg.
Fan, Y.O., Zhang, Y.H., Zhang, X.P., Liu, B., Ma, Y.X., Jin, Y.H., 2006. Comparative study of nanosized and microsized silicon dioxide on spermatogenesis function of male rats. Wei Sheng Yan Jiu 35, 549–553.
Farcal, L., Torres, A.F., Di, C.L., Rotoli, B.M., Bussolati, O., Bergamaschi, E., et al., 2015. Comprehensive in vitro toxicity testing of a panel of representative oxide nanomaterials: first steps towards an intelligent testing strategy. PLoS One 10, e0127174.
Fedulov, A.V., Leme, A., Yang, Z., Dahl, M., Lim, R., Mariani, T.J., et al., 2008. Pulmonary exposure to particles during pregnancy causes increased neonatal asthma susceptibility. Am. J. Respir. Cell. Mol. Biol. 38, 57–67.
Gao, X., Yin, S., Tang, M., Chen, J., Yang, Z., Zhang, W., et al., 2011. Effects of developmental exposure to TiO(2) nanoparticles on synaptic plasticity in hippocampal dentate gyrus area: an in vivo study in anesthetized rats. Biol. Trace Elem. Res. 143, 1616–1628.
Gupta, S.K., Baweja, L., Gurbani, D., Pandey, A.K., Dhawan, A., 2011. Interaction of C60 fullerene with the proteins involved in DNA mismatch repair pathway. J. Biomed. Nanotechnol. 7, 179–180.

Han, J.W., Jeong, J.K., Gurunathan, S., Choi, Y.J., Das, J., Kwon, D.N., et al., 2015. Male- and female-derived somatic and germ cell-specific toxicity of silver nanoparticles in mouse. Nanotoxicology, 1–13.
Hemmingsen, J.G., Hougaard, K.S., Talsness, C., Wellejus, A., Loft, S., Wallin, H., et al., 2009. Prenatal exposure to diesel exhaust particles and effect on the male reproductive system in mice. Toxicology 264, 61–68.
Hong, J.S., Park, M.K., Kim, M.S., Lim, J.H., Park, G.J., Maeng, E.H., et al., 2014a. Prenatal development toxicity study of zinc oxide nanoparticles in rats. Int. J. Nanomedicine 9 (Suppl. 2), 159–171.
Hong, J.S., Park, M.K., Kim, M.S., Lim, J.H., Park, G.J., Maeng, E.H., et al., 2014b. Effect of zinc oxide nanoparticles on dams and embryo-fetal development in rats. Int. J. Nanomedicine 9 (Suppl. 2), 145–157.
Hougaard, K.S., Jensen, K.A., Nordly, P., Taxvig, C., Vogel, U., Saber, A.T., et al., 2008. Effects of prenatal exposure to diesel exhaust particles on postnatal development, behavior, genotoxicity and inflammation in mice. Part. Fibre Toxicol. 5, 3.
Hougaard, K.S., Jackson, P., Jensen, K.A., Saber, A.T., Vogel, U., Wallin, H., 2010a. Carbon nano-tubes: effects on gestation and reproduction. Reprod. Toxicol. 30, 239.
Hougaard, K.S., Jackson, P., Jensen, K.A., Sloth, J.J., Loschner, K., Larsen, E.H., et al., 2010b. Effects of prenatal exposure to surface-coated nanosized titanium dioxide (UV-Titan). A study in mice. Part. Fibre Toxicol. 7, 16.
Hougaard, K.S., Fadeel, B., Gulumian, M., Kagan, V.E., Savolainen, K., 2011. Developmental toxicity of engineered nanoparticles. In: Gupta, R. (Ed.), Reproductive and Developmental Toxicology. Academic Press/Elsevier, San Diego, pp. 269–290.
Hougaard, K.S., Jackson, P., Kyjovska, Z.O., Birkedal, R.K., De Temmerman, P.J., Brunelli, A., et al., 2013. Effects of lung exposure to carbon nanotubes on female fertility and pregnancy. A study in mice. Reprod. Toxicol. 41, 86–97.
Hougaard, K.S., Campagnolo, L., Chavatte-Palmer, P., Tarrade, A., Rousseau-Ralliard, D., Valentino, S., et al., 2015. A perspective on the developmental toxicity of inhaled nanoparticles. Reprod. Toxicol 56, 118–140.
Iavicoli, I., Fontana, L., Leso, V., Bergamaschi, A., 2013. The effects of nanomaterials as endocrine disruptors. Int. J. Mol. Sci. 14, 16732–16801.
Jackson, P., Hougaard, K.S., Boisen, A.M., Jacobsen, N.R., Jensen, K.A., Moller, P., et al., 2011. Pulmonary exposure to carbon black by inhalation or instillation in pregnant mice: effects on liver DNA strand breaks in dams and offspring. Nanotoxicology 6, 484–500.
Jackson, P., Hougaard, K.S., Vogel, U., Wu, D., Casavant, L., Williams, A., et al., 2012. Exposure of pregnant mice to carbon black by intratracheal instillation: toxicogenomic effects in dams and offspring. Mutat. Res. 745, 73–83.
Jackson, P., Halappanavar, S., Hougaard, K.S., Williams, A., Madsen, A.M., Lamson, J.S., et al., 2013. Maternal inhalation of surface-coated nanosized titanium dioxide (UV-Titan) in C57BL/6 mice: effects in prenatally exposed offspring on hepatic DNA damage and gene expression. Nanotoxicology 7, 85–96.
Kim, J.S., Yoon, T.J., Yu, K.N., Kim, B.G., Park, S.J., Kim, H.W., et al., 2006. Toxicity and tissue distribution of magnetic nanoparticles in mice. Toxicol. Sci. 89, 338–347.
Klein, J.P., Boudard, D., Cadusseau, J., Palle, S., Forest, V., Pourchez, J., et al., 2013. Testicular biodistribution of 450 nm fluorescent latex particles after intramuscular injection in mice. Biomed. Microdevices 15, 427–436.
Kobyliak, N.M., Falalyeyeva, T.M., Kuryk, O.G., Beregova, T.V., Bodnar, P.M., Zholobak, N.M., et al., 2015. Antioxidative effects of cerium dioxide nanoparticles ameliorate age-related male infertility: optimistic results in rats and the review of clinical clues for integrative concept of men health and fertility. EPMA J. 6, 12.
Komatsu, T., Tabata, M., Kubo-Irie, M., Shimizu, T., Suzuki, K., Nihei, Y., et al., 2008. The effects of nanoparticles on mouse testis Leydig cells in vitro. Toxicol. In Vitro 22, 1825–1831.
Krug, H.F., 2014. Nanosafety research—are we on the right track? Angew. Chem. Int. Ed. Engl. 53, 12304–12319.
Kwon, J.T., Hwang, S.K., Jin, H., Kim, D.S., Minai-Tehrani, A., Yoon, H.J., et al., 2008. Body distribution of inhaled fluorescent magnetic nanoparticles in the mice. J. Occup. Health. 50, 1–6.
Kyjovska, Z.O., Boisen, A.M., Jackson, P., Wallin, H., Vogel, U., Hougaard, K.S., 2013. Daily sperm production: application in studies of prenatal exposure to nanoparticles in mice. Reprod. Toxicol. 36, 88–97.
Lamoureux, D.P., Kobzik, L., Fedulov, A.V., 2010. Customized PCR-array analysis informed by gene-chip microarray and biological hypothesis reveals pathways involved in lung inflammatory response to titanium dioxide in pregnancy. J. Toxicol. Environ. Health A 73, 596–606.
Lan, Z., Yang, W.X., 2012. Nanoparticles and spermatogenesis: how do nanoparticles affect spermatogenesis and penetrate the blood-testis barrier. Nanomedicine 7, 579–596.
Lawson, C.C., Schnorr, T.M., Daston, G.P., Grajewski, B., Marcus, M., McDiarmid, M., et al., 2003. An occupational reproductive research agenda for the third millennium. Environ. Health Perspect. 111, 584–592.
Lee, Y., Choi, J., Kim, P., Choi, K., Kim, S., Shon, W., et al., 2012. A transfer of silver nanoparticles from pregnant rat to offspring. Toxicol. Res. 28, 139–141.

Li, P.W., Kuo, T.H., Chang, J.H., Yeh, J.M., Chan, W.H., 2010. Induction of cytotoxicity and apoptosis in mouse blastocysts by silver nanoparticles. Toxicol. Lett. 197, 82–87.
Lim, J.H., Kim, S.H., Shin, I.S., Park, N.H., Moon, C., Kang, S.S., et al., 2011. Maternal exposure to multi-wall carbon nanotubes does not induce embryo-fetal developmental toxicity in rats. Birth Defects Res. B Dev. Reprod. Toxicol. 92, 69–76.
Liu, X., Qin, D., Cui, Y., Chen, L., Li, H., Chen, Z., et al., 2010. The effect of calcium phosphate nanoparticles on hormone production and apoptosis in human granulosa cells. Reprod. Biol. Endocrinol. 8, 32.
Lucas, B., Fields, C., Hofmann, M.C., 2009. Signaling pathways in spermatogonial stem cells and their disruption by toxicants. Birth Defects Res. C. Embryo. Today 87, 35–42.
Makhluf, S.B., bu-Mukh, R., Rubinstein, S., Breitbart, H., Gedanken, A, 2008. Modified PVA-Fe3O4 nanoparticles as protein carriers into sperm cells. Small 4, 1453–1458.
Melnik, E.A., Buzulukov, Y.P., Demin, V.F., Demin, V.A., Gmoshinski, I.V., Tyshko, N.V., Tutelyan, V.A., 2013. Transfer of silver nanoparticles through the placenta and breast milk during in vivo experiments on rats. Acta. Naturae. 5 (3), 107–115.
Moretti, E., Terzuoli, G., Renieri, T., Iacoponi, F., Castellini, C., Giordano, C., et al., 2013. In vitro effect of gold and silver nanoparticles on human spermatozoa. Andrologia 45, 392–396.
Morgane, P.J., Austin-LaFrance, R.J., Bronzino, J.D., Tonkiss, J., Galler, J.R., 1992. Malnutrition and the developing central nervous system. In: Isaacson, R.L., Jensen, K.F. (Eds.), The Vulnerable Brain and Environmental Risks. Plenum Press, New York, pp. 3–44.
Morishita, Y., Yoshioka, Y., Satoh, H., Nojiri, N., Nagano, K., Abe, Y., et al., 2012. Distribution and histologic effects of intravenously administered amorphous nanosilica particles in the testes of mice. Biochem. Biophys. Res. Commun. 420, 297–301.
Myllynen, P.K., Loughran, M.J., Howard, C.V., Sormunen, R., Walsh, A.A., Vahakangas, K.H., 2008. Kinetics of gold nanoparticles in the human placenta. Reprod. Toxicol. 26, 130–137.
Nel, A., Xia, T., Madler, L., Li, N., 2006. Toxic potential of materials at the nanolevel. Science 311, 622–627.
Park, M.V., Annema, W., Salvati, A., Lesniak, A., Elsaesser, A., Barnes, C., et al., 2009. In vitro developmental toxicity test detects inhibition of stem cell differentiation by silica nanoparticles. Toxicol. Appl. Pharmacol. 240, 108–116.
Philbrook, N.A., Winn, L.M., Afrooz, A.R., Saleh, N.B., Walker, V.K., 2011. The effect of TiO(2) and Ag nanoparticles on reproduction and development of Drosophila melanogaster and CD-1 mice. Toxicol. Appl. Pharmacol 257, 429–436.
Pietroiusti, A., Massimiani, M., Fenoglio, I., Colonna, M., Valentini, F., Palleschi, G., et al., 2011. Low doses of pristine and oxidized single-wall carbon nanotubes affect mammalian embryonic development. ACS Nano 5, 4624–4633.
Pietroiusti, A., Campagnolo, L., Fadeel, B., 2013. Interactions of engineered nanoparticles with organs protected by internal biological barriers. Small 9, 1557–1572.
Powers, C.M., Badireddy, A.R., Ryde, I.T., Seidler, F.J., Slotkin, T.A., 2011. Silver nanoparticles compromise neurodevelopment in PC12 cells: critical contributions of silver ion, particle size, coating, and composition. Environ. Health Perspect. 119, 37–44.
Pryor, J.L., Hughes, C., Foster, W., Hales, B.F., Robaire, B., 2000. Critical windows of exposure for children's health: the reproductive system in animals and humans. Environ. Health Perspect. 108 (Suppl. 3), 491–503.
Qi, W., Bi, J., Zhang, X., Wang, J., Wang, J., Liu, P., et al., 2014. Damaging effects of multi-walled carbon nanotubes on pregnant mice with different pregnancy times. Sci. Rep. 4, 4352.
Rattanapinyopituk, K., Shimada, A., Morita, T., Sakurai, M., Asano, A., Hasegawa, T., et al., 2014. Demonstration of the clathrin- and caveolin-mediated endocytosis at the maternal-fetal barrier in mouse placenta after intravenous administration of gold nanoparticles. J. Vet. Med. Sci. 76, 377–387.
Ritz, C., Ruminski, W., Hougaard, K.S., Wallin, H., Vogel, U., Yauk, C.L., 2011. Germline mutation rates in mice following in utero exposure to diesel exhaust particles by maternal inhalation. Mutat. Res. 712, 55–58.
Sadauskas, E., Wallin, H., Stoltenberg, M., Vogel, U., Doering, P., Larsen, A., et al., 2007. Kupffer cells are central in the removal of nanoparticles from the organism. Part. Fibre Toxicol. 4, 10.
Scientific Committee on Occupational Exposure Limits (SCOEL), 2009. Methodology for the Derivation of Occupational Exposure Limits: Key Documentation (Version 6). European Commission, Bruxelles.
Semmler-Behnke, M., Lipka, J., Wenk, A., Hirn, S., Schaffler, M., Tian, F., et al., 2014. Size dependent translocation and fetal accumulation of gold nanoparticles from maternal blood in the rat. Part. Fibre Toxicol. 11, 33.
Shah, P.S., Balkhair, T., 2011. Air pollution and birth outcomes: a systematic review. Environ. Int. 37, 498–516.
Simko, M., Mattsson, M.O., 2010. Risks from accidental exposures to engineered nanoparticles and neurological health effects: a critical review. Part. Fibre Toxicol. 7, 42.

Singer, T.M., Yauk, C.L., 2010. Germ cell mutagens: risk assessment challenges in the 21st century. Environ. Mol. Mutagen. 51, 919–928.
Srdjenovic, B., Milic-Torres, V., Grujic, N., Stankov, K., Djordjevic, A., Vasovic, V., 2010. Antioxidant properties of fullerenol C60(OH)24 in rat kidneys, testes, and lungs treated with doxorubicin. Toxicol. Mech. Methods 20, 298–305.
Stelzer, R., Hutz, R.J., 2009. Gold nanoparticles enter rat ovarian granulosa cells and subcellular organelles, and alter in-vitro estrogen accumulation. J. Reprod. Dev. 55, 685–690.
Takahashi, S., Matsuoka, O., 1981. Cross placental transfer of 198Au-colloid in near term rats. J. Radiat. Res. 22, 242–249.
Takeda, K., Suzuki, K., Ishihara, A., Kubo-Irie, M., Fujimoto, R., Tabata, M., et al., 2009. Nanoparticles transferred from pregnant mice to their offspring can damage the genital and cranial nerve systems. J. Health Sci. 55, 95–102.
Tassinari, R., Cubadda, F., Moracci, G., Aureli, F., D'Amato, M., Valeri, M., et al., 2014. Oral, short-term exposure to titanium dioxide nanoparticles in Sprague-Dawley rat: focus on reproductive and endocrine systems and spleen. Nanotoxicology 8, 654–662.
Taylor, U., Garrels, W., Barchanski, A., Peterson, S., Sajti, L., Lucas-Hahn, A., et al., 2014. Injection of ligand-free gold and silver nanoparticles into murine embryos does not impact pre-implantation development. Beilstein J. Nanotechnol. 5, 677–688.
Thompson, J., Bannigan, J., 2008. Cadmium: toxic effects on the reproductive system and the embryo. Reprod. Toxicol. 25, 304–315.
Tian, F., Razansky, D., Estrada, G.G., Semmler-Behnke, M., Beyerle, A., Kreyling, W., et al., 2009. Surface modification and size dependence in particle translocation during early embryonic development. Inhal. Toxicol. 21, 92–96.
Tian, X., Zhu, M., Du, L., Wang, J., Fan, Z., Liu, J., et al., 2013. Intrauterine inflammation increases materno-fetal transfer of gold nanoparticles in a size-dependent manner in murine pregnancy. Small 9, 2432–2439.
Tiedemann, D., Taylor, U., Rehbock, C., Jakobi, J., Klein, S., Kues, W.A., et al., 2014. Reprotoxicity of gold, silver, and gold-silver alloy nanoparticles on mammalian gametes. Analyst 139, 931–942.
Tijsterman, M., Pothof, J., Plasterk, R.H., 2002. Frequent germline mutations and somatic repeat instability in DNA mismatch-repair-deficient Caenorhabditis elegans. Genetics 161, 651–660.
Tran, D.N., Ota, L.C., Jacobson, J.D., Patton, W.C., Chan, P.J., 2007. Influence of nanoparticles on morphological differentiation of mouse embryonic stem cells. Fertil. Steril. 87, 965–970.
Tsuchiya, T., Oguri, I., Yamakoshi, Y.N., Miyata, N., 1996. Novel harmful effects of [60] fullerene on mouse embryos in vitro and in vivo. FEBS Lett. 393, 139–145.
United States Environmental Protection Agency, 1991. Guidelines for developmental toxicity risk assessment. Fed. Regist. 56, 63798–63826.
Usenko, C.Y., Harper, S.L., Tanguay, R.L., 2007. In vivo evaluation of carbon fullerene toxicity using embryonic zebrafish. Carbon NY 45, 1891–1898.
Vance, M.E., Kuiken, T., Vejerano, E.P., McGinnis, S.P., Hochella, Jr., M.F., Rejeski, D., et al., 2015. Nanotechnology in the real world: Redeveloping the nanomaterial consumer products inventory. Beilstein J. Nanotechnol. 6, 1769–1780.
Veras, M.M., eno-Rodrigues, N.R., Guimaraes Silva, R.M., Scoriza, J.N., Saldiva, P.H., Caldini, E.G., et al., 2009. Chronic exposure to fine particulate matter emitted by traffic affects reproductive and fetal outcomes in mice. Environ. Res. 109, 536–543.
Wang, Y., Seebald, J.L., Szeto, D.P., Irudayaraj, J., 2010. Biocompatibility and biodistribution of surface-enhanced Raman scattering nanoprobes in zebrafish embryos: in vivo and multiplex imaging. ACS Nano 4, 4039–4053.
Warheit, D.B., Boatman, R., Brown, S.C., 2015. Developmental toxicity studies with 6 forms of titanium dioxide test materials (3 pigment-different grade & 3 nanoscale) demonstrate an absence of effects in orally-exposed rats. Regul. Toxicol. Pharmacol. 73, 887–896.
Wells, P.G., McCallum, G.P., Chen, C.S., Henderson, J.T., Lee, C.J., Perstin, J., et al., 2009. Oxidative stress in developmental origins of disease: teratogenesis, neurodevelopmental deficits, and cancer. Toxicol. Sci. 108, 4–18.
Wick, P., Malek, A., Manser, P., Meili, D., Maeder-Althaus, X., Diener, L., et al., 2010. Barrier capacity of human placenta for nanosized materials. Environ. Health Perspect. 118, 432–436.
Wiwanitkit, V., Sereemaspun, A., Rojanathanes, R., 2009. Effect of gold nanoparticles on spermatozoa: the first world report. Fertil. Steril. 91, e7–e8.
Xu, Y., Wang, N., Yu, Y., Li, Y., Li, Y.B., Yu, Y.B., et al., 2014. Exposure to silica nanoparticles causes reversible damage of the spermatogenic process in mice. PLoS One 9, e101572.

Yadav, B.C., Kumar, R., 2008. Structure, properties and applications of fullerenes. Int. J. Nanotech. Appl. 2, 15–24.
Yamashita, K., Yoshioka, Y., Higashisaka, K., Mimura, K., Morishita, Y., Nozaki, M., et al., 2011. Silica and titanium dioxide nanoparticles cause pregnancy complications in mice. Nat. Nanotechnol. 6, 321–328.
Yang, H., Sun, C., Fan, Z., Tian, X., Yan, L., Du, L., et al., 2012. Effects of gestational age and surface modification on materno-fetal transfer of nanoparticles in murine pregnancy. Sci. Rep. 2, 847.
Yauk, C., Polyzos, A., Rowan-Carroll, A., Somers, C.M., Godschalk, R.W., Van Schooten, F.J., et al., 2008. Germ-line mutations, DNA damage, and global hypermethylation in mice exposed to particulate air pollution in an urban/industrial location. Proc. Natl. Acad. Sci. USA 105, 605–610.
Yoshida, S., Sagai, M., Oshio, S., Umeda, T., Ihara, T., Sugamata, M., et al., 1999. Exposure to diesel exhaust affects the male reproductive system of mice. Int. J. Androl. 22, 307–315.
Yoshida, S., Hiyoshi, K., Ichinose, T., Takano, H., Oshio, S., Sugawara, I., et al., 2009a. Effect of nanoparticles on the male reproductive system of mice. Int. J. Androl. 32, 337–342.
Yoshida, S., Hiyoshi, K., Oshio, S., Takano, H., Takeda, K., Ichinose, T., 2009b. Effects of fetal exposure to carbon nanoparticles on reproductive function in male offspring. Fertil. Steril. 93, 1695–1699.
Yu, W.J., Son, J.M., Lee, J., Kim, S.H., Lee, I.C., Baek, H.S., et al., 2013. Effects of silver nanoparticles on pregnant dams and embryo-fetal development in rats. Nanotoxicology 8, 85–91.
Zhang, H., Yang, D., Yang, H., Liu, H., 2008. Effect on conception and offspring development in female prenatal rats following intratracheal instillation of nano-C/ZnO and C-ZnO composite nanoparticles. Wei Sheng Yan Jiu 37, 654–656.
Zhu, X., Zhu, L., Li, Y., Duan, Z., Chen, W., Alvarez, P.J., 2007. Developmental toxicity in zebrafish (Danio rerio) embryos after exposure to manufactured nanomaterials: buckminsterfullerene aggregates (nC60) and fullerol. Environ. Toxicol. Chem. 26, 976–979.

CHAPTER

18

Genotoxicity and Cancer

*Shareen H. Doak**, *Ying Liu***, *Chunying Chen****

***Institute of Life Science, Swansea University Medical School, Swansea, Wales, United Kingdom; **National Centre for Nanoscience and Technology, Beijing, China**

OUTLINE

Introduction 423

Primary Mechanisms for Genotoxicity 424

Secondary Mechanisms for Genotoxicity 426

From Genotoxicity to Cancer 427

Carbon–Based Nanomaterials 428

Fullerene (C_{60}) and its Derivatives 428

Carbon Nanotubes (CNTs) 433

Graphene and Graphene Oxide 434

Other Carbon-Based Materials 435

Metallic Nanoparticles 436

Metal Oxides 437

Conclusions and Future Directions 439

References 440

INTRODUCTION

Genetic toxicology is the study of chemical and physical perturbations in DNA following exposure to endogenous or exogenous agents. The damage may consist of small lesions arising at very specific sites within the DNA molecule, such as DNA adducts, DNA cross-links, abasic sites, and point mutations. Alternatively, gross abnormalities can arise at the chromosomal level including aneuploidy (alterations in chromosome copy number), clastogenicity (chromosome fragmentation), and structural chromosomal rearrangements. The type of DNA damage induced by a genotoxic substance is dependent upon their mechanism of action. This has been well characterized for many chemical or pharmaceutical products humans may be exposed to, for example, SN2 alkylating agents have a tendency to target highly nucleophilic centers within the DNA macromolecule and therefore largely induce DNA alkyl-adducts at

Adverse Effects of Engineered Nanomaterials. http://dx.doi.org/10.1016/B978-0-12-809199-9.00018-5
Copyright © 2017 Elsevier Inc. All rights reserved.

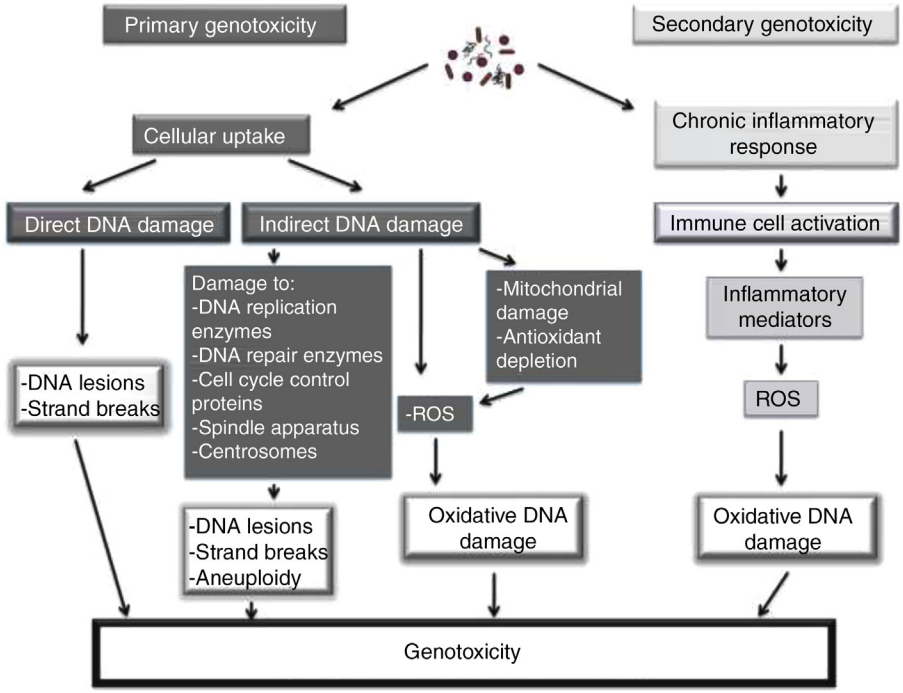


FIGURE 18.1 **Mechanisms of nanomaterial-induced DNA damage (genotoxicity).**

sites, such as the N7 position in guanine bases or the N3 position in adenine (Doak et al., 2007; Jenkins et al., 2005). However, for nanomaterials this level of understanding is lacking at present.

The mechanisms likely to be responsible for nanomaterial induced genotoxicity fall into two main categories, namely primary and secondary mechanisms, the former of which can be further subdivided into agents which act directly and indirectly to trigger DNA damage (Fig. 18.1).

In this chapter, we discuss mechanisms of genotoxicity, the progression from genotoxicity to cancer (tumorigenesis), and the potential of different classes of nanomaterials to elicit genotoxicity.

PRIMARY MECHANISMS FOR GENOTOXICITY

Primary mechanisms for genotoxicity are those that are imparted by the nanomaterials themselves at the level of the single cell. It requires cellular internalization of the nanomaterials and often their subsequent interaction with biomolecules.

Direct acting agents are those that are DNA reactive, that is, they come into direct contact with the genetic material causing physical or chemical damage. This might include the formation of DNA lesions at specific sites within the DNA molecule, which can lead to mutagenesis

as they may destabilize the nucleotide they damage, resulting in its loss and therefore an abasic site that are often subject to error prone repair. Alternatively, during replication the damaged base may not be recognized by the polymerase enzymes, which results in a random selection of nucleotides placed opposite to enable duplication of the genetic material to continue, thus leading to point mutations or strand breakages (Doak et al., 2007; Jenkins et al., 2005). Direct acting agents may also have the capacity to intercalate into the DNA double helix creating a structure that stalls DNA replication either leading to strand breaks or the insertion of additional nucleotides (Ferguson and Denny, 2007). Alternatively, these agents may cause physical damage to the DNA in the form of single- or double-strand breaks. Direct DNA damage can therefore only be induced if the nanomaterials are able to penetrate the cell nucleus, or those free in the cytoplasm (i.e., not contained within membrane bound vesicles) may have the opportunity to come into direct contact with DNA during mitosis when the nuclear membrane breaks down. Specific targeting of nanomaterials to the cell nucleus can be achieved by modifying their surfaces with nuclear localizing signals (Berry et al., 2007; Nativo et al., 2008). Nonetheless, most nanomaterials associated with human exposure would not have such surface functionalization and thus their uptake into the nucleus would be substantially limited, but a number of studies have observed that some nanomaterials including titanium dioxide (TiO_2), zinc oxide (ZnO), quantum dots, and silica are able to enter the nuclear region in the absence of targeting moieties (Chen and von Mikecz, 2005; Hackenberg et al., 2010, 2011; Nabiev et al., 2007; Shukla et al., 2011). However, it has yet to be established whether or not nanomaterials directly interact with DNA to induce damage.

Indirect acting agents are those that induce genetic damage via intermediate biomolecules, which are often components of the cell division machinery. DNA replication and cell division is a complex multifactorial process that involves a large number of proteins, which are potential targets for indirect acting genotoxins. For example, damage may occur to proteins required for the mechanical segregation of chromosomes (including the centrioles, microtubules, kinetochore structures on the chromosomes); enzymes involved in replication of the DNA (such as polymerases, topoisomerases); DNA repair enzymes or signal transduction proteins involved cell cycle checkpoints that monitor the chromosome replication and segregation process (Jenkins et al., 2005). Consequently, damage to such components often results in structural or numerical chromosomal aberrations. Aside from interfering with the DNA replication process, indirect DNA damage may arise as a result of the induction of oxidative stress. A normal by-product of aerobic metabolism is reactive oxygen species (ROS), which readily oxidize biomolecules, such as proteins, lipids, and DNA. To prevent oxidative damage, cellular antioxidants neutralize ROS but if there is an imbalance between the generation of ROS and the level of antioxidants within a cell, then the result is oxidative stress. This imbalance could be caused by enhanced generation of ROS after exposure to an exogenous agent or depletion of cellular antioxidants, the result of which can be the formation of oxidative DNA lesions that are subsequently fixed as mutations (Cooke et al., 2003). Currently, there is more evidence to support an indirect mode of action for nanomaterials with regards to DNA damage. For example, single-walled carbon nanotubes (SWCNTs) have been found to mechanically interfere with the spindle apparatus, culminating in centrosome fragmentation and multipolar mitotic spindles, which were associated with aneuploidy (Sargent et al., 2009). Nanosilica is able to induce nuclear protein aggregation, which is of concern as some of these proteins include enzymes important in DNA

replication processes, particularly topoisomearase I (Chen and von Mikecz, 2005). However, most studies have demonstrated that oxidative stress is a central cause for indirect DNA damage, with in vivo studies detecting oxidative DNA lesions (8-hydroxy-deoxyguanosine; 8-OHdG) in the liver and lungs of rodents following oral administration of nanoparticles (Folkmann et al., 2009; Trouiller et al., 2009). Furthermore, several reports have now shown that DNA damage can be reduced or prevented by preexposure of the human test cells to *N*-acetylcystein (a ROS scavenger); thus, providing direct evidence for the important role of oxidative stress in the genotoxic responses induced by nanoparticles (Nabeshi et al., 2011; Singh et al., 2012).

SECONDARY MECHANISMS FOR GENOTOXICITY

Secondary mechanisms for genotoxicity largely refers to the ability of a substance to induce a (chronic) inflammatory response in vivo that results in excessive generation of ROS by macrophage and neutrophil cells recruited to the exposure site (Chapter 13). This is a defense mechanism within the body to protect against invading pathogens—acute inflammation often enables the removal of foreign bodies by the immune cells, but if this fails due to biopersistence then the result is chronic inflammation, promoting oxidative stress that is subsequently responsible for damaging surrounding cells within the tissue. As described in the previous section, oxidative stress generates free radicals that damage biomolecules including DNA and consequently chronic inflammation is associated with carcinogenesis (Federico et al., 2007). In this model, the nanomaterial itself may exhibit no genotoxic potential within an in vitro testing system, but their physicochemical characteristics may be sufficient to promote a chronic immune response in vivo that in turn generates ROS responsible for inducing genotoxicity within the tissue cells at the nanomaterial deposition site. The classical example of such secondary genotoxicity involves inhalation of asbestos—these fibers are comprised of biopersistent materials that cannot be degraded by immune cells, their dimensions (very long and thin) prevent efficient phagocytosis, and they often contain redox-active iron. Consequently, inhalation of these fibers is associated with a chronic inflammatory response, and the persistent oxidative stress leads to secondary genotoxicity (as evidenced by 8-OHdG lesions with concomitant elevated mutagenicity) culminating in progressive fibrosis and ultimately mesothelioma (Marczynski et al., 2000; Rihn et al., 2000; Unfried et al., 2002) (and see Chapter 9). Many parallels have been drawn between asbestos and CNTs due to their high aspect ratio and biopersistence. Consequently, multiwalled CNTs (MWCNTs) have been reported to induce an asbestos-like inflammatory response and the formation of granulomas in vivo following intraperitoneal administration (Poland et al., 2008). However, carcinogenicity studies are limited and those in the literature are conflicting with some evidence that MWCNTs promote the development of mesothelioma following the administration of an intraperitoneal dose in Wistar rats (Rittinghausen et al., 2014), while another failed to find a carcinogenic response to MWCNTs (both with and without structural defects) in rats after a 2-year period following an intraperitoneal exposure (Muller et al., 2005). Although, evidence specifically involving nanomaterials is limited at present, this concept of secondary genotoxicity is important because it explains why nonmutagenic materials in vitro are still able to induce carcinogenesis in vivo—an example of which is carbon black. In vitro studies

indicated carbon black did not induce genotoxicity (Zhong et al., 1997), yet in vivo investigations indicated that inhalation of the dust was able to promote tumor formation (Driscoll et al., 1996; Schins, 2002).

While we have an understanding of the potential mechanism by which nanomaterials may induce DNA damage, it must be noted that there are only a limited number of studies that have experimentally demonstrated the means by which genotoxicity is induced by specific nanomaterials. Therefore, more studies are needed to fully elucidate the factors that govern the induction of genotoxicity by nanomaterials.

FROM GENOTOXICITY TO CANCER

Genetic instability involving gross chromosomal abnormalities through to comparatively minute aberrations, such as single base pair substitutions are closely associated with carcinogenesis and thus have long been detected in all malignancies. Thus, it is now widely accepted that cancer is a phenotype—the result of an accumulation of multiple genome-wide mutations that drive tumorigenesis in a multistep fashion, progressively transforming normal cells into premalignant foci, then subsequently to localized tumors that develop into invasive and metastatic lesions (Vogelstein et al., 2013). Cells are continuously subjected to potential mutagenic assaults through their exposure to environmental carcinogens, or simply as a result of normal metabolism. Genetic alterations are therefore constantly arising within a cells' genome, but stringent monitoring usually enables repair of such damage before it is transmitted to subsequent progeny cells. In extreme cases where the genetic damage is beyond repair, the cell commits suicide (apoptosis) to prevent the advent of abnormal cell populations.

The first step in tumorigenesis (initiation) involves a carcinogen interacting with DNA and creating a permanent alteration in the cells' genome. Tumors can be initiated by a single mutation arising in an indispensable gene within an individual cell as a consequence of an exogenous insult (e.g., mediated by carcinogens) or an endogenous strike (caused by metabolic by-products, such as ROS). Cells that have inherited a defective gene may then be more susceptible to tumor initiation depending upon the signaling pathway affected. If the initiating genetic abnormalities arise in vital genes involved in pathways required for maintenance of genomic integrity and cell cycle control, a cascade of further mutational events potentially result in malignant transformation.

Several types of mutations exist varying from gross chromosomal abnormalities involving numerical and structural alterations to single base pair changes, all of which have the potential to significantly alter the gene products' function. Not all genetic aberrations involved in carcinogenesis directly affect a gene's nucleotide sequence. Disruptions that alter gene expression or biochemical properties of protein products without altering the gene's DNA sequence are also important factors known as epigenetic mutations. One of the most prominent epigenetic changes that arise in tumorigenesis is hyper-methylation of gene control regions that act to silence their expression. The 5′ regions of genes are relatively rich in CpG dinucleotides when compared to the rest of the human genome and these sites are unmethylated in genes that are actively expressed. However, if aberrant methylation patterns arise as a consequence of exposure to an exogenous agent, the transcriptional status of the gene may be altered resulting in a reduction or complete loss of expression (Sharma et al., 2010). Indeed,

nanosized materials have been found to induce epigenetic changes through hypoacetylation of histones and altered DNA methylation patterns, which affect chromatin condensation and ultimately modify global gene expression patterns (Choi et al., 2008; Stoccoro et al., 2013).

Consequently, nanomaterials that are able to induce genotoxicity have the capacity to initiate carcinogenesis due to the alterations imposed on the genetic material. The ramifications are substantial and highlight the necessity to establish the genotoxic and carcinogenic potential of nanomaterials to which humans may be exposed (Table 18.1).

CARBON–BASED NANOMATERIALS

Carbon-based nanomaterials have become an important focus of the nanotechnology industry with many derivatives, such as fullerenes, carbon nanotubes, graphene, carbon black, and carbon diamond. Consequently, in recent years a great deal of attention has been given to their toxicity, genotoxicity, and carcinogenesis assessments. The properties of carbon-based nanomaterials, including size, shape, solubility, charge, and surface modification, are believed to be important determinants of toxicity and genotoxicity (Kumar et al., 2012).

Fullerene (C_{60}) and its Derivatives

Fullerene (C_{60}) and its derivatives have attracted increasing attention because of its unique structural, physical, chemical, and electric properties, with large-scale production of C_{60} representing a milestone in the development of nanomaterials. The Nobel Prize in Chemistry for 1996 was awarded to Kroto, Curl and Smalley for their discovery of C_{60} in 1985 (Kroto et al., 1985). C_{60} now have several technological and medicinal applications including cosmetics (Kato et al., 2010), lubricants, food additives, building materials, fuel cells, microreactors, drug delivery devices (Liang et al., 2010), and they have demonstrated antitumor effects in several tumor xenograft bearing mice models (Chen et al., 2005; Liang et al., 2010; Liu et al., 2009a,b).

Fullerenes have largely been found to induce limited toxicity and in some cases demonstrate antioxidant properties, but there is evidence that C_{60} also has the capacity to generate singlet oxygen that induces lipid peroxidation of linoleate, which leads to oxidative DNA damage (Sera et al., 1996). Indeed, Oberdörster found that fullerenes were lipophilic, localized into lipid-rich regions, such as cell membranes in vitro, and were redox active (Oberdorster, 2004). Several studies have reported C_{60} and its derivatives generate ROS with some evidence indicating these nanomaterials are able to damage DNA possibly via an oxidative stress-based mechanism (Markovic and Trajkovic, 2008).

C_{60} and its derivatives can also form complexes with DNA, induce DNA strand breakages, mutagenicity, and chromosomal damage. For example, the induction of mutagenesis by C_{60} has been demonstrated in gpt delta transgenic mouse primary embryo fibroblasts (Xu et al., 2009); colloidal C_{60} suspensions in ethanol, and water induced genotoxicity in human lymphocytes using single-cell gel electrophoresis assay (Comet assay) (Dhawan et al., 2006); and long-term exposure (80 days) of the water-soluble $C_{60}(OH)_{24}$ to Chinese hamster ovary cells, human epidermoid-like carcinoma (Hela) cells and human embryonic kidney (HEK293) cells increased structural chromosomal damage (Yasuharu, 2006).

TABLE 18.1 Genotoxicity and Toxicity Assays of Nanomaterial-Induced DNA Damage

Nanoparticle			In vitro/in vivo	Genotoxicity and toxicity assays	Findings	References
Carbon-based nanomaterials	C_{60}	$Gd@C_{82}(OH)_{22}$	In vitro and in vivo • HepG2 cells • LLC cells • MCF7 cells	CCK-8	[Gd@C82(OH)22]n nanoparticles have no toxicity	Liang et al. (2010); Chen et al. (2005); Liu et al. (2009a); Liu et al. (2009b)
		C_{60}	In vitro and in vivo	ROS	C_{60} has the capacity to generate singlet oxygen that induces lipid peroxidation of linoleate which leads to oxidative DNA damage	Sera et al. (1996); Oberdorster (2004); Markovic and Trajkovic (2008)
		C_{60} suspension	In vitro • *gpt* delta transgenic mouse primary embryo fibroblasts (MEF cells)	• MTT • Mutation analysis	C_{60} induced cytotoxicity in transgenic MEF cells. Increased mutation frequencies at red/gam gene loci in response to C_{60} exposure.	Xu et al. (2009)
		Aqu/nC_{60} and EthOH/nC_{60} suspensions	In vivo (PBL)	Comet assay	Aqu/nC_{60} suspensions elicited higher genotoxic response than EthOH/nC_{60} at the same dose	Dhawan et al. (2006)
		$C_{60}(OH)_{24}$	In vitro • CHO cells • Hela cells • HEK293 cells	• CCK-8 • micronucleus (MN) test	Structural chromosomal damage was increased	Yasuharu (2006)
		C_{60}	In vitro • FE1-Mutatrade mark-mouse lung epithelial cells	• Comet assay • FE1-MML Mutagenicity Analysis	The mutant frequency was slightly decreased; noncytotoxic concentrations did not result in increased levels of strand breaks.	Jacobsen et al. (2008)
	CNTs	SWCNTs	In vitro • FE1-Mutatrade mark-mouse lung epithelial cells	• Comet assay • FE1-MML Mutagenicity Analysis	Concentrations below cytotoxicity did not result in increased levels of strand breaks.	Jacobsen et al. (2008)
			In vitro • Lung fibroblast V79 cells	• Comet assay • MN test • Salmonella gene mutation assay	No DNA damage in the Ames and Mn tests, but some significance was detected by the comet assay.	Kisin et al. (2007)

(*Continued*)

TABLE 18.1 Genotoxicity and Toxicity Assays of Nanomaterial-Induced DNA Damage (*cont.*)

Nanoparticle		In vitro/in vivo	Genotoxicity and toxicity assays	Findings	References
		In vitro • alveolar macrophage-containing granuloma	Gene expression profiling	The expression levels of Atp6v0d2, Lpo, Mmp7, Mmp12 and Rnase9 were significantly upregulated after 754 days postinstillation of the SWCNT therefore demonstrating a sustained effect.	Fujita et al. (2014)
		In vivo	• Inflammatory effects • Fibrogenic effects • Genotoxic effects	The inhaled SWCNT being more highly dispersed with greater structure surface, as compared to the SWCNTs of bolus pharyngeal aspiration which were more agglomerated and had a lower structure surface area.	Shvedova et al. (2014)
		In vitro • The mouse macrophage cell line RAW 264.7	• ROS • Both necrosis and apoptosis • Chromosomal aberrations • Cellular ultrastructural alteration	SWCNTs are cyto and geno-toxic to RAW 264.7	Giorgio et al. (2011)
	MWCNTs	In vitro • Epithelial cells(Type II pneumocytes (AT-II); MCF-7; RLE cells)	Ex vivo and in vitro Mn assay	Significant dose-dependent increase in Mn	Muller et al. (2008a)
		In vitro • Mouse embryonic stem cells	Double strand break repair protein assay	Cellular apoptosis and activation of p53, and increased mutation frequency.	Zhu et al. (2007)
		In vivo • p53 heterozygous mice	—	MWCNT induced mesothelioma in p53 heterozygous mice	Takagi et al. (2008)
		In vivo • Male Fischer 344 rats	—	Mesothelioma observed	Sakamoto et al. (2009)
		In vivo • Female C57BL/6 mouse	• DNA microarrays • Comet assay • Dichlorodihydrofluorescein assay	The large-CNT were also found to elicit an earlier onset of inflammation and DNA damage, they induced more fibrosis and a unique fibrotic gene expression.	Poulsen et al. (2015)

			In vitro • Epithelial cells • Endothelial cells • Stromal cells	• Mitotic disruption • DNA breaks • Free radical generation • Tumor-promoting cell pathway • Chronic inflammation	CNTs-exposure could initiate and promote carcinogenesis and induce tumorigenesis	Møller et al. (2014)
			In vitro • The mouse macrophage cell line RAW 264.7	• ROS • Both necrosis and apoptosis • Chromosomal aberrations • Cellular ultrastructural alteration	SWCNTs are cyto and geno-toxic to RAW 264.7	Giorgio et al. (2011)
	Others	Nanodiamonds	In vitro • Embryonic stem cells	• Expression of DNA repair proteins	Oxidized nanodiamonds induced more DNA damage than the pristine/raw forms, showing the surface chemistry specific genotoxicity.	Xing et al. (2011)
Metallic nanoparticles	QDs	QDs	Plasmid DNA	• Gel electrophoreses analyses • Assay of reactive oxygen intermediates	Photoactivation of QD-conjugated nucleic acids for an extended period of time is not favorable for their stability	Anas et al. (2008)
		QDs modified with various molecules	In vitro	• Comet assay	The properties of QDs are not related to those of QD-core materials but to molecules covering the surface of QDs	Hoshino et al. (2004)
	Au-NPs	PDDAC/CTAB/PSS-coated Au NRs	In vitro and in vivo • MCF-7 • Mesenchymal stem cells	/	PDDAC-coated Au NRs have high cellular uptake efficiency. CTAB-coated Au NRs have distinct effects on cell viability of cancer cells while posing negligible impacts on normal and mesenchymal stem cells	Qiu et al. (2010); Wang et al. (2010); Wang et al. (2011)
		AuNP (20 m)	In vitro • Human fetal lung fibroblast cell line (MRC-5)	HPLC to measure 8-OHdG Cell proliferation (trypan blue)	Significant oxidative DNA damage; DNA repair genes downregulated (cyclin C, Hus1, BRCAI/BRCC1)	Li et al. (2008)
	Ag-NPs	AgNP (25 nm)	In vitro • Mouse embryonic stem cells (MES) and fibroblasts (MEF)	• Expression of DNA repair proteins • H_2AX phosphorylation • MTT assay	Increased expression of Rad51, p53 and phospho- H2AX proteins; time-dependent decrease in cell viability.	Ahamed et al. (2008)

(*Continued*)

TABLE 18.1 Genotoxicity and Toxicity Assays of Nanomaterial-Induced DNA Damage (*cont.*)

Nanoparticle			In vitro/in vivo	Genotoxicity and toxicity assays	Findings	References
Metal oxides	TiO_2	5 nm 40 nm	In vitro • *gpt* delta transgenic mouse primary embryo fibroblasts	• MTT • Mutation analysis	TiO_2 induced cytotoxicity in transgenic MEF cells; increased mutation frequencies at red/gam gene loci detected.	Xu et al. (2009)
		10 * 40 nm <25 nm <5 µm	In vitro • Human bronchial epithelial BEAS 2B cells	• Mn test • Comet assay	Uncoated nanosized anatase TiO_2 and fine rutile TiO_2 are more efficient than SiO_2-coated nanosized rutile TiO_2 in inducing DNA damage, whereas only nanosized anatase is able to slightly induce micronuclei	Falck et al. (2009)
		< 25 nm <100 nm	In vitro • Human hepatoma HepG2 cells	• Comet assay • DNA damage responsive genes	The toxic potential of TiO_2 nanoparticles varies not only with particle size but also with crystalline structure	Petkovic et al. (2011)
		25 nm	In vivo • PBL	• Mn test • Comet assay	Dose-dependent increase in Mn frequency and ROS generation; time-dependent up-regulation of phospho- and total-p53	Kang et al. (2008)
		<100 nm	In vitro • Human lymphoblastoid cells	• MTT assay • Population growth assay • Flow cytometry • Cytokinesis block micronucleus assay • Comet assay • Gene mutation assay	TiO_2 can cause genotoxicity and cytotoxicity in cultured human cells	Wang et al. (2007a)
	Zinc oxide	40–70 nm	In vitro and in vivo • PBL • Human sperm cells	• Comet assay • Trypan blue staining	Dose-dependent increase in DNA damage	Gopalan et al. (2009)
		100 nm	In vitro • CHO cells	• Chromosomal aberration test	Photoclastogenicity detected	Park et al. (2011)
	SiO_2	6.57, 8.2, and 196.52 nm	In vitro • Human lymphoblastoid cells	• Mn assay • Comet assay • HPRT assay	Mn induced and significant increase in HPRT mutations at 120 mg/mL, but no increases in comet tail detected.	Wang et al. (2007b)

Despite these studies reporting significant DNA damaging responses and toxicity following exposure to fullerenes, there are conflicting reports where no such damage has been observed. For example, Ema et al. evaluated the genotoxicity of fullerene C_{60} nanoparticles in vivo with the comet assays in the lung cells of rats. In this study they found that no increase in DNA damage (as measured by % Tail DNA) was detected in any group given the C_{60} nanoparticles, even at doses associated with inflammation (Ema et al., 2012). Jacobsen et al. (2008) also found that fullerenes did not induce significant increase in DNA strand breakages as detected by the comet assay. However, in contrast, Dhawan et al. (2006) reported positive genotoxic responses using the same test.

Surface derivatization and structural alterations can largely change the toxicity of fullerenes. Sayes et al. (2004) compared the cytotoxicity of C_{60} and water-soluble species in two different human cell lines and found the lethal dose of fullerene changed over seven orders of magnitude with relatively minor alterations in fullerene structure . Thus, discrepancies in the literature are likely to be due to slight physicochemical variations in the fullerenes studied (Singh et al., 2009).

Carbon Nanotubes (CNTs)

CNTs are molecular-scale tubes of graphitic carbon with unique characteristics, and among the stiffest and strongest fibers known. The discovery of CNTs is a promising breakthrough in the field of material science and has attracted huge academic and industrial interest. Some investigations reported that CNTs, both SWCNTs and MWCNTs have minimal or insignificant cellular responses (Davoren et al., 2007; Worle-Knirsch et al., 2006). However, others demonstrated that CNTs were cytotoxic to several cell types and due to the fibrous structure, the toxicity of CNTs is based on a high-aspect ratio and biopersistence, leading to prolonged inflammatory responses.

In recent years, an increasing number of investigations have considered the genotoxic potential of CNTs, and they have been found to promote structural and numerical chromosomal damage (both aneugenic and clastogenic events) (Kisin et al., 2007; Manshian et al., 2013; Muller et al., 2008a; Zhu et al., 2007), together with point mutations (Manshian et al., 2013; Unfried et al., 2002), which indicates potential carcinogenicity. SWCNTs not only enter the cytoplasm, but can also become localized in the cell nucleus, causing cell mortality in a dose-dependent manner (Porter et al., 2007). Significant DNA strand breakages are induced by different SWCNTs and MWCNTs exposure in a variety of model cell lines and in vivo.

In vivo, SWCNTs also promote time-dependent changes in genomic expression associated with pulmonary toxicity. The expression levels of the Ctsk, Gcgr, Gpnmb, Lilrb4, Marco, Mreg, Mt3, Padi1, Slc26a4, Spp1, Tnfsf4, and Trem2 genes have been reported to be persistently upregulated in a dose-dependent manner for up to 365 days postinstillation. Of further interest, the expression levels of Atp6v0d2, Lpo, Mmp7, Mmp12, and Rnase9 were significantly upregulated after 754 days postinstillation of the SWCNT therefore demonstrating a sustained effect. These persistently upregulated genes in the chronic-phase response might therefore act as potential biomarkers in lung tissue after SWCNT instillation (Fujita et al., 2014).

There are now two studies that have reported that MWCNTs promote mesothelioma in vivo following the administration of an intraperitoneal and intrascrotal dose (Sakamoto et al., 2009; Takagi et al., 2008). However, there is a contradictory report that failed to

detect a carcinogenic response to MWCNTs 2 years after an intraperitoneal exposure (Muller et al., 2005). In 2015, Poulsen et al. presented a toxicogenomic analysis of curled small-MWCNT or thick large-MWCNT. Both MWCNTs elicited strong acute phase and inflammatory responses that included the increasing of cellular influx in bronchoalveolar lavage fluid, interstitial pneumonia and gene expression changes. The large-CNT were also found to elicit an earlier onset of inflammation and DNA damage, they induced more fibrosis and a unique fibrotic gene expression (Poulsen et al., 2015).

It is evident from these investigations that it remains unclear on whether CNTs-exposure could initiate and promote carcinogenesis and induce tumorigenesis. Evidence is however accumulating to suggest that for some CNTs, this possibility may exist because generally prolonged CNTs-exposure activates several known (and possibly unknown) mechanisms associated with carcinogenicity including mitotic disruption, DNA breaks, free radical generation (i.e., ROS and NOS), stimulation of tumor-promoting cell signaling pathways, and chronic inflammation (Møller et al., 2014). In support of this, there exist reports that have found that long MWCNTs induced a more pronounced profibrotic and inflammatory response compared with shorter, more agglomerated MWCNTs (van Berlo et al., 2014). Long SWCNTs were significantly more potent in inducing the fibrogenic and toxic effects than short SWCNTs with respect to their ability to induce ROS, collagen production, and TGF-β release (Manke et al., 2014). Additionally, Shvedova and coworkers reported that inhalation exposure to SWCNTs showed significantly greater inflammatory, fibrotic, and genotoxic effects than bolus pharyngeal aspiration (Chapter 10). This effect was postulated to be due to the inhaled SWCNT being more highly dispersed with greater structure surface, as compared to the SWCNTs of bolus pharyngeal aspiration which were more agglomerated and had a lower structure surface area (Shvedova et al., 2014).

Thus, it appears that CNTs have genotoxic and carcinogenic potential, but the underlying mechanism requires further study. Most investigations have highlighted the importance of oxidative stress-mediated DNA damage; however, CNTs have a potential to interact with cellular biomolecules, such as the mitotic spindle, as well as the motor proteins that separate the chromosomes during cell division. Indeed, the disruption of centrosomes and mitotic spindles by CNTs has been found to result in monopolar, tripolar, and quadrapolar divisions of chromosomes (Sargent et al., 2010). The potential role of secondary genotoxicity as a consequence of chronic inflammation has yet to be assessed; however, this is likely to be an important mechanism given the high association with inflammation following CNTs exposure. Furthermore, the physicochemical characteristics of CNTs are important in governing the resultant biological response following exposure, with surface chemistry representing a key parameter that has a substantial effect on genotoxic potential (Ge et al., 2008).

Graphene and Graphene Oxide

Graphene is an allotrope of carbon in the form of a two-dimensional, atomic-scale, honeycomb lattice, which provides numerous possibilities for commercial applications due to its unique and superior mechanical, electrical, and physical properties. In the literature evaluating graphene for potential genotoxicity and subsequent tumor-associated pathologies, the size, surface functionalization, surface morphology, and dispersibility is thought to have both a direct and/or indirect impact the DNA damaging potential of graphene and graphene oxide (GO).

Hinzmann et al. (2014) demonstrated that pristine and reduced GO led to high cytotoxicity and induced DNA damage as measured by the Comet assay. PEG coated GO has been found to cause less oxidative stress in lung fibroblast cells than PEI coated or unmodified GO; thus indicating the GO surface chemistry is important in protecting cells against ROS-induced oxidative DNA damage (Wang et al., 2013). Furthermore, hydrophilic GO are able to become internalized in cells and induce ROS generation via NADPH oxidase, deregulation of DNA repair, and promote expression of oxidative stress response genes, with TGF-β being the key mediator of toxicological response (Chatterjee et al., 2014).

Some studies suggest that small graphene materials might bind to single strand DNA, during replication or transcription, which could cause DNA damage, failure of genetic inheritance, and block specific protein synthesis (Ren et al., 2010). Akhavan et al. (2012) exposed human mesenchymal stem cells to such small, nanosized GO at low doses and found that GO penetrated cell membranes to initiate genotoxicity, which was in the form of DNA fragmentation and chromosome aberrations, detected by the comet and chromosome aberration assays respectively. Nano-GO (130 nm) and micron-sized GO (1320 nm) have also been compared for their capacity to induce DNA damage in lung, intestinal, and kidney cells. Nano-GO was reported to be more potent at inducing genotoxicity than its micron counterpart at 10 μg/mL, again highlighting the importance of size in respect to induced cell damage following exposure (De Marzi et al., 2014).

The impact of dispersion is also a key determinant when considering genotoxicity studies conducted with graphene. Well-dispersed graphene has been associated with acute inflammation but with minimal long-term health impacts (Ma-Hock et al., 2013). Duch et al. (2011) evaluated aggregated graphene, dispersed graphene, and GO following intratracheal exposure, and found that only GO caused a significant increase in TUNEL positive nuclei in vivo and DNA fragmentation in alveolar macrophage exposure experiments in vitro. However, well-dispersed pristine graphene induced minimal biological disruption and was considered a safe material to handle. Interestingly, a recent report has also demonstrated that GO triggered an early onset of DNA melting in vitro, thus presenting a new mode of action to consider in the genotoxic capacity of these materials (Ivask et al., 2015).

Other Carbon-Based Materials

To date, there are limited reports examining the genotoxicity and carcinogenesis of other carbon-based materials, including carbon black, carbon nanofibers, nanorods, nanowires, and nanodiamonds. These carbon-based materials are all generating great interest in industrial sectors, such as energy production, and electronics, thus occupational exposure is likely. There is evidence demonstrating carbon black is cytotoxic and genotoxic to RAW 264.7 mouse macrophages (Giorgio et al., 2011). Cellular uptake, generation of oxygen radicals, gross chromosomal damage has also been observed in several cell types following exposure to carbon nanofibers. Nanodiamonds lead to mildly increased expression of DNA repair proteins (p53 and MOGG-1) in embryonic stem cells, but surface chemistry was important as oxidized nanodiamonds caused more DNA damage than unmodified nanodiamonds (Xing et al., 2011). In contrast, carbon-fiber-reinforced polybutylene terephthalate (CFRPBT) was nonhaemolytic, nonimmunogenic and showed no genotoxicity (Brooks et al., 2004). Evidence for the genotoxicity and carcinogenesis of carbon-based nanomaterials is accumulating with

a clear dependency on physicochemical features, but their long-term impact on human health has yet to be definitively established.

METALLIC NANOPARTICLES

A vast array of metallic nanoparticles (NPs) for a myriad of applications has been developed. Their genotoxicity has been reviewed in detail elsewhere, thus here we will only summarize the genotoxic potential of those in greatest industrial fabrication.

Quantum dots (QDs) are semiconductor nanocrystals that possess unique optical properties, making them promising tools for bioimaging. QDs can be conjugated with specific receptors to target particular cell types or molecules, such as in the directed labeling of neoplastic cells (Gao et al., 2004). However, QDs can transfer energy to nearby oxygen molecules, which could induce the generation of ROS, and in turn lead to cell damage or death (Bakalova et al., 2004). The main disadvantage of these nanoparticles is that they consist of highly toxic elements, such as, tellurium (Te), cadmium (Cd), lead (Pb), and selenium (Se), which is likely to hinder their use in vivo, as these dangerous materials could be released into the cellular environment, which may induce cyto- or genotoxicity (Gaponik et al., 2002). Indeed, uncoated CdTe QD elevated cellular ROS levels leading to plasma membrane, mitochondria, and nuclear damage (shrinkage and chromatin condensation), accompanied by upregulation of the antioxidant enzyme superoxide dismutase 1 (SOD-1) (Choi et al., 2008; Lovric et al., 2005). Capping the QDs with zinc sulfide (ZnS) can reduce this effect by protecting cells from the toxic core, but two groups have demonstrated that CdSe/ZnS QDs can cause DNA nicking and fragmentation in cell-free systems (Anas et al., 2008). Other methods to reduce QDs toxicity involve coating their surface with biocompatible molecules, such as polyethylene glycol (PEG). However, some functional groups or coating layers may also be cyto- or genotoxic, thus careful consideration should be given when selecting these modifications. Indeed, there are now several reports in the literature that demonstrate the importance of surface chemistry in governing the cell uptake, gene expression profiles, genotoxic and inflammatory response of QD (Al-Ali et al., 2015; Manshian et al., 2015a,b). In addition, purification of the material is an important factor in their toxicity, as crude QD coated with carboxyl groups prepared only by membrane filtration caused greater DNA damage than purified QD-COOH (Hoshino et al., 2004).

Gold nanoparticles (GNPs) have been utilized in imaging, biosensing, gene and drug delivery, cancer diagnostics and therapy, due to their unique optical properties, and biocompatibility. Their small size enables GNPs to easily penetrate into cells and translocate throughout the intracellular space (including the nucleus) causing a variety of responses. Size, shape, and surface properties (surface charge and functionalization) influence the biological effects of GNPs, particularly their Cytotoxicity (Qiu et al., 2010; Wang et al., 2010, 2011). However, GNP induced genotoxicity is controversial. In embryonic lung fibroblasts GNPs induced oxidative DNA damage (8-OHdG adducts) and decreased expression of DNA repair genes and the cell cycle checkpoint genes MAD2, cyclinB1, and cyclinB2, which could lower the cellular DNA damage response pathways leading to genetic instability (Li et al., 2008). Moreover, GNPs could induce DNA damage indirectly via an oxidative stress response in a size and cell type dependent manner. Of some concern, is the observation that exposure to GNPs causes failure

of nuclear chromatin decondensation in mouse sperm in vitro. Thus, some limited indication of GNPS genotoxicity has been presented, but there appears to be some nanoparticle-size and cell type dependency.

Silver nanoparticles (Ag NPs) are used in various consumer products including cosmetics, textiles, and health care products owning to its strong antimicrobial activity. However, there is accumulating evidence to suggest that Ag NPs are highly toxic to a variety of cells. As with many nanomaterials, physicochemical properties are important in defining cellular impact and polysaccharide-coated Ag NPs exhibit a more severe DNA damage response than uncoated Ag-NPs (Ahamed et al., 2008), although both types were able to increase p53 expression and phosphorylation, upregulate the DNA damage repair protein Rad51 and elevate the phosphorylation of H2AX. Ag-NPs have also been found to reduce the ATP content of cells which damages mitochondria, and increased production of ROS and DNA damage, indicating that Ag-NPs can disrupt the mitochondrial respiratory chain leading to the interruption of ATP synthesis and generation of oxidative stress, which in turn causes DNA damage (AshaRani et al., 2009). The level of bulky DNA adducts induced by Ag-NPs has not only been correlated with the level of cellular ROS, but is also inhibited by antioxidant pretreatment, suggesting Ag NPs mediate ROS-induced genotoxicity (Foldbjerg et al., 2010). Chen and colleagues systematically investigated the cellular uptake pathways, intracellular dissolution and chemical species, and genotoxicity of Ag NPs. Ag NPs, but also silver ions (Ag^+), induced bulky-DNA adducts, 8-oxodG and micronuclei formation in a concentration-dependent manner; however, there were quantitative and qualitative differences between the particulate and ionic form of silver (Jiang et al., 2013). The cytotoxicity mechanism of Ag NPs is related to the intracellular release of silver ions, followed by their binding to SH-groups, presumably coming from amino acids or proteins, and affecting protein functions and the antioxidant defense system of cells (Jiang et al., 2015).

Metallic NPs are promising nanomaterials in biomedical and commercial applications, but there is limited data on their genotoxic potential. Once inside cells, a related issue is the long-term fate of nanomaterials. One issue that has yet to be fully investigated is that the metallic NPs may itself not induce DNA damage, but they may corrode over time in biological environments to release metallic ions which could induce genotoxicity. Thus, the long-term genotoxic responses may be significantly different from short-term exposure datasets and further work in this area is required.

METAL OXIDES

To date, TiO_2 nanoparticles have been the focus in the majority of nanogenotoxicity studies due to its current use in sunscreens and cosmetics. These investigations have tested a range of TiO_2 forms with different sizes and crystalline structure, in an array of mammalian cell lines, using a variety of exposure times and assay systems. However, the primary factor that appears to be critical with regards to the genotoxicity of TiO_2 is its crystalline structure as anatase TiO_2 nanoparticles generally induce DNA strand breaks and chromosomal damage, while rutile TiO_2 is largely nongenotoxic in vitro (Falck et al., 2009; Gurr et al., 2005; Petkovic et al., 2011). Only a small number of studies have directly compared rutile to anatase TiO_2 genotoxicity and in these reports the rutile nanoparticles are larger than the anatase forms

investigated (Falck et al., 2009; Gurr et al., 2005; Petkovic et al., 2011). Additionally, many earlier studies exist that report significant increases in DNA damage but have either not detailed crystalline structure or used a mixed anatase/rutile sample (Kang et al., 2008; Karlsson et al., 2008; Wang et al., 2007a). Thus, further detailed investigation into the influence of this physicochemical parameter on TiO_2 genotoxicity is required.

Very few in vivo genotoxicity or carcinogenicity studies exist in the literature with TiO_2. Following oral administration, anatase TiO_2 has been demonstrated to induce DNA double strand breaks in bone marrow cells. However, it is important to note that the bone marrow micronucleus test in this study was negative; therefore, indicating that the double strand breaks detected were repairable lesions (Chen et al., 2014). This, therefore, is an important factor to take into consideration when interpreting DNA strand break assays (such as the Comet or y-H2Ax staining), as this form of DNA damage may be transient and only tests that measure fixed mutation (e.g., the micronucleus assay or point mutation assays) are truly indicative of induced genotoxicity.

Only two in vivo carcinogenicity studies have been performed to date with TiO_2, both of which yielded negative results but one study was performed with the rutile form (Xu et al., 2011), while the other does not specify the crystalline structure (Yokohira et al., 2009). Xu and colleagues topically applied rutile TiO_2 nanoparticles to the skin of C-Ha-*ras* proto-oncogen transgenic mice (which are more sensitive to chemically induced skin carcinogens than wild type mice), and observed that the nanoparticles were not able to penetrate through the epidermis to reach the dermis where skin tumors arise. Yokohira and colleagues failed to detect any histological lesions in the lungs of male rats that were exposed to TiO_2 nanoparticles via intratracheal instillation. However, mice that were exposed to mixed TiO_2 (75% anatase/25% rutile) in their drinking water were found to have 8-OHdG adducts in their livers, gross chromosomal damage in their peripheral blood cells, and the fetuses of the maternally exposed animals demonstrated an increased DNA deletion frequency (Trouiller et al., 2009). Hence, there is clear justification for further carcinogenicity studies focusing on the role of the TiO_2 crystalline structure.

Due to the use of TiO_2 in sunscreens, one concern has been the influence of UV irradiation on the induction of genotoxicity by these nanoparticles, which unfortunately has not been the subject of many studies. However, one investigation has demonstrated an enhanced level of clastogenicity in the presence of UV light in vitro when compared to its genotoxicity in the dark (Gopalan et al., 2009). Furthermore, ZnO nanoparticles, which are also utilized as an ingredient in sunscreens has also demonstrated the same response following simultaneous- and preirradiation (Dufour et al., 2009; Park et al., 2011).

Silica represents the other most rigorously studied metal oxide nanoparticle for genotoxic responses in vitro, but the evidence currently available is conflicting even where similar nanoparticles have been studied in the same cell line. For instance, 34 nm silica induced positive genotoxic responses in 3T3 mouse fibroblasts in one study (Park et al., 2011), while a second reported no genotoxicity in these cells (Barnes et al., 2008). A similar contradiction has also been seen in A549 lung carcinoma cells (Gonzalez et al., 2010; Jin et al., 2007). However, it appears that the conflict is due to the genotoxicity test applied as all those studies utilizing the comet assay to assess silica demonstrate no significant DNA damage (Barnes et al., 2008; Gonzalez et al., 2010; Jin et al., 2007), yet when the micronucleus assay is used, the authors report a genotoxic response (Barnes et al., 2008; Gonzalez et al., 2010). Furthermore, when

both test systems were used in parallel within the same investigation, the same pattern was observed whereby the silica induced significant genotoxicity in the micronucleus assay but no increase in comet tail was detected (Gonzalez et al., 2010; Wang et al., 2007b). The comet assay generally detects DNA strand breaks and alkali-labile sites that can be repaired, while the micronucleus assay reports on chromosome fragmentation or whole chromosome loss that are present postreplication and are therefore fixed mutations. The reason for the discrepancy between these two assays when testing silica is currently unclear but it suggests the micronucleus assay may be more appropriate in the genotoxicity testing of silica due to the type of DNA damage induced. Mutagenicity in vivo has not been observed to date with silica and very few carcinogenicity studies have been conducted with silica in nano form (Fruijtier-Pölloth, 2012; Sayes et al., 2010). There are some reports that indicate a weak tumor induction response, but this has occurred at very high doses where overload phenomena through high bolus administration are likely (Fruijtier-Pölloth, 2012).

A small number of studies have also been performed on several other metal oxide nanoparticles including iron oxide, aluminium oxide, and cerium oxide with all inducing genotoxic changes both in vitro and in vivo (Auffan et al., 2009; Balasubramanyam et al., 2009; Singh et al., 2010, 2012). However, the limited array of these studies makes it difficult to identify the physicochemical characteristics that influence genotoxic potential. However, it is becoming increasingly apparent that the physicochemical features of the materials are a key factor in their uptake mechanics, and the greater the level of internalization, the more likely it is for an inflammatory and potentially a genotoxic response to be promoted. It has therefore been clearly demonstrated that metal oxide nanoparticles have the capacity to induce a range of genotoxic responses, nevertheless it is yet to be established if these mutagenic alterations are sufficient to induce carcinogenesis in vivo.

CONCLUSIONS AND FUTURE DIRECTIONS

Our understanding of the genotoxicity induced by nanomaterials is slowly developing. As more nanomaterials are being investigated, there is increasing evidence supporting their ability to induce DNA damage primarily through the promotion of oxidative stress, although some direct interference with DNA and cell division machinery has been observed. However, it must be stressed that the genotoxic potential of nanomaterials is clearly dependent upon their physicochemical characteristics. Unfortunately, the high degree of variation in materials tested and genotoxicity assessment regimens utilized currently makes it very difficult to form firm conclusions on those specific features that are specifically responsible for inducing genotoxic responses. Further studies should consider the following:

1. Future studies should focus on investigating the genotoxicity of a range of nanomaterials in parallel, assessing several DNA damage endpoints to determine the true impact of specific physicochemical features and elucidate the mechanisms that govern the induction of genotoxicity by nanomaterials.
2. In the case of metal nanoparticles that are often located in lysosomes and endosomes once they have become internalized into cells, the importance of corrosion in biological environments and, thus, their long-term effects are poorly understood and require further investigation.

3. Consideration needs to be given to the importance of secondary genotoxicity as most studies to date have solely focused on mechanisms that are associated with primary genotoxicity.
4. In vivo carcinogenicity investigations are required to provide an understanding of the carcinogenic potential of nanomaterials.

Substances that promote genotoxicity are highly suspect carcinogens, but this needs to be confirmed in appropriate studies. Thus, carcinogenicity assessments are necessary to determine if the genotoxic impact of a given nanomaterial is sufficient to initiate tumorigenesis. It is, however, of importance to note that such investigations carry a heavy burden on the use of animals. It will therefore be pertinent to consider the development and implementation of in vitro alternative models and in silico systems to assist in understanding the genotoxic and carcinogenic potential of the wide array of nanomaterials that are currently being produced and designed by the nanotechnology industry. This will be critical to ensure the future protection of both humans and the environment and to facilitate a detailed understanding of the long-term health effects following exposure to nanomaterials.

References

Ahamed, M., Karns, M., Goodson, M., Rowe, J., Hussain, S.M., Schlager, J.J., Hong, Y., 2008. DNA damage response to different surface chemistry of silver nanoparticles in mammalian cells. Toxicol. Appl. Pharm. 233, 404–410.

Akhavan, O., Ghaderi, E., Akhavan, A., 2012. Size-dependent genotoxicity of graphene nanoplatelets in human stem cells. Biomaterials 33, 8017–8025.

Al-Ali, A., Singh, N., Manshian, B., Wilksinson, T., Wills, J., Jenkins, G.J.S., Doak, S.H., 2015. Quantum dot induced cellular perturbations involving varying toxicity pathways. Toxicol. Res. 4, 623–633.

Anas, A.A., Akita, H., Harashima, H., Itoh, T., Ishikawa, M., Biju, V., 2008. Photosensitized breakage and damage of DNA by CdSe- ZnS quantum dots. J. Phys. Chem. B 112, 10005–10011.

AshaRani, P.V., Low Kah Mun, G., Hande, M.P., Valiyaveettil, S., 2009. Cytotoxicity and genotoxicity of silver nanoparticles in human cells. ACS Nano 3, 279–290.

Auffan, M., Rose, J., Orsiere, T., De Meo, M., Thill, A., Zeyons, O., Proux, O., Masion, A., Chaurand, P., Spalla, O., Botta, A., Wiesner, M.R., Bottero, J.-Y., 2009. CeO2 nanoparticles induce DNA damage towards human dermal fibroblasts in vitro. Nanotoxicology 3, 161–171.

Bakalova, R., Ohba, H., Zhelev, Z., Ishikawa, M., Baba, Y., 2004. Quantum dots as photosensitizers? Nat Biotechnol 22, 1360–1361.

Balasubramanyam, A., Sailaja, N., Mahboob, M., Rahman, M.F., Hussain, S.M., Grover, P., 2009. In vivo genotoxicity assessment of aluminium oxide nanomaterials in rat peripheral blood cells using the comet assay and micronucleus test. Mutagenesis 24, 245–251.

Barnes, C.A., Elsaesser, A., Arkusz, J., Smok, A., Palus, J., Lesniak, A., Salvati, A., Hanrahan, J.P., de Jong, W.H., Dziubaltowska, E., Stepnik, M., Rydzynski, K., McKerr, G., Lynch, I., Dawson, K.A., Howard, C.V., 2008. Reproducible comet assay of amorphous silica nanoparticles detects no genotoxicity. Nano Lett. 8, 3069–3074.

Berry, C.C., de la Fuente, J.M., Mullin, M., Chu, S.W., Curtis, A.S., 2007. Nuclear localization of HIV-1 tat functionalized gold nanoparticles. IEEE Trans. Nanobiosci. 6, 262–269.

Brooks, R.A., Jones, E., Storer, A., Rushton, N., 2004. Biological evaluation of carbon-fibre-reinforced polybutyleneterephthalate (CFRPBT) employed in a novel acetabular cup. Biomaterials 25, 3429–3438.

Chatterjee, N., Eom, H.J., Choi, J., 2014. A systems toxicology approach to the surface functionality control of graphene-cell interactions. Biomaterials 35, 1109–1127.

Chen, M., von Mikecz, A., 2005. Formation of nucleoplasmic protein aggregates impairs nuclear function in response to SiO2 nanoparticles. Exp. Cell. Res. 305, 51–62.

Chen, C., Xing, G., Wang, J., Zhao, Y., Li, B., Tang, J., Jia, G., Wang, T., Sun, J., Xing, L., Yuan, H., Gao, Y., Meng, H., Chen, Z., Zhao, F., Chai, Z., Fang, X., 2005. Multihydroxylated nanoparticles: antineoplastic activity of high efficiency and low toxicity. Nano Lett. 5, 2050–2057.

Chen, Z., Wang, Y., Ba, T., Li, Y., Pu, J., Chen, T., Song, Y., Gu, Y., Quian, Q., Yang, J., Jia, G., 2014. Genotoxic evaluation of titanium dioxide nanoparticles in vivo and in vitro. Toxicol. Lett. 226, 314–319.
Choi, A.O., Brown, S.E., Szyf, M., Maysinger, D., 2008. Quantum dot-induced epigenetic and genotoxic changes in human breast cancer cells. J. Mol. Med. 86, 291–302.
Cooke, M.S., Evans, M.D., Dizdaroglu, M., Lunec, J., 2003. Oxidative DNA damage: mechanism, mutation, and disease. FASEB J. 17, 1195–1214.
Davoren, M., Herzog, E., Casey, A., Cottineau, B., Chambers, G., Byrne, H.J., Lyng, F.M., 2007. In vitro toxicity evaluation of single walled carbon nanotubes on human A549 lung cells. Toxicol. In Vitro 21, 438–448.
De Marzi, L., Ottaviano, L., Perrozzi, F., Nardone, M., Santucci, S., De Lapuente, J., Borras, M., Treossi, E., Palermo, V., Poma, A., 2014. Flake size-dependent cyto and genotoxic evaluation of graphene oxide on in vitro A549, Caco2 and vero cell lines. J. Biol. Regul. Homeost. Agents 28, 281–289.
Dhawan, A., Taurozzi, J.S., Pandey, A.K., Shan, W., Miller, S.M., Hashsham, S.A., Tarabara, V.V., 2006. Stable colloidal dispersions of C60 fullerenes in water: evidence for genotoxicity. Environ. Sci. Technol. 40, 7394–7401.
Doak, S.H., Johnson, G., Quick, E., Jenkins, G.J.S., Parry, J.M., 2007. Mechanistic influences for mutation induction curves after exposure to DNA-reactive carcinogens. Cancer Res. 67, 3904–3911.
Driscoll, K.E., Carter, J.M., Howard, B.W., Hassenbein, D.G., Pepelko, W., Baggs, R.B., Orberdorster, G., 1996. Pulmonary inflammatory, chemokine, and mutagenic responses in rats after sub-chronic inhalation of carbon black. Toxicol. Appl. Pharmacol. 136, 372–380.
Duch, M.C., Budinger, G.R., Liang, Y.T., Soberanes, S., Urich, D., Chiarella, S.E., Campochiaro, L.A., Gonzalez, A., Chandel, N.S., Hersam, M.C., Mutlu, G.M., 2011. Minimizing oxidation and stable nanoscale dispersion improves the biocompatibility of graphene in the lung. Nano Lett. 11, 5201–5207.
Dufour, E.K., Kumaravel, T., Nohynek, G.J., Kirkland, D., Toutain, H., 2009. Clastogenicity, photo-clastogenicity or pseudo-photo-clastogenicity: genotoxic effects of zinc oxide in the dark, in pre-irradiated or simultaneously irradiated Chinese hamster ovary cells. Mut. Res. 607, 215–224.
Ema, M., Tanaka, J., Kobayashi, N., Naya, M., Endoh, S., Maru, J., Hosoi, M., Nagai, M., Nakajima, M., Hayashi, M., Nakanishi, J., 2012. Genotoxicity evaluation of fullerene C60 nanoparticles in a comet assay using lung cells of intratracheally instilled rats. Regul. Toxicol. Pharmacol. 62, 419–424.
Falck, G.C.M., Lindberg, H.K., Suhonen, S., Vippola, M., Vanhala, E., Catalan, J., Savolainen, K., Norppa, H., 2009. Genotoxic effects of nanosized and fine TiO_2. Hum. Exp. Toxicol. 28, 339–352.
Federico, A., Morgillo, F., Tuccillo, C., Ciardiello, F., Loguercioc, C., 2007. Chronic inflammation and oxidative stress in human carcinogenesis. Int. J. Cancer 121, 2381–2386.
Ferguson, L.R., Denny, W.A., 2007. Genotoxicity of non-covalent interactions: DNA intercalators. Mut. Res. 623, 14–23.
Foldbjerg, R., Dang, D., Autrup, H., 2010. Cytotoxicity and genotoxicity of silver nanoparticles in the human lung cancer cell line, A549. Arch. Toxicol., 1–8.
Folkmann, J.K., Risom, L., Jacobsen, N.R., Wallin, H., Loft, S., Moller, P., 2009. Oxidatively damaged DNA in rats exposed by oral gavage to C60 fullerenes and single-walled carbon nanotubes. Env. Health Persp. 117, 703–708.
Fruijtier-Pölloth, C., 2012. The toxicological mode of action and the safety of synthetic amorphous silica—a nanostructured material. Toxicology 294, 61–79.
Fujita, K., Fukuda, M., Fukui, H., Horie, M., Endoh, S., Uchida, K., Shichiri, M., Morimoto, Y., Ogami, A., Iwahashi, H., 2014. Intratracheal instillation of single-wall carbon nanotubes in the rat lung induces time-dependent changes in gene expression. Nanotoxicology 9, 1–12.
Gao, X., Cui, Y., Levenson, R.M., Chung, L.W.K., Nie, S., 2004. In vivo cancer targeting and imaging with semiconductor quantum dots. Nat. Biotechnol. 22, 969–976.
Gaponik, N., Talapin, D.V., Rogach, A.L., Hoppe, K., Shevchenko, E.V., Kornowski, A., Eychmüller, A., Weller, H., 2002. Thiol-capping of CdTe nanocrystals: an alternative to organometallic synthetic routes. J. Phys. Chem. B 106, 7177–7185.
Ge, C., Lao, F., Li, W., Li, Y., Chen, C., Qiu, Y., Mao, X., Li, B., Chai, Z., Zhao, Y., 2008. Quantitative analysis of metal impurities in carbon nanotubes: efficacy of different pretreatment protocols for ICPMS spectroscopy. Anal. Chem. 80, 9426–9434.
Giorgio, M.L., Di Bucchianico, S., Ragnelli, A.M., Aimola, P., Santucci, S., Poma, A., 2011. Effects of single- and multi-walled carbon nanotubes on macrophages: cytotoxicity, genotoxicity and ultrastructural damage. Mutat. Res. 722, 20–31.

Gonzalez, L., Thomassen, L.C.J., Plas, G., Rabolli, V., Napierska, D., Decordier, I., Roelants, M., Hoet, P.H., Kirschhock, C.E.A., Martens, J.A., Lison, D., Kirsch-Volders, M., 2010. Exploring the aneugenic and clastogenic potential oin the nanosize range: A549 human lung carcinoma cells and amorphous monodisperse silica nanoparticles as models. Nanotoxicology 4, 382–395.

Gopalan, R.C., Osman, I.F., Amani, A., De Matas, M., Anderson, D., 2009. The effect of zinc oxide and titanium dioxide nanoparticles in the comet assay with UVA photo*activation of* human sperm and lymphocytes. Nanotoxicology 3, 33–39.

Gurr, J.R., Wang, A.S., Chen, C.H., Jan, K.Y., 2005. Ultrafine titanium dioxide particles in the absence of photoactivation can induce oxidative damage to human bronchial epithelial cells. Toxicology 213, 66–73.

Hackenberg, S., Friehs, G., Froelich, K., Ginzkey, C., Koehler, C., Scherzed, A., Burghartz, M., Hagen, R., Kleinsasser, N., 2010. Intracellular distribution, geno- and cytotoxic effects of nanosized titanium dioxide particles in the anatase crystal phase on human nasal mucosa cells. Toxicol. Lett. 195, 9–14.

Hackenberg, S., Scherzed, A., Technau, A., Kessler, M., Froelich, K., Ginzkey, C., Koehler, C., Burghartz, M., Hagen, R., Kleinsasser, N., 2011. Cytotoxic, genotoxic and pro-inflammatory effects of zinc oxide nanoparticles in human nasal mucosa cells in vitro. Toxicol. In Vitro 25, 657–663.

Hinzmann, M., Jaworski, S., Kutwin, M., Jagiełło, J., Kozi ski, R., Wierzbicki, M., Grodzik, M., Lipińska, L., Sawosz, E., Chwalibog, A., 2014. Nanoparticles containing allotropes of carbon have genotoxic effects on glioblastoma multiforme cells. Int. J. Nanomedicine 9, 2409–2417.

Hoshino, A., Fujioka, K., Oku, T., Suga, M., Sasaki, Y.F., Ohta, T., Yasuhara, M., Suzuki, K., Yamamoto, K., 2004. Physicochemical properties and cellular toxicity of nanocrystal quantum dots depend on their surface modification. Nano Lett. 4, 2163–2169.

Ivask, A., Voelcker, N.H., Seabrook, S.A., Hor, M., Kirby, J.K., Fenech, M., Davis, T.P., Ke, P.C., 2015. DNA melting and genotoxicity induced by silver nanoparticles and graphene. Chem. Res. Toxicol. 28, 1023–1035.

Jacobsen, N.R., Pojana, G., White, P., Moller, P., Cohn, C.A., Korsholm, K.S., Vogel, U., Marcomini, A., Loft, S., Wallin, H., 2008. Genotoxicity, cytotoxicity, and reactive oxygen species induced by singlewalled carbon nanotubes and C(60) fullerenes in the FE1-Mutatrade mark-Mouse lung epithelial cells. Environ. Mol. Mutagen. 49, 476–487.

Jenkins, G.J.S., Doak, S.H., Johnson, G., Quick, E., Waters, E., Parry, J., 2005. Assessment of the mechanistic basis for genotoxic thresholds induced by alkylating agents. Mutagenesis 20, 389–398.

Jiang, X., Foldbjerg, R., Miclaus, T., Wang, L., Singh, R., Hayashi, Y., Sutherland, D., Chen, C., Autrup, H., Beer, C., 2013. Multi-platform genotoxicity analysis of silver nanoparticles in the model cell line CHO-K1. Toxicol. Lett. 222, 55–63.

Jiang, X., Miclaus, T., Wang, L., Foldbjerg, R., Sutherland, D.S., Autrup, H., Chen, C., Beer, C., 2015. Fast intracellular dissolution and persistent cellular uptake of silver nanoparticles in CHO-K1 cells: implication for cytotoxicity. Nanotoxicology 9, 181–189.

Jin, Y., Kannan, S., Wu, M., Zhao, J.X., 2007. Toxicity of luminescent silica nanoparticles to living cells. Chem. Res. Toxicol. 20, 1126–1133.

Kang, S.J., Kim, B.M., Lee, Y.J., Chung, H.W., 2008. Titanium dioxide nanoparticles trigger p53-mediated damage response in peripheral blood lymphocytes. Env. Mol. Mutagen. 49, 399–405.

Karlsson, H.L., Cronholm, P., Gustafsson, J., Moller, L., 2008. Copper oxide nanoparticles are highly toxic: a comparison between metal oxide nanoparticles and carbon nanotubes. Chem. Res. Toxicol. 21, 1726–1732.

Kato, S., Taira, H., Aoshima, H., Saitoh, Y., Miwa, N., 2010. Clinical evaluation of fullerene-C60 dissolved in squalane for anti-wrinkle cosmetics. J. Nanosci. Nanotechnol. 10, 6769–6774.

Kisin, E.R., Murray, A.R., Keane, M.J., Shi, X.C., Schwegler-Berry, D., Gorelik, O., Arepalli, S., Castranova, V., Wallace, W.E., Kagan, V.E., Shvedova, A.A., 2007. Single-walled carbon nanotubes: geno- and cytotoxic effects in lung fibroblast V79 cells. J. Toxicol. Environ. Health A 70, 2071–2079.

Kroto, H.W., Heath, J.R., O'Brien, S.C., Smalley, R.E., 1985. C60: buckminsterfullerene. Nature 318, 162–163.

Kumar, V., Kumari, A., Guleria, P., Yadav, S.K., 2012. Evaluating the toxicity of selected types of nanochemicals. Rev. Environ. Contam. Toxicol. 215, 39–121.

Li, J.J., Zou, L., Hartono, D., Ong, C.N., Bay, B.H., Lanry Yung, L.Y., 2008. Gold nanoparticles induce oxidative damage in lung fibroblasts in vitro. Adv. Mater. 20, 138–142.

Liang, X.J., Meng, H., Wang, Y., He, H., Meng, J., Lu, J., Wang, P.C., Zhao, Y., Gao, X., Sun, B., Chen, C., Xing, G., Shen, D., Gottesman, M.M., Wu, Y., Yin, J.J., Jia, L., 2010. Metallofullerene nanoparticles circumvent tumor resistance to cisplatin by reactivating endocytosis. Proc. Natl. Acad. Sci. USA 107, 7449–7454.

Liu, Y., Jiao, F., Qiu, Y., Li, W., Lao, F., Zhou, G., Sun, B., Xing, G., Dong, J., Zhao, Y., Chai, Z., Chen, C., 2009a. The effect of $Gd@C_{82}(OH)_{22}$ nanoparticles on the release of Th1/Th2 cytokines and induction of TNF-alpha mediated cellular immunity. Biomaterials 30, 3934–3945.

Liu, Y., Jiao, F., Qiu, Y., Li, W., Qu, Y., Tian, C., Li, Y., Bai, R., Lao, F., Zhao, Y., Chai, Z., Chen, C., 2009b. Immunostimulatory properties and enhanced TNF- alpha mediated cellular immunity for tumor therapy by $C_{60}(OH)_{20}$ nanoparticles. Nanotechnology 20, 415102.

Lovric, J., Cho, S.J., Winnik, F.M., Maysinger, D., 2005. Unmodified cadmium telluride quantum dots induce reactive oxygen species formation leading to multiple organelle damage and cell death. Chem. Biol. 12, 1227–1234.

Ma-Hock, L., Strauss, V., Treumann, S., Küttler, K., Wohlleben, W., Hofmann, T., Gröters, S., Wiench, K., van Ravenzwaay, B., Landsiedel, R., 2013. Comparative inhalation toxicity of multi-wall carbon nanotubes, graphene, graphite nanoplatelets and low surface carbon black. Part. Fibre Toxicol. 10, 23.

Manke, A., Luanpitpong, S., Dong, C., Wang, L., He, X., Battelli, L., Derk, R., Stueckle, T.A., Porter, D.W., Sager, T., Gou, H., Dinu, C.Z., Wu, N., Mercer, R.R., Rojanasakul, Y., 2014. Effect of fiber length on carbon nanotube-induced fibrogenesis. Int. J. Mol. Sci. 15, 7444–7461.

Manshian, B.B., Jenkins, G.J.S., Williams, P.M., Wright, C., Barron, A.R., Brown, A.P., Hondow, N., Doak, S.H., 2013. Single walled carbon nanotubes: differential genotoxic potential associated with physico-chemical properties. Nanotoxicology 7, 144–156.

Manshian, B.B., Soenen, S.J., Al-Ali, A., Brown, A., Hondow, N., Wills, J.W., Jenkins, G.J.S., Doak, S.H., 2015a. Cell type dependent changes in CdSe/ZnS quantum dot uptake and toxic endpoints. Toxicol. Sci. 144, 246–258.

Manshian, B.B., Soenen, S.J., Brown, A., Hondow, N., Wills, J., Jenkins, G.J.S., Doak, S.H., 2015b. Genotoxic capacity of Cd/Se semiconductor quantum dots with differing surface chemistries. Mutagenesis 31, 97–106.

Marczynski, B., Kraus, T., Rozynek, P., Raithel, H.J., Baur, X., 2000. Association between 8-hyrdoxy-2-deoxyguanosine levels in DNA of workers highly exposed to asbestos and their clinical data, occupational and non-occupational confounding factors, and cancer. Mut. Res. 468, 203–312.

Markovic, Z., Trajkovic, V., 2008. Biomedical potential of the reactive oxygen species generation and quenching by fullerenes (C60). Biomaterials 29, 3561–3573.

Møller, P., Christophersen, D.V., Jensen, D.M., Kermanizadeh, A., Roursgaard, M., Jacobsen, N.R., Hemmingsen, J.G., Danielsen, P.H., Cao, Y., Jantzen, K., Klingberg, H., Hersoug, L.G., Loft, S., 2014. Role of oxidative stress in carbon nanotube-generated health effects. Arch. Toxicol. 88, 1939–1964.

Muller, J., Huaux, F., Moreau, N., Misson, P., Heilier, J.-F., Delos, M., Arras, M., Fonseca, A., Nagy, J.B., Lison, D., 2005. Respiratory toxicity of multi-walled carbon nanotubes. Toxicol. App. Pharmacol. 207, 221–231.

Muller, J., Decordier, I., Hoet, P.H., Lombaert, N., Thomassen, L., Huaux, F., Lison, D., Kirsch-Volders, M., 2008a. Clastogenic and aneugenic effects of multi-wall carbon nanotubes in epithelial cells. Carcinogenesis 29, 427–433.

Nabeshi, H., Yoshikawa, T., Matsuyama, K., Nakazato, Y., Tochigi, S., Kondoh, S., Hirai, T., Akase, T., Nagano, K., Abe, Y., Yoshioka, Y., Kamada, H., Itoh, N., Tsunoda, S., Tsutsumi, Y., 2011. Amorphous nanosilica induce endocytosis-dependent ROS generation and DNA damage in human keratinocytes. Part. Fibre Toxicol. 8, 1.

Nabiev, I., Mitchell, S., Davies, A., Williams, Y., Kelleher, D., Moore, R., Gun'ko, Y.K., Byrne, S., Rakovich, Y.P., Donegan, J.F., Sukhanova, A., Conroy, J., Cottell, D., Gaponik, N., Rogach, A., Volkov, Y., 2007. Nonfunctionalised nanocrystals can exploit a cell's active transport machinery delivering them to specific nuclear and cytoplasmic compartments. Nano Lett. 7, 3452–3461.

Nativo, P., Prior, I.A., Brust, M., 2008. Uptake and intracellular fate of surface-modified gold nanoparticles. ACS Nano 2, 1639–1644.

Oberdorster, E., 2004. Manufactured nanomaterials (fullerenes, C60) induce oxidative stress in the brain of juvenile largemouth bass. Environ. Health Perspect. 112, 1058–1062.

Park, M.V.D.Z., Verharen, H.W., Zwart, E., hernandez, L., van Benthem, J., Elsaesser, A., Barnes, C., McKerr, G., Howard, V., Salvati, A., Lynch, I., Dawson, K.A., de Jong, W.H., 2011. Genotoxicity evaluation of amorphous silica nanoparticles of different sizes using the micronucleus and the plasmid *lacZ* gene mutation assay. Nanotoxicology 5, 168–181.

Petkovic, J., Zegura, B., Stevanovic, M., Drnovsek, N., Uskokovic, D., Novak, S., Filipic, M., 2011. DNA damage and alterations in expression of DNA damage responsive genes induced by TiO_2 nanoparticles in human hepatoma HepG2 cells. Nanotoxicology 5, 341–353.

Poland, C.A., Duffin, R., Kinlock, I., Maynard, A., Wallace, W.A.H., Seaton, A., Stone, V., Brown, S., MacNee, W., Donaldson, K., 2008. Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study. Nat. Nanotech. 3, 423–428.

Porter, A.E., Gass, M., Muller, K., Skepper, J.N., Midgley, P.A., Welland, M., 2007. Direct imaging of single-walled carbon nanotubes in cells. Nat. Nanotechnol. 2, 713–717.

Poulsen, S.S., Saber, A.T., Williams, A., Andersen, O., Købler, C., Atluri, R., Pozzebon, M.E., Mucelli, S.P., Simion, M., Rickerby, D., Mortensen, A., Jackson, P., Kyjovska, Z.O., Mølhave, K., Jacobsen, N.R., Jensen, K.A., Yauk, C.L., Wallin, H., Halappanavar, S., Vogel, U., 2015. MWCNTs of different physicochemical properties cause similar inflammatory responses, but differences in transcriptional and histological markers of fibrosis in mouse lungs. Toxicol. Appl. Pharmacol. 284, 16–32.

Qiu, Y., Liu, Y., Wang, L., Xu, L., Bai, R., Ji, Y., Wu, X., Zhao, Y., Li, Y., Chen, C., 2010. Surface chemistry and aspect ratio mediated cellular uptake of Au nanorods. Biomaterials 31, 7606–7619.

Ren, H., Wang, C., Zhang, J., Zhou, X., Xu, D., Zheng, J., Guo, S., Zhang, J., 2010. Dna cleavage system of nanosized graphene oxide sheets and copper ions. ACS Nano 4, 7169–7174.

Rihn, B., Coulais, C., Kauffer, E., Bottin, M.C., Martin, P., Yvon, F., Vigneron, J.C., Binet, S., Monoven, N., Steiblen, G., Keith, G., 2000. Inhaled crocidolite mutagenicity in lung DNA. Environ. Health Perspect. 108, 341–346.

Rittinghausen, S., Hackbarth, A., Creutzenberg, O., Ernst, H., Heinrich, U., Leonhardt, A., Schaudien, D., 2014. The carcinogenic effect of various multi-walled carbon nanotubes (MWCNTs) after intraperitoneal injection in rats. Part. Fibre Toxicol. 11, 59.

Sakamoto, Y., Nakae, D., Fukumori, N., Tayama, K., Maekawa, A., Imai, K., Hirose, A., Nishimura, T., Ohashi, N., Ogata, A., 2009. Induction of mesothelioma by a single intrascrotal administration of multi-wall carbon nanotube in intact male Fischer 344 rats. J. Toxicol. Sci. 34, 65–76.

Sargent, L.M., Shvedova, A.A., Hubbs, A.F., Salisbury, J.L., Benkovic, S.A., Kashon, M.L., Lowry, D.T., Murray, A.R., Kisin, E.R., Friend, S., 2009. Induction of aneuploidy by single-walled carbon nanotubes. Environ. Mol. Mutagen. 50, 708–717.

Sargent, L.M., Reynolds, S.H., Castranova, V., 2010. Potential pulmonary effects of engineered carbon nanotubes: in vitro genotoxic effects. Nanotoxicology 4, 396–408.

Sayes, C.M., Fortner, J.D., Guo, W., Lyon, D., Boyd, A.M., Ausman, K.D., Tao, Y.J., Sitharaman, B., Wilson, L.J., Hughes, J.B., West, J.L., Colvin, V.L., 2004. The differential cytotoxicity of water-soluble fullerenes. Nano Lett. 4, 7.

Sayes, C.M., Reed, K.L., Glover, K.P., Swain, K.A., Ostraat, M.L., Donner, E.M., Warheit, D.B., 2010. Changing the dose metric for inhalation toxicity studies: short-term study in rats with engineered aerosolized amorphous silica nanoparticles. Inhal. Toxicol. 22, 348–354.

Schins, R.P.F., 2002. Mechanisms of genotoxicity of particles and fibres. Inhal. Toxicol. 14, 57–78.

Sera, N., Tokiwa, H., Miyata, N., 1996. Mutagenicity of the fullerene C60-generated singlet oxygen dependent formation of lipid peroxides. Carcinogenesis 17, 2163–2169.

Sharma, S., Kelly, T.K., Jones, P.A., 2010. Epigenetics in cancer. Carcinogenesis 31, 27–36.

Shukla, R.K., Sharma, V., Pandey, A.K., Singh, S., Sultana, S., Dhawan, A., 2011. ROS-mediated genotoxicity induced by titanium dioxide nanoparticles in human epidermal cells. Toxicol. In Vitro 25, 231–241.

Shvedova, A.A., Yanamala, N., Kisin, E.R., Tkach, A.V., Murray, A.R., Hubbs, A., Chirila, M.M., Keohavong, P., Sycheva, L.P., Kagan, V.E., Castranova, V., 2014. Long-term effects of carbon containing engineered nanomaterials and asbestos in the lung: one year postexposure comparisons. Am. J. Physiol. Lung Cell Mol. Physiol. 306, L170–L182.

Singh, N., Manshian, B., Jenkins, G.J., Griffiths, S.M., Williams, P.M., Maffeis, T.G., Wright, C.J., Doak, S.H., 2009. NanoGenotoxicology: the DNA damaging potential of engineered nanomaterials. Biomaterials 30, 3891–3914.

Singh, N., Jenkins, G.J.S., Asadi, R., Doak, S.H., 2010. Potential toxicity of superparamagnetic iron oxide nanoparticles (SPION). Nano Rev. 1, 5358.

Singh, N., Jenkins, G.J.S., Nelson, B.C., Marquis, B.J., Maffeis, T.G.G., Brown, A.P., Williams, P.M., Wright, C.J., Doak, S.H., 2012. The role of iron redox state in the genotoxicity of ultrafine superparamagnetic iron oxide nanoparticles. Biomaterials 33, 163–170.

Stoccoro, A., Karlsson, H.L., Coppedè, F., Migliore, L., 2013. Epigenetic effects of nano-sized materials. Toxicology 313, 13–14.

Takagi, A., Hirose, A., Nishimura, T., Fukumori, N., Ogata, A., Ohashi, N., Kitajima, S., Kanno, J., 2008. Induction of mesothelioma in p53+/- mouse by intraperitoneal application of multi-wall carbon nanotube. J. Toxicol. Sci. 33, 105–116.

Trouiller, B., Reliene, R., Westbrook, A., Solaimani, P., Schiestl, R.H., 2009. Titanium dioxide nanoparticles induce DNA damage and genetic instability in vivo in mice. Cancer Res. 69, 8784–8789.

Unfried, K., Schurkes, C., Abel, J., 2002. Distinct spectrum of mutations induced by crocidolite asbestos: clues for 8-hydroxydeoxyguanosine-dependent mutagenesis in vivo. Cancer Res. 62, 99–104.

van Berlo, D., Wilhelmi, V., Boots, A.W., Hullmann, M., Kuhlbusch, T.A., Bast, A., Schins, R.P., Albrecht, C., 2014. Apoptotic, inflammatory, and fibrogenic effects of two different types of multi-walled carbon nanotubes in mouse lung. Arch. Toxicol. 88, 1725–1737.

Vogelstein, B., Papadopoulos, N., Velculescu, V.E., Zhou, S., Diaz, Jr., L.A., Kinzler, K.W., 2013. Cancer Genome Landscapes. Science 399, 1546–1558.

Wang, J.J., Sanderson, B.J., Wang, H., 2007a. Cyto- and genotoxicity of ultrafine TiO_2 particles in cultured human lymphoblastoid cells. Mut. Res. 628, 99–106.

Wang, J.J., Sanderson, B.J., Wang, H., 2007b. Cytotoxicity and genotoxicity of ultrafine crystalline SiO2 particulate in cultured human lymphoblastoid cells. Env. Mol. Mutagen. 48, 151–157.

Wang, L., Li, Y.F., Zhou, L., Liu, Y., Meng, L., Zhang, K., Wu, X., Zhang, L., Li, B., Chen, C., 2010. Characterization of gold nanorods in vivo by integrated analytical techniques: their uptake, retention, and chemical forms. Anal. Bioanal. Chem. 396, 1105–1114.

Wang, L., Liu, Y., Li, W., Jiang, X., Ji, Y., Wu, X., Xu, L., Qiu, Y., Zhao, K., Wei, T., et al., 2011. Selective targeting of gold nanorods at the mitochondria of cancer cells: implications for cancer therapy. Nano Lett. 11, 772–780.

Wang, A., Pu, K., Dong, B., Liu, Y., Zhang, L., Zhang, Z., Duan, W., Zhu, Y., 2013. Role of surface charge and oxidative stress in cytotoxicity and genotoxicity of graphene oxide towards human lung fibroblast cells. J. Appl. Toxicol. 33, 1156–1164.

Worle-Knirsch, J.M., Pulskamp, K., Krug, H.F., 2006. Oops they did it again! Carbon nanotubes hoax scientists in viability assays. Nano Lett. 6, 1261–1268.

Xing, Y., Xiong, W., Zhu, L., Osawa, E., Hussin, S., Dai, L., 2011. DNA damage in embryonic stem cells caused by nanodiamonds. ACS Nano 5, 2376–2384.

Xu, A., Chai, Y., Nohmi, T., Hei, T.K., 2009. Genotoxic responses to titanium dioxide nanoparticles and fullerene in gpt delta transgenic MEF cells. Part. Fibre Toxicol. 6, 3.

Xu, J., Sagawa, Y., Futakuchi, M., Fukamachi, K., Alexander, D.B., Furukawa, F., Ikarashi, Y., Uchino, T., Nishimura, T., Morita, A., Suzui, M., Tsuda, H., 2011. Lack of promoting effect of titanium dioxide particles on ultraviolet B-initiated skin carcinogenesis in rats. Food Chem. Toxicol. 49, 1298–1302.

Niwa, Y., Iwai, N., 2006. Genotoxicity in cell lines induced by chronic exposure to water soluble fullerenes using micronucleus test. Environ. Health Prev. Med. 11 (6), 292–297.

Yokohira, M., Hashimoto, N., Yamakawa, K., Suzuki, S., Saoo, K., Kuno, T., Imaida, K., 2009. Lung carcinogenic bioassay of CuO and TiO_2 nanoparticles with intratracheal instillation using F344 male rats. J. Toxicol. Pathol. 22, 71–78.

Zhong, B.Z., Whong, W.Z., Ong, T.M., 1997. Detection of mineral-dust-induced DNA damage in two mammalian cell lines using the alkaline single cell gel/comet assay. Mut. Res. 393, 181–187.

Zhu, L., Chang, D.W., Dai, L., Hong, Y., 2007. DNA damage induced by multiwalled carbon nanotubes in mouse embryonic stem cells. Nano Lett. 7, 3592–3597.

Glossary

Absorption, Distribution, Metabolism, Excretion (ADME) Terms used to describe the disposition of a pharmaceutical compound in an organism. The route of administration critically influences ADME.

Adverse effect Changes in an organism that are caused by exposure to a substance that result in impairment of functional capacity or impairment of the capacity to compensate for additional stress or that results in an increase in susceptibility to the harmful effects of other environmental influences.

Adverse outcome pathways (AOPs) Structured representation of biological events leading to adverse effects. AOPs are a conceptual construct linking existing knowledge regarding a molecular initiating event to an adverse outcome occurring at a level of biological organization relevant to risk assessment.

Aerodynamic diameter The notional dimension that is a function of the actual diameter, the shape and the density that determines where in the respiratory tract that particles will deposit. ENM deposit in the distal tract of the respiratory system.

Agglomerate Collection of weakly bound nanoparticles. The forces holding an agglomerate together are weak forces, for example, van der Waals forces, or simple physical entanglement.

Aggregate Particle comprising strongly bonded or fused particles where the resulting external surface area may be significantly smaller than the sum of calculated surface areas of the individual components. The forces holding an aggregate together are strong forces, for example, covalent bonds.

Airways (bronchi, bronchioles) The tubes that connect the outside air to the lung parenchyma.

Allergen Type of antigen that produces an abnormally vigorous immune response (antigen, a substance capable of inducing an immune response). Can be found in a variety of sources, for example, food, pollen.

Allergic reactions (or hypersensitivity reactions) The result of exaggerated and inappropriate immune reactions that are triggered by substances which usually cause little or no problems in most people. Hay fever, atopic dermatitis, allergic asthma, and anaphylaxis are all examples of allergic reactions.

Alveolar lipoproteinosis Severe type of lung injury where the alveolar spaces are filled by lipoprotein.

Alveoli The very distal portion of the lungs, devoted to gas exchange with the blood.

Ames test Widely employed method that uses certain strains of bacteria to test whether a xenobiotic causes mutations.

Anaphylaxis Severe and potentially life-threatening allergic reaction. Common triggers include insect bites and stings, foods, and medication.

Aneuploidy A state where a cell has gained or lost individual or multiple chromosomes leading to numerical changes in chromosome copy number.

Antigen sampling Uptake and delivery of proteins and/or peptides to subepithelial lymphocytes to generate immune responses. This process may be relevant for nanoparticles in the gastrointestinal tract.

Apoptosis Form of programmed cell death, that is, an energy-consuming, genetically regulated program of cell "suicide," which leads to removal of dying cells without damage to the surrounding tissues.

Asthma Chronic inflammatory disorder of the lungs that is characterized by recurrent episodes of airflow obstruction, bronchial inflammation, mucus hypersecretion, and airway hyperresponsiveness.

Atherosclerosis (or hardening of the arteries) A disease in which plaques build up inside the arteries.

Autophagy (self-eating) A process that allows the degradation and recycling of cellular components. In autophagy, targeted cytoplasmic constituents are isolated in a vesicle known as the autophagosome that eventually fuses with lysosomes whereupon the contents are degraded and recycled.

Bayesian methods Methods of statistical inference in which some evidence or observations are used to calculate the probability that a hypothesis may be true, or else to update its previously calculated probability. Bayesian comes from its use of the Bayes' theorem in the computational process.

Benchmark materials Materials which have been tested and evaluated according to standard criteria and to which new materials may reliably be compared.

Adverse Effects of Engineered Nanomaterials. http://dx.doi.org/10.1016/B978-0-12-809199-9.00019-7
Copyright © 2017 Elsevier Inc. All rights reserved.

Bioavailability The extent to which a compound (e.g., nanomaterials) reaches the systemic circulation.

Bio-corona Adsorbed material, such as proteins, lipids, ions, organic matter, on a nanoparticle surface. It is believed that all nanoparticles entering the human body are rapidly coated with a bio-corona.

Biological identity (of nanomaterials) The adopted physicochemical properties of nanomaterials once they come in contact with biomolecules, leading to bio-corona formation; the primary nano-biointerface.

Biological monitoring Continuous or repeated measurement of potentially toxic substances, their metabolites, or their biochemical effects in tissues, secreta, excreta, expired air, or any combination of these. Its purpose is to evaluate occupational or environmental exposure and health risk by comparison with appropriate reference values based on knowledge of the probable relationship between ambient exposure and resultant adverse health effects. This approach is in its early infancy for nanomaterials.

Biomarker An indicator signaling an event or a condition in a biological system and giving a measure of exposure, effect, or susceptibility. In reference to biological monitoring, a biomarker is the presence of any substance, or a change in any biological structure or process that can be measured as a result of exposure.

Biopersistence The extent to which compounds remain in an organism while retaining their structure.

Blood–brain barrier (BBB) Physical barrier between the central nervous system (CNS) and the circulating blood, consisting of tight junctions in the endothelial cells of the capillaries that restrict the diffusion of large molecules.

Bronchoalveolar lavage (BAL) Minimally invasive technique that may provide information about diagnosis and yield insights into immunologic, inflammatory, and infectious processes taking place at the alveolar level. BAL fluid, the liquid that is obtained through this procedure (in humans or animals).

Brush-border Surface coating of intestinal epithelial cells formed by densely packed microvilli. This structure greatly enlarges the surface of these cells.

Carbon nanotubes Seamless tubular structures of graphite that can be either single-walled carbon nanotubes (SWCNT), comprising a single layer of carbon atoms, or multiwalled carbon nanotubes (MWCNT), comprising multiple concentric tubes. These materials are among the most important ENM.

Carcinogenicity Process of induction, promotion, and progression of cancer.

Central Nervous System (CNS) The part of the nervous system consisting of the brain and spinal cord.

Chemokines Cytokines identified on the basis of their ability to induce chemotaxis (migration) of immune cells.

Chronic obstructive pulmonary disease (COPD) Lung disease characterized by long-term poor airflow. Tobacco smoking is the most common cause, while other factors, such as air pollution also play a role. In contrast to asthma, the airflow reduction does not improve with the use of a bronchodilator.

Circadian rhythm Any biological process that displays an endogenous oscillation of about 24 h.

Clastogenicity Giving rise to or inducing breakage of chromosomes.

Clearance The processes whereby particles that deposit in biologic compartments (tissues) are removed and lost from the body.

Colony forming efficiency assay (CFE) In the CFE assay single cells are seeded at low density, and colonies (with minimum 50 cells) are counted at fixed time points after exposure. Thus both viability (colony number) and proliferative capacity (colony size) are measured in this viability assay.

Combustion-derived nanoparticles Particles originating from a number of sources including diesel soot and welding fumes. A substantial body of literature demonstrates that these pose a hazard to the lungs.

Comet assay (or single cell gel electrophoresis assay, SCGE) One of the most common tests for exploring the genotoxic potential of nanomaterials. The assay detects single and double-stranded DNA breaks by measuring the migration of DNA from individual cell nuclei following alkaline treatment. The term "comet" refers to the pattern of DNA migration through the gel, which often resembles a comet.

Complement System of serum proteins that collectively enhances (complements) the ability of antibodies to promote phagocytic clearance of microbes from the organism. Complement-mediated mechanisms are proinflammatory and play an important role for early and unspecific immune responses.

Condensation particle counter (CPC) Provides real-time number concentration measurements within their particle diameter detection limits. Without a nanoparticle preseparator, these counters are not specific to the nanometer size range. This instrument, used to assess nanoparticle concentrations in occupational settings, is generally sensitive to particles greater than 10–20 nm in diameter.

Contact dermatitis A type of inflammation of the skin resulting either from exposure to allergens or irritants.

Cytokines Collection of small proteins that play a role in cell signaling especially in the immune system.

Danger signals Compounds that activate the immune system; can be endogenous—released by tissues undergoing stress, damage, or abnormal death—and exogenous signals, expressed by pathogens.

Danger-associated molecular patterns (DAMPs) Host biomolecules that initiate and perpetuate a noninfectious (sterile) immune response, in contrast to pathogen-associated molecular patterns (PAMPs).

Dendritic cells Type of mononuclear leukocytes or white blood cells specialized in antigen presentation.

Descriptor A number, used to identify molecules, allowing for mathematical treatment of the chemical information contained in the molecule; a molecular property that QSAR can calculate. The identification of "nanoparticle-specific" descriptors represents an important aspect of computational nanotoxicology.

Developmental toxicity Any effect interfering with normal development before and after birth, up to and including puberty.

Diffusion chargers Provide estimates of external aerosol surface area when airborne particles are smaller than 100 nm. Instrument used to measure nanoparticle concentration in an occupational setting.

DNA adducts Formation of a complex between a DNA base and a chemical group through covalent bonding.

DNA lesion Altered form of DNA or a base that forms following exposure to a genotoxic substance.

Dose Total amount of a substance administered to, taken up or absorbed by an organism. The dose is a crucial aspect in determining if and to what extent engineered nanomaterials cause adverse effects in humans.

Dose–effect relationship Association between dose and the magnitude of a continuously graded effect, either in an individual or in a population or in experimental animals.

Dose–response relationship Association between dose and the incidence of a defined biological effect in an exposed population.

Dustiness The propensity of a material to generate airborne dust during its handling.

Dynamic light scattering (DLS) Commonly used nondestructive technique that measures particle size in liquid dispersions.

Electron energy loss spectroscopy (EELS) Provides valuable information on ENM surface chemistry.

Electrophoretic light scattering (ELS) Used to determine the zeta potential of dispersed nanomaterials.

Embryonic stem cell test (EST) An alternative test validated by ECVAM for testing embryotoxicity. EST uses two permanent cell lines: murine 3T3 fibroblasts and murine embryonic stem cells (ESCs).

Endocrine disruptors (EDs) Chemicals or compounds that can interfere with endocrine (hormone) systems.

Endotoxins Heat-stable components of the outer membranes of certain Gram-negative bacteria (see LPS).

Engineered nanomaterials (ENMs) Nanosized materials intentionally engineered and produced with very specific properties or compositions (e.g., shape, size, surface properties, and chemistry) that distinguish them from other man-made (welding fume, diesel exhaust) and/or environmental particulates.

Endocytosis Generic term for the uptake of material by cells through invagination of the plasma membrane.

Eosinophils Type of leukocyte (white blood cell) that is involved in inflammatory and allergic type reactions.

Epigenetics The study of heritable changes in gene expression that do not involve changes to the DNA sequence.

Ex vivo (tissue cultures) Represent a model between in vitro and in vivo, where whole tissue slices are cultured outside the body. In such models the cyto-architecture is retained as well as many of the intercellular connections.

Fenton chemistry Oxidation of materials by hydrogen peroxide using an iron catalyst.

Fiber paradigm Structure-activity paradigm in toxicology predicting adverse effects of a man-made fiber on the basis of the length (> 20 μm), diameter (< 3 μm), and biopersistence. Some multiwalled carbon nanotubes, and some other man-made materials, for example, asbestos fibers, have these characteristics.

Fibrosis The formation of excess connective tissue in an organ or tissue in a reparative or reactive process.

Field-flow fractionation (FFF) Very powerful chromatographic method and can, in principle, separate particles from 1 nm up to several microns, regardless of their chemical composition.

Fourier transformed infrared spectroscopy (FTIR) Nondestructive, qualitative, and in some cases quantitative method that can provide information about organic and inorganic structures by analyzing their vibrational modes.

Frustrated phagocytosis The incomplete removal of fiber-like material (see fiber paradigm) by (alveolar) macrophages.

Fullerene Closed-cage structure having more than 20 carbon atoms, about 1 nm in diameter. Spherical fullerenes, also referred to as Buckminsterfullerenes or buckyballs, resemble a (soccer) football.

Functionalization The purposeful insertion of chemical groups to the surface of nanomaterials in order to modify their physicochemical properties. Generally performed for nanomaterials for biomedical applications, for instance, to increase biocompatibility and enable targeted delivery (see stealth).

Gastrointestinal tract (GIT) (or digestive tract) Consists of the esophagus, stomach, and small and large intestines.

Genotoxicity Toxicity related to genetic endpoints, for example, DNA damage, chromosomal aberrations.

Glial cells Supportive cells in the central nervous system; the most abundant type of glia is the astrocyte.

Glycocalyx Glycoprotein-polysaccharide covering on the surface of the cell membrane of epithelial cells.

Gonads The ovaries in the female, that produce eggs and the testicles in the male, that produce sperms.

Gut-associated lymphoid tissues (GALT) The immune system of the gastrointestinal tract consisting of: the tonsils, Peyer's patches, and other lymphoid aggregates or diffusely distributed lymphoid cells.

Hazard Set of inherent properties of a substance, mixture of substances, or a process involving substances that, under production, usage or disposal conditions, make it capable of causing adverse effects.

Hemolysis The rupturing of red blood cells.

High-aspect ratio nanoparticles (HARN) Engineered nanomaterials with one dimension that greatly differs from the other conferring to the nanomaterial a "fiber-like" aspect (see fiber paradigm).

High-throughput screening (HTS) A process which leverages automation to quickly assay the biological or biochemical activity of large numbers of compounds, especially in the drug discovery process.

Hormones Signaling molecules that are secreted into the blood and carried to distant target organs to regulate physiology and behavior.

Hyperreactivity (or, airway hyperreactivity, AHR) State characterized by an easily triggered bronchospasm (contraction of the small airways). AHR is a hallmark of asthma, but also occurs frequently in patients suffering from COPD.

In silico A process carried out by a computer.

Inductively coupled plasma mass spectrometry (ICP-MS) Highly sensitive type of mass spectrometry. Inductively coupled plasma produces ions and the mass spectrometer separates and detects the ions. Can be used to determine the levels of a range of metal (and some nonmetal) nanomaterials down to nanogram per gram.

Inflammasome Intracellular multiprotein complex which regulates the activation of caspase-1. Activation of this protease leads to cleavage of the proforms of the inflammatory cytokines interleukin (IL)-1β and IL-18, which results in release of their active forms and, consequently, in inflammation.

Inflammation (from the Latin, *to set on fire***)** An adaptive response involving soluble factors and cells of the immune system that is triggered in response to infection, trauma, ischemia, toxic, or other injury.

Interstitium The anatomic compartment underlying the epithelial layer containing mesenchymal tissue and blood vessels.

LC_{50} Concentration that is lethal for 50% of the cells. One of the standard concentrations used to assess the toxicity of a substance.

Lipopolysaccharide (LPS) (also known as endotoxin) A heat-stable compound from the outer membrane of Gram-negative bacteria. LPS is one of the most powerful proinflammatory stimuli and LPS contamination of nanomaterials is common, and should not be overlooked when assessing for immune effects of such materials. Limulus amebocyte lysate (LAL) is a common test for LPS content.

Lung parenchyma The parts of the lung where gas exchange occurs.

M cells Epithelial cells found in the gut-associated lymphoid tissues (see earlier) which have high capacity for transporting microbes and particles as part of their antigen-sampling function. Also known as microfold cells or membranous cells, but neither of these terms provides a fully satisfactory description of their morphology so both terms have been usurped by the abbreviated name.

Machine learning Explores the study and construction of algorithms that can learn from and make predictions on data.

Macrophages Mononuclear leukocytes (white blood cells) with a major role in eliminating foreign or dangerous entities. Macrophages are scavengers, able to phagocytose, and degrade foreign material. The term macrophage is usually adopted for tissue resident cells (such as alveolar macrophages in the lungs), while the term "monocyte" designates the immature cells circulating in the blood.

Magnetic resonance imaging (MRI) Medical imaging test that uses strong magnetic fields and radio waves to create anatomical images of the body and physiological processes in both health and disease.

Microbiome The collection of microorganisms and their genomes living in symbiotic relationship with humans on and inside the body. The gut microbiome is often referred to as the "forgotten organ."

Micronucleus test Test for genotoxic carcinogens that detects both clastogenic and aneugenic effects and is suitable for detection of genotoxicity of a wide range of compounds including nanomaterials.

Microvilli Hair-like projections on the surface of cells formed by membrane protrusions with cytoskeletal scaffold. In the intestine, these increase the surface area for absorption of nutrients.

Molecular dynamics (MD) Method for modeling the dynamic behavior of molecules or materials using Newtonian mechanics where molecules are treated as masses and bonds as springs.

Muco-ciliary escalator The cellular processes that waft mucus and any particles that are trapped in it from the airways toward the mouth where it is either swallowed or expelled via coughing.

Mutations Permanent, structural, transmissible changes to the genetic material (usually cellular DNA or RNA).

Myeloid derived suppressor cells Heterogenous group of immune cells defined by their myeloid origin, immature state, and ability to potently suppress T cell responses. They rapidly expand during inflammation, infection, and cancer.

Nanomaterial (EC definition) Natural, incidental, or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1–100 nm.

Nanomedicine The medical application of nanotechnology.

Nanoparticle (NP) Material which has all three dimensions in the nanoscale (of the order of 100 nm or less).

Nanotechnology Design, characterization, production and application of structures, devices, and systems by controlling shape and size at the nanoscale.

Nanotoxicology Study of the toxicity of nanomaterials. Nanotoxicology may also be conceptualized as "the study of the undesirable interference between man-made nanomaterials and cellular nanostructures or nanomachines" (Shvedova et al., 2010. Annu. Rev. Pharmacol. Toxicol. 50, 63–88).

Neutrophil extracellular traps (NETs) Networks of extracellular fibers, primarily composed of nuclear chromatin (DNA and histones) decorated with granule proteins which bind bacteria or fungi.

Nominal size The mean size of a given (nano)material.

Neutron activation Process whereby neutron radiation induces radioactivity in materials, occurs when atomic nuclei capture free neutrons. Methods using radioactive tracers can track movement of NPs in the body.

Neural network Method for generating a QSAR relationship using an algorithm that learns the relationship in an analogous way to how the human brain learns. A key method in computational nanotoxicology.

Neurons Key cells of the nervous system that process and transmit information by electrical and chemical signaling.

Nuclear magnetic resonance (NMR) Diagnostic technique which can detect the presence of contaminants and monomers in the case of organic polymeric nanoparticles. In some cases it can also provide information on the surface functionalization and on the kinetics of functional group release.

Occupational exposure limits (OEL) an upper limit on the acceptable concentration of a hazardous substance in workplace air for a particular material or class of materials. Normally set by regulatory agencies.

Omics analysis The mapping of expression data of a global set of biomolecules (genes, proteins, etc) relevant for life, by means of the identification of specific interrelationships, and the detection of changes in these relationships after physiological or pathological perturbations. Omics studies might thus allow for a more precise understanding of the mechanisms underlying nanomaterial effects.

Optical particle counter (OPC) Provides real time number concentration measurements between their particle diameter detection limits. Particle size diameters begin at 300 nm and may go up to 10.000 nm.

Organisation for Economic Co-operation and Development (OECD) International economic organisation of 34 countries with headquarters in Paris which aims to stimulate economic progress and world trade.

Organ-on-a-chip devices Sophisticated, microfluidics based in vitro models, implementing not only structural, but also mechanical features of the whole living organ. May be amenable to high-throughput testing.

Overload Animal (rat)-specific response whereby normal particle clearance is overcome at high lung burden of low toxicity dust culminating in fibrosis and cancer. Its relevance for human disease is questionable.

Oxidative stress Imbalance between the generation of harmful reactive oxygen species (ROS) and the cells' ability to neutralize these oxidants, resulting in damage to cellular components including proteins, lipids, and DNA. It is considered by many to be the main pathogenetic mechanism of ENMs.

Pathogen-associated molecular patterns (PAMPs) Molecular motifs associated with groups of pathogens, that are recognized by the immune system. One example is LPS, expressed by Gram-negative bacteria.

Pattern recognition receptors (PRRs) Class of innate immune cell-expressed proteins (membrane bound or cytoplasmic) that respond to pathogen-associated molecular patterns (PAMP) and danger-associated molecular patterns (DAMP). Toll-like receptors (TLRs) are one well studied class of PRRs.

Phagocytosis The cellular process of engulfing microorganisms, particles, cell debris by invagination of the cell membrane to form an internal phagosome. Macrophages are professional phagocytes.

Pharmacokinetics and pharmacodynamics Pharmacokinetics is what the body does to a molecule or material as opposed to pharmacodynamics, which is what the molecule or material does to the body.

Photocatalysis The ability of a material to increase the speed of a chemical reaction when exposed to light.

Photon correlation spectroscopy (PCS) Commonly used nondestructive technique that measures particle size in liquid dispersions.

Peyer's patches Organized lymphoid follicles usually found in the lower portion of the small intestine.

Placenta The organ that develops during pregnancy of most mammalian species on the interface between the pregnant organism and the fetus. It functions as an exchange surface for gases and nutrients between the mother and the fetus, without the mixing of maternal and fetal blood.

Pleura The layer that covers the lungs (visceral pleura) and inside of the chest wall (parietal pleura) such that the two pleura are opposed each other during normal breathing. Some fiber-like ENM may be unable to cross the stomata (see this term) and thus become trapped between visceral and parietal placenta. The main concern linked to this event is the potential for the development of mesothelioma, a malignant tumor of the pleura associated with other fiber-like particles, such as asbestos.

Polymorphonuclear leukocytes (PMN) Short-lived white blood cells equipped with an arsenal of oxidative and proteolytic processes for the killing of microorganisms that usually die rapidly upon encounter with the offending agent. PMNs, or neutrophils, can kill microbes intra- and extracellularly.

Positron emission tomography (PET) Medical imaging technique used to obtain images of functional processes in the body through the detection of pairs of photons emitted indirectly by a positron-emitting radionuclide (i.e., ^{11}C, ^{18}F, ^{64}Cu, ^{94m}Tc, ^{124}I, etc) which are introduced to biomolecules.

Primary cell culture Cells taken directly from living tissues or blood and established for growth in vitro.

Quantitative structure-activity relationship (QSAR) Computational modeling method that finds relationships between the intrinsic properties of molecules and their physical or biological properties.

Quantum chemistry Method for calculating properties of molecules or materials by solving Schrodinger's equation; the application of quantum mechanical principles and equations to the study of molecules.

Quantum dots Crystalline, semiconductor nanoparticles that exhibit size-dependent properties due to quantum confinement effects on the electronic states; they exhibit properties that are intermediate between those of bulk semiconductors and those of discrete molecules.

Raman spectroscopy Nondestructive qualitative and in some cases quantitative method that can provide information about organic and inorganic structures by analyzing their vibrational modes. Named after the Indian physicist, Sir. C.V. Raman who was awarded the Nobel Prize in Physics in 1930.

Reference material A material which is used for calibration, method validation, the establishment of metrological traceability, method development, and other quality control purposes. The use of appropriate reference materials is very important for a correct evaluation of experimental studies on ENM.

REACH Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH) is a European Union Regulation that addresses the production and use of chemical substances, and their potential impacts on both human health and the environment. REACH is the strictest law to date regulating chemical substances and it affects industries throughout the world. REACH entered into force in 2007.

Reticuloendothelial system (RES) Consists of the phagocytic cells located in reticular connective tissue. The cells primarily include the Kupffer cells, tissue histiocytes, monocytes, and macrophages in lymph nodes and spleen. Tissues rich in RES cells are preferential sites of accumulation of nanoparticles.

Risk Possibility or expected frequency of occurrence of a harmful event arising from exposure to a chemical or physical agent.

Risk assessment Identification and quantification of the hazard resulting from a specific use or occurrence of a chemical or physical agent. Risk assessment considers any possible harmful effects on individual people or on society of using the chemical or physical agent in the amount and manner proposed and via all possible routes of exposure. Quantification ideally requires the establishment of dose–effect and dose–response relationships in likely target individuals and populations.

Reactive oxygen species (ROS) Oxygen-based ions or radicals that are very chemically reactive. Their excess may cause cellular damage, so a balance is maintained by antioxidants that scavenge or neutralize ROS (see also oxidative stress).

Scanning electron microscopy (SEM) Imaging technique that provides highly magnified images of surface morphology (10–50 nm resolution).

Scanning mobility particle sizer (SMPS) This instrument measures particle diameter from 2.5 to 1.000 nm and can display data as a size and number distribution.

Scanning probe microscopy (SPM) Branch of microcsopy that forms images of surfaces using a physical probe that scans the specimen; includes atomic force microscopy (AFM) and scanning tunneling microscopy (STM). In contrast to SEM and TEM, in SPM nanoparticles and aggregates/agglomerates can in principle be measured along all three dimensions (maximum length, width, and height).

Secondary ion mass spectroscopy (SIMS) Technique which allows the identification of trace elements, organic molecules, or polymers on the surface of nanomaterials with sensitivity higher than 1 ppm.

Single particle (sp) ICP-MS Provides information on size and size distribution of NPs based on measurement of highly diluted nanoparticle dispersions by ICP-MS operated in time resolved mode for a preselected mass-to-charge ratio.

Single photon emission computed tomograpy (SPECT) Nuclear imaging technique using gamma rays emitted by radionuclides. Similar to conventional nuclear planar imaging, SPECT imaging is performed by using a gamma camera to acquire multiple 2D projection images from multiple angles. Its performance may be improved by ENM and, conversely, may be useful for ENM tracking in vivo.

Size selective sampler (SSS) Cascade impactors are currently the only devices giving a direct estimation of mass concentration with a cut-off around 100 nm and are used to detect nanomaterials in occupational settings.

Small angle X-ray scattering (SAXS) Method based on elastic scattering of X-rays of wavelength 0.1–0.2 nm. Provides information about the shape and size of nanoparticles, characteristic distances of partially ordered materials, pore sizes, and many other types of data.

Standard operating procedures (SOPs) Step-by-step instructions of key processes aimed to ensure consistent and quality output.

Stealth (in nanomedicine) Conferring nanoparticle avoidance of immune recognition and clearance from the blood circulation; this is frequently accomplished by surface coating with hydrophilic polymers, for example, poly(ethylene glycol) that prevent particle opsonization or bio-corona formation and phagocytosis.

Stomata Small holes in the parietal pleura involved in clearance of particles and fibers (see pleura).

Stratum corneum The outermost layer of the epidermis, providing a protective barrier against the outside world.

Surface area Ideally, the surface area of particles involved in biological interactions (biologically relevant particle surface area). This is perhaps the best metric associated with the biological activity of ENM.

Surface plasmon field Property of certain metal nanoparticles, such as gold nanoparticles to generate oscillating electrons around the metal surface with the application of light. This property is very useful to improve the performance of some diagnostic imaging techniques, such as MRI (see earlier).

Surfactant (pulmonary) Phospholipid and protein layers located in the lungs at the air-liquid interface and made up of 85%–90% phospholipids and about 10% surfactant proteins. Surfactant reduces surface tension and increases pulmonary compliance. Inhaled ENM are coated with surfactant.

Surfactant (surface active agent) Compound that lowers the surface tension of a liquid, allowing dispersion of insoluble compounds in solution. Frequently used in experiments involving ENM, thereby posing the problem of interpretation of the data, due to the potential intrinsic toxicity of some surfactants.

Synthetic identity (of nanomaterials) The intrinsic physicochemical properties of pristine (naked) ENMs.

Teratogens Chemicals or environmental agents that are able to interfere with embryonic or fetal development.

Test guideline (TG) The OECD *Guidelines for the Testing of Chemicals* is a collection of about 150 of the most relevant internationally agreed testing methods used by government, industry, and independent laboratories to identify and characterize potential health and environmental hazards of chemicals.

Theranostic Describes a nanoparticle that can be used both for diagnostic and therapeutic applications.

Thermogravimetric analysis (TGA) Destructive method that can provide information on phase changes (both amorphous and crystalline).

Tight junctions Areas of close membrane contact between adjacent epithelial cells, underpinned by protein complexes, that form a barrier to the movement of material between cells and separate the distinct apical and basolateral membrane domains. They may nevertheless be crossed by ENM.

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) A surface-sensitive analytical method that uses a pulsed ion beam to remove molecules from the very outermost surface of the sample. ToF-SIMS provides elemental, chemical state, and molecular information from surfaces of solid materials. The average depth of analysis for a TOF-SIMS measurement is approximately 1 nm.

ToxCAST (toxicity forecaster) Part of the toxicology in the 21st century (Tox21) federal collaboration in the United States and comprises high-throughput toxicity data on thousands of chemicals.

Toxicokinetics and toxicodynamics The branch of toxicology that studies how toxicants are absorbed by, distributed in, metabolized by, and eliminated from the living systems. The pathogenetic mechanisms by which a toxin causes toxicological effects are called toxicodynamics. The terms particokinetics and particodynamics may be used when studying these processes for ENM.

Transepithelial electrical resistance (TEER) Method which allows an indirect evaluation of the integrity of a cell barrier.

Transmission electron microscopy (TEM) Imaging technique that uses a beam of highly energetic electrons to examine objects very closely, on a fine scale (resolution <10 nm). Very useful for the detection of ENM in biological samples.

Ultrafine particles (UFP) Particles with a nominal diameter (such as geometric, aerodynamic, mobility, projected area, or otherwise) of 100 nm or less. The term is often used in the context of particles produced as a by-product of a process, such as fuel combustion, for example, incidental nanoparticles.

Working party on manufactured nanomaterials (WPMN) Established in 2006 under the OECD Chemicals Committee, focuses on issues related to nanomaterials impacting human and environmental health and safety. A key endeavor is the Sponsorship Programme for the Testing of Manufactured Nanomaterials, through which the OECD WPMN agreed on a list of 14 (initially, later 13) "commercially relevant" representative manufactured nanomaterials (RMNs), that is, nanomaterials which are already on or will soon enter the market, to be investigated under the WPMN.

X-Ray diffraction One of the most commonly used techniques to resolve the crystal structure of ENMs.

X-ray photon spectroscopy (XPS) Nondestructive technique commonly used for the determination of surface elemental composition of ENMs.

Zeta potential (surface charge) The potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed particle. Provides information about stability of colloidal dispersions. It is not measurable directly but can be calculated using theoretical models and an experimentally determined electrophoretic mobility or dynamic electrophoretic mobility.

Index

A

N-Acetylcystein, 427
ACGIH. *See* American Conference of Governmental Industrial Hygienists (ACGIH)
Acoustic startle reaction, 412
ACTH. *See* Adrenocorticotropic hormone (ACTH)
ADH. *See* Antidiuretic hormone (ADH)
Adrenocorticotropic hormone (ACTH), 343
Adsorbing proteins, 6
Aerodynamic equivalent diameter (D_{ae}), 227–228
  translocation, 227
Aged ENM, 108, 291
Air pollution, as chronic source of neuroinflammation, 302
  neuropathology instigating CNS disease, 302
  ROS production, 302
Airway inflammation, 244
  chronic allergen-induced, 248
  endogenous reactive oxygen species, 244
  exogenous reactive oxygen species, 244
  reactive nitrogen species, role in, 244
Allergens, 243, 327
  birch pollen, 327
  birch pollen, 245
  grass pollen, 327
  house dust mite, 327
  house dust mite, 245
  timothy grass pollen, 245
Allergic airway inflammation, 246, 247, 250
  characteristic features of
    eosinophilia, 246
    goblet cell hyperplasia, 246
Allergic contact dermatitis, 370
Allergic pulmonary inflammation, 246–247
Allergic reactions
  asthma and, 243–244
Allergy, 244–246. *See also* Hypersensitivity reactions
  engineered nanoparticles (ENP) and, 244–246
A549 lung carcinoma cells, 440
Alzheimer's disease, 87
American Conference of Governmental Industrial Hygienists (ACGIH), 361
Ames test, 67
Amyloid-beta peptide, 87
Amyloidogenic peptides
  autocatalytic self-assembly, 93
Aneuploidy, 68, 425
Animal testing, 212
Antidiuretic hormone (ADH), 343
Antigen-sampling M cells, 386
Antimicrobial Ag ions, 375
Antioxidant defense system, 439
Antioxidant enzymes, 403
Apoptosis, 328, 429
Argyria, 374
Asthma, 243–244
  allergic asthma, 244
    ovalbumin for study of, 248
    single walled carbon nanotubes (SWCNT) induced, 248
  bronchial asthma, 244
  oxidative stress in, 244
  pathogenesis of, 244
Astrocytes, 295
Atherosclerosis, 263
Atherosclerotic plaques, 261
Atherothrombosis, 256
Atomic force microscopy (AFM), 24
Atopic eczema, 362
ATPase, 292
ATP-mediated intercellular signaling, 405
Attached ENM, 108
Australian Therapeutics Goods Administration, 367, 368
Autoimmune diseases
  role of nanoparticles in pathogenesis of, 319
  treatment of, 331
Autoimmunity, 319
Autophagosomes, 282
Autophagy, ENM induced, 282

B

Bacterial endonuclease III (Endo III), 65
Bacterial lipopolysaccharide, as potent activator of innate immunity, 330
Badimon chamber, 268, 269
  porcine aortic strips from, 269
BAL. *See* Bronchoalveolar lavage (BAL)
Bayesian neural networks, 89

BBB. *See* Blood-brain barrier (BBB)
BED. *See* Biologically active dose (BED)
Biocidal products, 170
Biodistribution, 52–58
Biokinetics, 212
Biological barriers, 52
Biologically active dose (BED), 105
Biologically relevant entity, 94
Biological monitoring (BM) strategy, application of, 127
  epidemiology, 141–144
  in health practice, 127
  health surveillance, 141–144
  for occupational health risk assessment, 127
Biomarkers
  assessing exposure to harmful substances, used in, 127
  development for engineered nanomaterials, issues in, 128
    toxicokinetic considerations, 128
    toxicology research, 128
  epigenetic biomarkers, 135–137
    epigenetic mechanisms, 136
    intercellular communication, 137
      biogenesis, 137
      exosomes, 137
      microvesicles, 137
      target cells, 137
  functional categories
    of effect, 127
    of exposure, 127
    of susceptibility, 127
  genetic biomarkers, 135–136
  quality of, 147
  systemic biomarkers, 134–135
    adhesion of endothelial cells, 134
    fibrinolysis, regulation of, 134–135
    tissue factor, 134
  as tools to protect workers, 127
Biomarkers of effects, 131
  definition of, 131
  inflammatory response, 131
Biomarkers of exposure, 128
  factors affecting, 128–131
    deagglomeration, 130
    zinc concentrations, 129
Biomolecule corona, 4
  composition, 5
  dynamic formation of, 6
  impact on human systems, 7
    entry routes for NPs, 9
    microbiome interactions, 10
    in vitro studies, 8
    in vivo studies, 8
    nanotoxicity and, 10
    protein-binding kinetics during, temporal evolution of, 7
Biomolecules, 128
Biomonitoring programs
  ethical aspects of, 146–147
  practical aspects of, 146–147
Biopersistence, 428
Blood-brain barrier (BBB), 277, 278, 300, 343
Blood-placental barrier (BPB), 344
Blood-testis-barrier (BTB), 400
BM. *See* Biological monitoring
BMEC. *See* Brain microvascular endothelial cell (BMEC)
Bone marrow cells, 440
BPEI coated silver nanoparticles. *See* Branched polyethylenimine (BPEI) coated silver nanoparticles
Brain microvascular endothelial cell (BMEC), 283
Branched polyethylenimine (BPEI) coated silver nanoparticles, 96
Bronchial epithelial cells
  metal oxide nanoparticles, effect of, 92
Bronchial hyperresponsiveness, 244
Bronchiolitis obliterans organizing pneumonia, 112
Bronchoalveolar lavage (BAL), 70
Brownian motion, 109, 407
Brunauer-Emmet-Teller (BET) method, 27
BTB. *See* Blood-testis-barrier (BTB)
Buckyballs, 372

C

Caco-2 cells, 384, 388
  brush-border differentiation, 387
Caco-2 monolayers
  drawback, 388
Cadmium containing particles
  industrial applications, 409
  medical applications, 409
  renal dysfunction induction, 409
Cadmium telluride (CdTe) NP, 61
Caenorhabditis elegans, 347
Cancer genotoxicity, relation with, 429–430
Capillary hydrodynamic fractionation (CHDF), 25
Carbon-based nanomaterials, properties, 430
Carbon black (CB), 62, 371
  inhalation, effect of, 414
  lung inflammation, 412
  maternal pulmonary exposure, 412
Carbon black particles
  effects on male reproductive parameters, 402
Carbon fiber dermatitis, 370
Carbon-fiber-reinforced polybutylene terephthalate (CFRPBT), 437
Carbon nanomaterials, applications, 366

Carbon nanotubes (CNT), 246–247, 322
  allergen-induced airway inflammation by, 248–249
  allergic responses induced by, mechanism, 249–250
  exposure carcinogenicity activating mechanisms, 436
  multiwalled carbon nanotube (MWCNT), 285, 286, 322
  rod-shaped (rCNT), 246
  single-walled carbon nanotube (SWCNT), 285, 286, 321
Carcinogenesis, 231, 428
Carcinogenicity, 225, 231–232
Cardiovascular disease
  combustion-derived nanoparticles in, 264
  diagnostic applications of nanoparticles in, 256–262
    gold nanoparticles, 262
    lipid-based nanoparticles, 261
    nanotechnology in medical imaging, 257
    superparamagnetic iron oxide nanoparticles, 260–261
  toxicity of engineered nanoparticles in, 265–268
    ex vivo studies, 268
    in vitro studies, 265–267
    in vivo studies, 267
Cardiovascular medicine, role of nanoparticles in, 255
CARS microscopy. *See* Coherent anti-Stokes Raman scattering (CARS) microscopy
Caveoli-dependent endocytosis, 386
CB. *See* Carbon black (CB)
CDNP. *See* Combustion-derived nanoparticles (CDNP)
CdSe quantum dots, 389
  toxicity, 389
CEA. *See* Comprehensive environmental assessment
Cell proliferation, 143, 198
Cells brick and mortar structure, 360
Cell transformation assays (CTA), 67
Cellular oxidative stress, 235
Cellular stress, 233
Cellular uptake visualization methods, 52–58
Cell viability assessment, 62
Central nervous system (CNS), 277
  barriers
    blood-brain barrier, 279, 292
    blood-cerebrospinal fluid, 279
    blood-nerve barriers, 279
    blood-retina barrier, 279
  effects in vivo ENM exposure on, 288–292
    nanoalumina, 289
      adverse effects on mental development, 289
      contribution to Alzheimer's disease, 289
    nanocadmium and quantum dots, 289
    nanocarbon, 290
    nanoceria, 287, 292
    nanocopper, 287, 291
    nanogold, 291
    nanoiron, 291
    nanolead, 291
    nanomanganese, 292
    nanoscale zinc oxide, 292
    nanosilica, 292
    nanosilver, 288
    nanotitania, 282, 286, 288
  ENM interaction with, 283–285
  ENM translocation to and within, 279–282
CeO_2 NPs, 405
Chemical compounds, OECD guidelines
  in vitro tests, 53
  in vivo tests, 55
Cheminformatics methods, 88
Chemotactic factors, 318
Chinese hamster ovary cells, 430
Chitosan, 386
Chromosomal aberrations, 67
  assays, 65
Chromosome painting, 65
Chronic obstructive pulmonary disease (COPD), 333
Citrate-capped nanoparticles, 408
Citrate-coated gold nanoparticles, 94
Clastogenesis, 72
Clastogenicity, 68, 425
Clathrin-dependent endocytosis, 386
C_{60}-NLS-FITC NP cytoplasm bioaccumulation TEM, 372
CNS. *See* Central nervous system (CNS)
CNT. *See* Carbon nanotubes (CNT)
Coagulation, 135
Coagulation/aggregation phenomena, 113
Cobalt-doped tungsten carbide nanoparticle, 390
Cobalt ferrite ($CoFe_2O_4$) magnetic NPs, 344
Coherent anti-Stokes Raman scattering (CARS) microscopy, 56
Colony forming efficiency assay (CFE), 64
Colorimetric chemical markers, 371
Combustion-derived nanoparticles (CDNP), 255, 264, 268
Combustion-derived ultrafine particles, 128
Comet assay, 65
Complement activation, 323
Comprehensive environmental assessment (CEA), 161
Computed tomography (CT), 59
Condensation particles counter (CPC), 113
Consumer Product Safety Commission (CPSC), 167
Consumer products inventory (CPI), 399
Contrast agents, 261
  gadolinium, 261, 265
COPD. *See* Chronic obstructive pulmonary disease (COPD)

Corona
coating, 94
formation, 94
Cosmetic Products Regulation, 174
cosmetic ingredients, testing of, 174
labeling obligation, 174
CPC. *See* Condensation particles counter (CPC)
CPI. *See* Consumer products inventory (CPI)
CPSC. *See* Consumer Product Safety Commission
CrGnRHl. *See* Gonadotropin-releasing hormone gene (CrGnRHl)
Crohn's disease, 390
CT. *See* Computed tomography (CT)
CTA. *See* Cell transformation assays (CTA)
Cultured tail fin biopsy (C-fin) assay, 344
Cumulus-cell-oocyte-complex, 404
Cytochalasin B, 66
Cytochrome P450scc. *See* Cytochrome P450 side-chain cleavage (P450scc)
Cytochrome P450 side-chain cleavage (P450scc), 353
Cytokines, 126, 320
Cytotoxic reactions, immunoglobulin M (IgM) mediated, 243

D

D_{ae}. *See* Aerodynamic equivalent diameter
Damage-associated molecular patterns (DAMPS), 244
DAMPS. *See* Damage-associated molecular patterns (DAMPS)
Danger associated molecular patterns (DAMP), 317
3D cell cultures systems, 74
DCs. *See* Diffusion chargers (DCs)
Dendritic cells, 69, 319
function of, 324
Derived no effect level (DNEL), 97
Desmosomes, 363
Desorbing proteins, 6
Developmental toxicity
nanoparticles, role of, 405–418
carbonaceous particles, 410–412
carbon black, 412
carbon nanotubes, 410
fullerenes, 412
fulleroles, 412
metal-based nanoparticles, 406–409
cadmium containing particles, 409
gold (Au) NPs, 407
silver (Ag) NPs, 408
titanium dioxide (TiO_2), 406
zinc oxide nanoparticles, 409
polystyrene nanoparticles, 413
DHM. *See* Digital holographic microscopy (DHM)
Differential mobility analyzer (DMA), 115
Diffusion chargers (DCs), 115
Diffusion size classifiers (DiSCs), 115
Digital holographic microscopy (DHM), 62
Dimethlybenzanthracene (DMBA), 372
Dimyristoylphosphatidylcholine (DMPC) membrane bilayer, 92
DiSCs. *See* Diffusion size classifiers (DiSCs)
DMA. *See* Differential mobility analyzer (DMA)
DMPC membrane bilayer. *See* Dimyristoylphosphatidylcholine (DMPC) membrane bilayer
DNA alkyl-adducts, 425
DNA damage
comet assay, measurement by, 437
nanomaterials, role of, 429
oxidative stress-mediated, 436
DNA fragmentation, 438
DNA nicking, 438
DNA repair enzymes, 427
DNEL. *See* Derived no effect level (DNEL)
Dose modeling, 209
Dose-response relationship, 285
Drosophila melanogaster, 347
Drug delivery, 257, 261, 277
application of ENM for, 277
receptor-mediated, 277
Dynamic light scattering (DLS), 24

E

EBC. *See* Exhaled breath condensate
EC. *See* European Commission (EC)
ECHA. *See* European Chemicals Agency
EDs. *See* Endocrine disrupters (EDs)
Effective hydrogen bond acidity, 91
Effective hydrogen bond basicity, 91
EFSA. *See* European Food Safety Authority
Electrical low pressure impactor (ELPI), 116
Electric low pressure impactor (ELPI), 113
Electrothermal atomic absorption spectroscopy (ETAAS), 130
ELPI. *See* Electric low pressure impactor (ELPI)
Embryo-fetal mortality, 410
Embryonic stem cells, 410
Embryotoxicity, 405
Endocrine disrupters (EDs), 342
by nanomaterials *vs.* classical endocrine disrupters, 349
Endocrine-disrupting effects, 343
Endocrine functions, 349, 353, 354
biological mechanisms of NMs, 354

Endocrine organs
nanomaterials (NMs), effect of, 342–349
adrenal gland, 348
biological mechanisms, 353–355
gonads, 345–347
mammals, 345–347
other animals, 347
hypothalamus, 343
pancreas, 348
parathyroid gland, 344
pineal gland, 343–344
pituitary gland, 343
thymus, 349
thyroid gland, 344
nanomaterials (NMs) function, effect of, 350–353
exposure patterns, 351–353
physicochemical properties, 351
tissue distribution, 353
Endocytosis
clathrin-dependent, 281
clathrin-independent, 281
Endotoxins, 126
Energy-dispersive X-ray spectroscopy (EDS), 23, 114
Engineered nanomaterials (ENM), 108, 255, 266, 277, 300
adverse effects, 278, 285, 287–303
effects of in vivo ENM exposure, 288–292
on human health, 225, 301–303
pathological reactions, 225
respiratory system, effects on, 225
related to nervous system functions and disease, 287–303
in silico studies of ENM effects, 299–300
in vitro studies of ENM effects, 292–299
behavior characterization, 106–107
biotransformation and persistence of, in nervous system, 286–287
cellular uptake, 56
containing aerosols, 109
effects on blood-brain barrier permeability and function, 280
electron-dense, 56
exposure, 225
routes, 105–106, 278
dermal exposure, 278
gastrointestinal pathway, 278
inhalation, 278
systemic administration, 279
scenarios, 109
to workers, 104
exposure assessment, 255
challenges, 108–111
in workplaces, 111–119
characterization, 117
harmful effects on workers, 112
health surveillance, 119
indirect estimates giving instruments, 116
mass concentration measuring instrumen ts, 116
modifications after release, 117–118
nanoparticles measurement, metrics, 113
nanoparticles measurement, strategies, 113–114
number concentration measuring instruments, 114–115
sample collection, 117
surface concentration measuring instruments, 115–116
workers protection, 118–119
hazards of, 225
health risk assessment, 110
interactions with biological structures, 283–285
life cycle stages, 109
monitoring, 110
occupational exposure limits (OEL), 110
release, 107
respiratory tract, effect on, 225
in silico studies of ENM effects, 299–300
transport across cell membranes and intercellular transport of, 281–282
transport and uptake
in vitro evaluation, 58–60
in vivo evaluation, 58–60
in vitro studies of ENM effects, 292–299
nanocarbon, 296
nanoceria, 297
nanoiron, 295–296
nanomanganese, 298
nanosilica, 297–298
nanosilver, 294–295
nanotitania, 293
quantum dots, 279, 298–299
Engineered nanoparticles (ENP), 255, 266. *See also* Engineered nanomaterials (ENM)
developmental toxicity, 414
hypothalamus-hypothesis-gonad axis, interaction with, 414
resistant to excretion, 416
risk assessment of, 255
ENM. *See* Engineered nanomaterials (ENM)
ENP. *See* Engineered nanoparticles (ENP)
Environmental protection, 176–177
Hazardous Waste Directive, 177
Epigenetic mutations, 429
ER. *See* Estrogen receptor (ER)
Escherichia coli, 89, 375
Estrogen receptor (ER), 354
Estrous cyclicity, 405
ETAAS. *See* Electrothermal atomic absorption spectroscopy
5-Ethylisopropyl amiloride (EIPA), 58

EU-OSHA. *See* European Agengy for Safety and Health at Work (EU-OSHA)
European Agengy for Safety and Health at Work (EU-OSHA), 107
European Chemicals Agency (ECHA), 172, 191
European Commission (EC), 103
European Food Safety Authority (EFSA), 172
European Scientific Committee on Consumer Products, 368
Exhaled breath condensate (EBC), 130
Exposure assessment, 190
Exposure to nanomaterials, risk assessment, 190, 191
Extraembryonic tissues (EET), 345, 408
Ex vivo organotypical slice cultures, 60
Ex vivo tissue slice cultures, 74

F
FAE. *See* Follicle-associated epithelium (FAE)
Fao cells, 348
Fast mobility particle sizer (FMPS), 113
Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), 166
Federal safety standards, for workers, 168
Female germ cells, 400
Fertility, 400–405
  female fertility, 404–405
  male reproductive function, 400–404
Fibrillar keratin, 363
Fibrosis, 139, 231
Fibrotic gene expression, 435
Field flow fractionation (FFF) techniques, 25
FIFRA. *See* Federal Insecticide, Fungicide, and Rodenticide Act
Fluorescence in situ hybridization, 65
Fluorescence-mediated tomography (FMT), 257
FMPS. *See* Fast mobility particle sizer (FMPS)
FMT. *See* Fluorescence-mediated tomography (FMT)
Follicle-associated epithelium (FAE), 386
Follicle-stimulating hormone (FSH), 343
Food legislation, 174–176
  for contact materials, 175
  for food additives, 175
  for food supplements, 175
  Novel Food Regulation, 174
  toxicity testing, 175
Food packaging safety, 383
Food Safety, 167
  Medical Applications, 167
Foreign body scavenging systems, 52
Fractionation techniques, 25
Franz cells, 368
Franz diffusion cells, 370
Free radical scavenger, 245
FSH. *See* Follicle-stimulating hormone (FSH)
Fullerene (C_{60})
  amino acid-derivatized peptide NP, 363
  antioxidant properties, 430
  antitumor effects, 430
  application, 372
  based therapies, 370
  basophilic allergic responses, role in, 370
  complex formation with DNA, 430
  functionalized, 371
  mast cell inhibition, role in, 370
  medicinal applications, 430
  technological applications, 430

G
GALT. *See* Gut-associated lymphoid tissues (GALT)
Gametogenesis, 400
Gastrointestinal tract
  barriers, 385–386
  conditions, 385–386
  nanomaterials
    potential toxicity, 389–390
  nanomaterials uptake, 386–389
    in vitro models to study uptake, 387–389
Gene expression, 136
Gene mutation assays, 66, 67
Genotoxicity, 198, 230–232
  carbon-based nanomaterials, 430–437
    carbon nanotubes (CNTs), 435–436
    fullerene (C_{60}) and derivatives, 430–435
    graphene, 436–437
    graphene oxide, 436–437
    other carbon-based materials, 437
  direct acting agents, 426
  indirect acting agents, 427
  metallic nanoparticles, 438–439
  metal oxides, 439–441
  primary mechanisms, 426–427
  secondary mechanisms, 428–429
GH. *See* Growth hormone (GH)
GLP. *See* Good laboratory practice (GLP)
Glycocalyx, 385
  nanoparticles entrapment, 385
Glycosidases, 360
Glycosylated proteins, 385
GNPs. *See* Gold nanoparticles (GNPs)
Gold-binding peptides, 94
Gold nanoparticles (GNPs), 345, 438
  protein interactions, 94
Gold NPs (AuNPs). *See* Gold nanoparticles (GNPs)
Gonadotropin-releasing hormone gene (CrGnRHl), 347
Good laboratory practice (GLP), 43
G-protein-coupled receptor, 374
Granuloma formation, 321
Granulomatous inflammation, 211

Granulosa cells, 404
Graphene oxide (GO), 250
Growth hormone (GH), 343
Gut-associated lymphoid tissues (GALT), 386
Gut-on-a-chip model, 388

H

HaCaT cells. *See* Immortalized nontumorigenic human epidermal (HaCaT) cells
Hard protein corona, 4
HARN. *See* High aspect ratio nanoparticles (HARN)
H2AX assay, 66
HepG2 cells, 91
High aspect ratio nanoparticles (HARN), 117
High-efficiency particulate air (HEPA)-filtered vacuum cleaner, 118
High-resolution transmission electron microscopy (HRTEM), 23
HPRT gene, 66
HRTEM. *See* High-resolution transmission electron microscopy (HRTEM)
Human adenocarcinoma cell-line, 387
Human colon adenocarcinoma mucus-secreting cells (HT29-MTX cells), 388
Human digestion models, 388
Human embryonic kidney (HEK293) cells, 430
Human epidermoid-like carcinoma (Hela) cells, 430
Human intestine microfold (M) cells, 388
Human macrophages, activation by GMCSF, 88
Human test cells, 427
Human umbilical vein endothelial cells (HUVEC), 88
HUVEC. *See* Human umbilical vein endothelial cells (HUVEC)
Hydroxyapatite chromatography, 65
Hypersensitivity reactions, 243, 247
  antibody-mediated, 243
  cell-mediated, 243. *See also* Hypersensitivity reactions; delayed type
  nanoparticles, role in, 244
Hypersensitivity reactions, delayed type, 243

I

IARC. *See* International Agency for Research on Cancer (IARC)
ICP-MS. *See* Inductively coupled plasma mass spectrometry (ICP-MS)
ICRP. *See* International Commission on Radiological Protection (ICRP)
IgE-mediated allergic response, 370
Immortalized nontumorigenic human epidermal (HaCaT) cells, 373
Immune cells, 428
  B cells, 315
  phagocytic, 329
  T cells, 315
Immune cells activation, 126
Immune complex mediated reactions, 243
Immune responses
  adaptive, 324–326
  innate, 317–322
    mast cells, 322
Immune system, 322
  adverse effects of nanomaterials on, 315
Immunosafety assays, 332
Immunosuppression, 328–329
Inductively coupled plasma mass spectrometry (ICP-MS), 130, 353, 368
  tissue levels of metal NPs determination, use in, 59
Industrial innovation, 125
Inflammasome, 319, 320
Inflammation, 128, 132, 225, 230, 317, 318
  airway inflammation, 132
  fractional exhaled nitric oxide, 132
  neutrophils, role in, 320
  pulmonary inflammation, 132
  reactive oxygen species, 132
Inflammatory mediators, 243
Inflammatory responses, 319, 321
Inhalation exposure, of lung, 226
Intercellular lipid bilayers
  solvents, effect of, 363
Interleukin 33 (IL-33), 249
Interleukin (IL) 1β, 319
International Agency for Research on Cancer (IARC), 104
International Commission on Radiological Protection (ICRP), 116
International Standards Organisation (ISO), 42
Intratracheal instillation, 70, 440
In vitro alveolar macrophage assay, 207
In vitro genotoxicity assays, 65
In vitro M cell model, 390
In vitro toxicity tests, 60–68
  cytotoxicity tests, 62
  ex vivo models, 68
  genotoxicity tests, 65–68
  limitations, 62–63
  principles, 63–64
  validated cytotoxicity tests, 64
In vivo toxicity tests, 68–73
  carcinogenicity, 72–73
  chronic toxicity, 72–73
  immunological responses, 72
  toxicokinetic, 69–71

Ion beam microscopy (IBM) techniques, 60
  microproton-induced X-ray emission (μPIXE), 60
  micro-Rutherford backscattering (μRBS), 60
Irregular particle, illustration of, number of possible external dimensions of, 17
Irritant contact dermatitis, 362
ISO. *See* International Standards Organisation (ISO)

K
Keratinocytes, 359
Kidney cells, 437

L
Lactate dehydrogenase (LDH) assay, 62
Langerhans cells, 69, 360
Laser scanning confocal microscopy, 366
LCA. *See* Life cycle framework
LC-MS-based proteomics, 6
Leydig cells, 406
LH. *See* Luteinizing hormone (LH)
Life cycle framework (LCA), 161
Limulus amebocyte lysate (LAL) assay, 330
Lipid-based nanoconstructs, 269
Liposomes, 261, 278
LOAEL. *See* Lowest observable adverse effect level (LOAEL)
Lowest observable adverse effect level (LOAEL), 73
Lung biopsy, 112
Lung burden, 73
Lung fibroblasts, 244
Luteinizing hormone (LH), 343
Lymphatic drainage, 71

M
Macrophages, 319, 325
  as target for molecular imaging agents, 260
Macropinocytosis, 281, 293
Magnetic resonance imaging (MRI), 58, 257, 260
Manufactured nanomaterials
  ecotoxicity of, 181
  environmental impact of, 181
Mass spectrometry (MS), 60
Mast cells, 243, 247, 322
Master endocrine organ, 343
MDSC. *See* Myeloid-derived suppressor cells (MDSC)
MEA. *See* Multielectrode arrays (MEA)
MEAD. *See* Modified electrical aerosol detector (MEAD)
Mechanical stressors, 363
Mechanism-based toxicity models, 85
Medicinal products
  innovation task force, 176
  and medical devices, 176
16-Mercaptohexadecyltrimethylammonium bromide (MTAB) coated gold nanoparticles, 94
Merkel cells, 360
Mesothelioma, MWCNTs, effect of, 435
Metabolite identification, 71
Methicillin-resistant *Staphylococcus aureus* (MRSA), 375
Micelles, 278, 284
Microbiome interactions, 10
Micronucleus assay, 440
MicroRNA (miRNA) expression, 136
Microvillus brush-border, 385
Modified electrical aerosol detector (MEAD), 116
Molar refractivity, 91
Molecular patterns
  danger associated, 317
  pathogen associated, 317
Monoclonal antibodies, 93
Mononuclear phagocyte system, 72
Mouse spermatogonial stem cell line, 404
Mouse TM3 Leydig cell line, 404
MRI. *See* Magnetic resonance imaging (MRI)
mRNA assay, 371
MRSA. *See* Methicillin-resistant *Staphylococcus aureus* (MRSA)
MS. *See* Mass spectrometry (MS)
Muco-ciliary escalator cells, 105
Multiangle light scattering (MALS), 24
Multielectrode arrays (MEA), 293
Multiple genome-wide mutations, 429
Multiwalled carbon nanotubes (MWCNT), 91, 104, 246, 247, 285, 322, 323
  intravenous administration, 401
Mutagenesis, 426, 440
Mutual acceptance of data (MAD), 19
MWCNT. *See* Multiwalled carbon nanotube (MWCNT)
Myeloid-derived suppressor cells (MDSC), 329
Myeloperoxidase (MPO), 237
Myocardial infarction, 265

N
NADPH oxidase, 437
NALP3 inflammasome activation, 235
Nanocarriers, 278, 384
Nanoenabled products, 103
Nanomaterials (NMs), 3, 125, 258, 342. *See also* Engineered nanomaterials (ENM)
  allergies and, 326–328
  application in biotechnology and biomedicine, 3
  bioassay on, 160
  biological activity, 4, 126
  biological pathway of, 201
  biomedical applications, 331, 362
  biopersistence, 202
  biophysical properties of, 5

carbon nanotubes, 321
characterization, 43
chemical composition, 27–29
classification, nanotoxicological fingerprints, 91–92
in clinical use
  diagnosis, 258, 262
  imaging, 258
  therapy, 258, 262
components, 125
crystal structure (crystallinity), 27–29
data collection, 41–43
definition of, 15, 162
deposition site, 428
dispersibility, 31
dispersion media, 32–41
dosimetry, OECD guidance document, 41
dustiness, 32
ecotoxicological tests, 41
engineered nanomaterials
  adverse effects of, 265
environmental contaminants, impact of, 160
exposure, 190, 385
  risk assessment, 190, 208
  risk management, 190, 208
external exposure, 191
genotoxic effects of, 135
grouping, framework for, 201–206
  apical effects, 206–208
  cellular effects, 206–208
  DF4nanoGrouping, 206
hazard assessment
  by carcinogenesis, 198–200
    bulk substances, 198
    chronic inflammation, 198
  by local toxicity, 194
    Bovine Corneal Opacity, 194
    EpiOcular eye-irritation test, 194
    permeability, 194
  by reproductive and developmental toxicity, 201
    prenatal toxicity, 201
    reproduction toxicity, 201
  by short-term toxicity, 195–198
    inhalation route of exposure, 195
    inhalation toxicity, 195
    oral exposure, 198
health effects of, 125
high aspect ratio of, 202
human health hazards of, 160, 190
hydrophobicity, 206
identification, 43
immunosuppression by, 328–329
induced DNA damage
  genotoxicity and toxicity assays, 431
induced DNA damage, mechanism, 426
internal exposure, 191
interpretation, 41–43
legislations for, 160
  Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), 166
  Food, Drug and Cosmetic Act (FDCA), 166
  Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), 160
    industrial chemicals, applications in, 170
  Toxic Chemical Control Act and the Chemical Registration and Assessment Act, 160
  Toxic Substances Control Act (TSCA), 160
life cycle stages of, 191
in nanomedicine, 257
occupational exposure to, 126
OECDWPMN Proposed Endpoints, 18
organ burden, estimation of, 192–193
particokinetics of, for diagnostic use, 263
physicochemical
  endpoints for, 17
  parameters, 20
    pristine form, 20
physicochemical data, in-house measurement, 43
physicochemical properties, 16, 126
  characterization
    analytical methods, 35
    suitability of methods, 33
and proteins, interactions between, 94–96
purity, 27–29
regulation of, 160
  Biocidal Products Regulation, 163
  in European Union, 163, 168
  in Japan, 180
  in Korea, 177
risk assessment of, 303
risks of exposure to, 160
safety, 43, 160
sample preparation, 32–41
  OECD guidance document, 41
solubility, 31
specific surface area and porosity, 26–27
stability in dispersion, 30
surface charge density, 30
surface chemistry (and contamination), 29–30
system-dependent properties of, 203
systemic exposure, estimation of, 193–194
testing of, 190
toxicity, QSAR modeling, 88
transgenerational effects, 355
usage, 167
  in consumer products, 167
    Directive on General Safety of Consumer Products, 174
    product legislation, 174
  in the United States, legislative authorities for, 164
water-octanol partition coefficient for, 16

Nanoobject
  aggregates and agglomerates (NOAA), 125
  hierarchical relationship, 108
Nanoparticle, 244, 320
  alergic effect of, 326
  biological effects
    challenges in modeling, 85
  blood-borne nanoparticles, interactions of, 266
  combustion-derived nanoparticles (CDNP), 255
  deposition, 226–227
    clearance from the lungs, 226
      airways, 226
      lymph nodes, 227
      pleura, 227
      proximal alveolar region, 226
    mechanisms, effect of
      diffusion, 226
      impaction, 226
      sedimentation, 226
  detection methods, 56
    ex vivo, 57
    in vitro, 57
  engineered nanoparticles (ENP), 255, 328
  gold nanoparticles, 245
  lipid-based nanoparticles, 261
  magnetic core-shell, 245
  metal oxide nanoparticles, 245
  multiwalled carbon nanotubes, 246
  penetration, 361–370
    barrier modifications, 363
    carbon-based nanomaterial, 366
    gold (Au)NPs, 369
    mechanical action, 363
    quantum dots, 366–367
    silver (Ag)NPs, 369–370
    TiO_2, 367–368
    UVB-damaged skin, 364
    ZnO, 367–368
  polyethylene glycol modified, 245
  preseparator, 114
  silica nanoparticles (SNP), 245
  superparamagnetic iron oxide nanoparticles, 257
  toxicological properties of, used for diagnosis or therapy, 324
  transport prediction
    random forest classification, 91
    regression methods, 91
Nanosafety management, master plans on, 177
NanoSAR models, 92
Nanosilica, 427
Nanotechnology, 162, 255
  development, 125
    sustainability of, 125
  in medical imaging, 257
  regulatory acts in Korea, 178
  related policy in the United States, 163
    National Nanotechnology Initiative (NNI), 163
  standard development, 177
  standardization of, 181
Nanotoxicology, 51
  other modeling approaches, 97
Nanotoxicology modeling approaches
  examples, 85–93
  machine learning methods, 88–91
  molecular dynamics methods, 92–93
  molecular modeling, 92–93
  nanotoxicological fingerprints, 91–92
  quantum chemical studies, 86–87
  statistical modeling, 88–91
National Institute for Occupational Safety and Health (NIOSH), 168
National Institute of Health Sciences (NIHS), 182
National Institute of Occupational Health and Safety (NIOSH), 110
National Nanotechnology Initiative (NNI), 163
Nerve growth factor (NGF), 296
Neutral red assay, 64
Neutrophilic inflammation, 211
NGF. *See* Nerve growth factor (NGF)
Nickel nanoparticles (NiNPs). *See* Nickel (Ni) NPs
Nickel (Ni) NPs, 345, 370, 375
NIHS. *See* National Institute of Health Sciences
NIOSH. *See* National Institute for Occupational Safety and Health
NMs. *See* Nanomaterials (NMs)
NNI. *See* National Nanotechnology Initiative
NOAEL. *See* No observable adverse effect level (NOAEL)
NOEL. *See* No observable effect level (NOAEL)
Nonfluorescent NP suspension, 60
Nongold-binding peptides, 94
Noninvasive molecular cardiovascular imaging, 257
  fibre-optic microscopy, 257
  fluorescence-mediated tomography (FMT), 257
  fluorescence reflectance imaging, 257
  optical frequency-domain imaging, 257
  positron-emission tomography (PET), 257
  single-photon-emission computed tomography (SPECT), 257
No observable adverse effect level (NOAEL), 73
No observable effect level (NOEL), 73
Nose-only inhalation, 70
NP-induced cellular stress, pathways for, 233
NP-rich diesel exhaust (NR-DE), 352
NPs. *See* Polymeric nanoparticles (NPs)
NR-DE. *See* NP-rich diesel exhaust (NR-DE)
Nuclear magnetic resonance (NMR) methods, 27
Numerical chromosomal damage, 435

O

Obstructive pneumonia, 71
Occupational exposures, 225
  limits, 208
Occupational Safety and Health Act, 168
Occupational Safety and Health Administration (OSHA), 168
OECD. *See* Organisation for Economic Co-operation and Development (OECD)
Olfactory bulb, 279
OPC. *See* Optical particle counter (OPC)
Opsonization, 323, 324
Optical particle counter (OPC), 113, 115
Orally ingested nanoparticles, 388
Organisation for Economic Co-operation and Development (OECD), 42, 104, 160
Organ-on-a-chip microdevices, 61
  models, 388
Organotypical slice cultures, 68
OSH. *See* Occupational safety and health
OSHA. *See* Occupational Safety and Health Administration
Ovalbumin, aerosolized model allergen, 407
Ovarian lymphocytosis, 353
Oxidative stress, 128, 198, 233, 234, 282, 289, 403
  detection, 62
  ENM-induced, 62
  particulate matter, health effects of, 133
  response genes, 437

P

PaCa2. *See* Pancreatic adenocarcinoma cells (PaCa2)
PaCa2 nanoparticle uptake model
  test set performance, 90
  training set performance, 90
PAMP. *See* Pathogen associated molecular patterns (PAMP)
Pancreatic adenocarcinoma cells (PaCa2), 88
Parathyroid hormone (PTH), 344
Particle morphology, 202
Particles with small D_{ae}, 228
  fibers, 228
  platelets, 228
    graphene family nanomaterials, 228
Particle toxicology, 150
Passive diffusion, 362
Pathogen associated molecular patterns (PAMP), 317, 318
Pattern recognition receptors (PRR), 319
PBPD model. *See* Pharmaco-Dynamics (PBPD) model
PEG. *See* Polyethylene glycol (PEG)
PEG-SWCNT-750
  biodistribution analysis, 411
PEGylated Au NPs, 408
PEGylation, 389
Peroxynitrite ($ONOO^-$), 237
Personal breathing devices, 111
Personal protective equipment (PPE), 119
Persorption, 387
PET. *See* Positron-emission tomography (PET)
Peyer's patches, 386
Phagocytosis, 244, 263, 282, 317, 320
Phagosomes, 282
Pharmaco-Dynamics (PBPD) model, 97
Phospholipid bilayer
  carbon nanotube insertion, effect of, 93
Photon correlation spectroscopy (PCS), 24
Physicochemical characterization, 15
  modalities of, 22
    agglomeration, 22
    aggregation, 22
    particle size, 22
    size distribution, 22
Physiologically-based-pharmaco-kinetics (PBPK) model, 97
Pier's patch, 69
Pigments, particle size distribution measurements, 42
Pineal body, 343
Plant Protection Products (PPP), 171–172
Plaque rupture, 257
  inflammatory processes
    degradation of the fibrous cap, 257
    endothelial dysfunction, 257
    leukocyte recruitment and migration, 257
    matrix metalloproteinase activity, 257
    platelet activation, 257
Plasma-mass spectrometry (ICP-MS), 114
Platelet-derived growth factor (PDGF), 248
Pleuro-pulmonary system, particle-induced effects in, 233
  mechanisms, 233
  processes, 233
    biopersistence, 237–238
    cellular stress, 233
    frustrated phagocytosis, 235–236
    inflammasome activation, 235
    oxidative stress, 233–235
  processes and mechanisms, 233
    biopersistence, 237–238
    cellular stress, 233
    frustrated phagocytosis, 235–236
    inflammasome activation, 234
    oxidative stress, 233–235
Point mutation assays, 440
Pollutants, clastogenic effects, 66
Polyester powder paint, 112
Polyethylene glycol (PEG), 263, 438

Polylactide coglycolide, 386
Polymerase enzyme, 426
Polymeric nanoparticles (NPs), 343
Polystyrene, 386
Polystyrene beads, 413
  DNA transfection, role in, 413
  drug delivery, role in, 413
  protein adsorption, role in, 413
Porosity, 26–27
Positron-emission tomography (PET), 59, 257
PPE. *See* Personal protective equipment (PPE)
PPP. *See* Plant Protection Products
Proinflammatory cytokine, 62
Proinflammatory mediator, 373
Proinflammatory response evaluation, 62
Protein corona, 4
  development of, 4
  hard *vs.* soft, 4, 5
Protein corona models, 5
Protein expression assays, 371
Proteomics, 141
PRR. *See* Pattern recognition receptors (PRR)
Pseudoallergy, 244. *See also* Allergic reactions
Pseudo-pregnant females, 408
Psoriasis, 362
Pulmonary responses, 230
  fibrosis, 231
  genotoxicity and carcinogenesis, 231
  inflammation, 230
Pulmonary toxicity, 435

Q

QDs. *See* Quantum dots (QDs)
QSAR. *See* Quantitative structure activity relationships (QSAR)
Quantitative structure activity relationships (QSAR), 84, 300
Quantum dots (QDs), 344, 438
  cytotoxicity, 374
  immunotoxicity, role in, 374
  proinflammatory potential, 374
Quasi-elastic light scattering (QELS), 24

R

Raman spectroscopy, 60
Rana catesbeiana, 344
Rat brain microvessel endothelial cells (rBMEC), 280
rBMEC. *See* Rat brain microvessel endothelial cells (rBMEC)
REACH. *See* Registration, evaluation, authorization, and restriction of chemicals (REACH)
Reactive nitrogen species (RNS), 234
Reactive oxygen species (ROS), 236, 249, 282, 300, 319, 345, 410, 427
  endogenous, 244
  exogenous, 244
Recommended exposure limit (REL), 146
Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), 51, 160
Regulatory hazard, 191
Relaxation rates, 261
Respiratory system
  clearance rate, 71
Resting human macrophages (RestMph), 88
RestMph. *See* Resting human macrophages (RestMph)
Reticuloendothelial system (RES), 72, 264
Rhodium (Rh)NP, 375
ROS. *See* Reactive oxygen species (ROS)

S

Safety of nanomaterials, research activities, 179
Saturated hydrocarbons, 132
Scaled-down nanosize material, 51
Scanning electron microscopy (SEM), 23, 114
Scanning mobility particle size (SMPS), 113
  spectrometry, 25
Scanning tunnelling microscopy (STM), 24
Screening information dataset (SIDS), 16
Self-cleaning surfaces, 83
SEM. *See* Scanning electron microscopy (SEM)
Seminal paper, 86
Sertoli cells, 401, 406
Sex hormones, 345
Sham instilled females, 404
SHE cells. *See* Syrian hamster embryo (SHE) cells
Short-term toxicity inhalation assay, 73
Signal transduction proteins, 427
Silica nanoparticles, 96, 401
Silver flux penetration, 369
Silver nanoparticles (Ag NPs), 439
  absorption, 369
  antibacterial effects, 389
  consumer products, use in, 374
  embryotoxic effects, 408
  personal healthcare fields, use in, 374
Single base pair substitutions, 429
Single-cell gel electrophoresis assay, 65, 430
Single-photon-emission computed tomography (SPECT), 59, 257

Single-walled carbon nanotubes (SWCNTs), 62, 104, 246, 285, 321, 345
SiO_2 NPs. *See* Silica nanoparticles
Size selective sampler (SSS), 116
Skin
  anatomy, 359–360
  cells, 359
  as environmental barrier, 360
  functions, 360
  as immunological effector axis, 360
  keratin biosynthesis, role in, 360
  morphology, 360
  neurosensory properties, 360
  notation, 361
Small-angle X-ray scattering (SAXS), 24
Small-scale whole-body inhalation systems, 70
Smooth muscle cells, 244
SOD-1. *See* Superoxide dismutase 1 (SOD-1)
Soft protein corona, 4
Specific surface area (SSA), 16, 26–27
SPECT. *See* Single-photon-emission computed tomography (SPECT)
Spermatogenesis, NPs, adverse effects of, 401
Spermatogonial cells, 401
Sperm DNA damage, 401
SPION. *See* Superparamagnetic iron oxide nanoparticles (SPION)
ST. *See* System toxicology
π−π Stacking interactions, 96
Standard operating procedures (SOPs), 23
Staphylococcus aureus, 375
StAR. *See* Steroidogenic acute regulatory protein (StAR)
Steroidogenic acute regulatory protein (StAR), 353
Stratified squamous keratinized epithelium, 360
Stratum corneum cell, 360
Structure activity relationships (SAR), 299. *See also* Quantitative structure activity relationships (QSAR)
Sunscreens, formulations
  nanoparticles, biological effects, 86
  TiO_2NP penetration, 368
  ZnONP penetration, 368
Superoxide dismutase 1 (SOD-1), 438
Superparamagnetic iron oxide nanoparticles (SPION), 257
  applications of
    assessment of myocardial perfusion, 260
    characterization of aortic aneurysm formation, 260
    in labelling gene constructs for localization in gene therapy studies, 260
    in molecular imaging, 260
    in receptor-directed imaging, 260
Surface charge density, 85
SWCNTs. *See* Single-walled carbon nanotubes
α−Synuclein
  citrate-coated gold nanoparticles, binding with, 95
  MTAB-coated gold nanoparticles, binding with, 95
Syrian hamster embryo (SHE) cells, 67
System toxicology (ST), 139

T

Tapered element oscillating microbalance (TEOM), 116
Tape-stripped human skin
  exposed to QD565-COOH
    laser confocal microscopy of, 364
TBARS. *See* Thiobarbituric acid reactive substances
TEER. *See* Transepithelial electrical resistance (TEER)
TEM. *See* Transmission electron microscopy (TEM)
TEOM. *See* Tapered element oscillating microbalance (TEOM)
Terpenes, 366
Testicular damage, NPs, role of, 403
Tetramethylammonium hydroxide (TMAOH), 375
Tetrazolium assays, 62
TH. *See* Thyroid hormone (TH)
Thiobarbituric acid reactive substances (TBARS), 132
Thrombosis, 257
Thymopoietins, 349
Thymosins, 349
Thyroid hormone (TH), 342
Thyroid-stimulating hormone (TSH), 343
Thyroxine (T4), 344
Time-of-flight secondary ion mass spectrometry (TOF-SIMS), 364
Time-to-first F2 litter, 402
Time-weighted average (TWA), 168
TiO_2 nanoparticles, 343
  environmentally sensitive phase map, 86
  within intercellular lipid space of stratum corneum transmission electron micrograph (TEM), 365
  relative phototoxicity
    as function of particle size, 87
TLR. *See* Toll-like receptors (TLR)
TMAOH. *See* Tetramethylammonium hydroxide (TMAOH)
TNT. *See* Tunneling nanotubes (TNT)
TOF-SIMS. *See* Time-of-flight secondary ion mass spectrometry (TOF-SIMS)
Toll-like receptors (TLR), 319, 320, 330
Topoisomearase I, 427
Toxicity studies, 370–375. *See also* Toxicity tests
  carbon-based nanomaterials, 371
  C_{60} fullerenes, 372
  irritation, 370
  MWCNTs, 373
  other metals, 375

Toxicity studies (*cont.*)
quantum dots, 374
sensitization, 370
silver (Ag)NPs, 374–375
SWCNTs, 373
Toxicity tests
considerations for selection, 73–75
in vitro *vs.* in vivo, 73–75
Toxicokinetics, 191
Toxicology, 226
Toxic Substances Control Act (TSCA), 160
Trans-epithelial/endothelial electrical resistance (TEER), 58, 387, 389
Transforming growth factor (TGF), 250
Transmission electron microscopy (TEM), 23, 107, 360
Triiodothyronine (T3), 344
TSCA. *See* Toxic Substances Control Act
TSH. *See* Thyroid-stimulating hormone (TSH)
Tumor formation
dust inhalation, role of, 429
Tumorigenesis, 426
Tumor-promoting cell signaling pathways, 436
Tunneling nanotubes (TNT), 282
TWA. *See* Time-weighted average

U
Ubiquitin-gold nanoparticle interactions, 94
Ultrasmall paramagnetic iron oxide nanoparticles (USPION), 260
USPION. *See* Ultrasmall paramagnetic iron oxide nanoparticles (USPION)

V
Valence band electrons, 86
Volatile organic compounds (VOCs), 132
Vroman-effect, 6

W
Water photolysis reactions, 83
Watson-Crick interactions, 94
Wet wiping methods, 118
Whole body inhalation, 70
Wide-angle X-ray scattering (WAXS), 24
Workers, protection of, 172–173
Working Party on Manufactured Nanomaterials (WPMN), 16, 160
Workplace
engineered nanoparticles release
evidence for, 111–112
exposures, 168
occupational exposure limits, 416
WPMN. *See* Working Party on Manufactured Nanomaterials

X
Xenobiotic-detoxifying enzyme, 405
Xenobiotic-metabolizing enzyme, 405

Z
Zeta potential (ZP), 235
Zn tracer, 368
Zona pellucida, 404