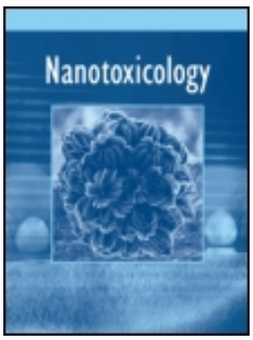

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REVIEW

There's plenty of room at the forum: Potential risks and safety assessment of engineered nanomaterials

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Abstract
The celebrated physicist and Nobel laureate Richard Feynman was the first to predict the opportunities presented by the manipulation of matter at the level of individual atoms and molecules. Today, almost 50 years after his classic lecture on the wonders of the small world, the evolving nanotechnologies have the potential to bring about major changes in the lives of citizens. However, the very same properties that make engineered nanomaterials so promising from a technological perspective, such as their high degree of reactivity and the ability to cross biological barriers, could also make these novel materials harmful to human health and the environment. Therefore, exploitation of the full potential of the nanotechnologies requires close attention to safety issues. The 1st Nobel Forum mini-symposium on nanotoxicology was recently held in Stockholm, Sweden, and the program was devoted to the topic of definitions and standardization in nanotoxicological research, as well as nano-specific risk assessment and regulatory/legislative issues. Examples of recent and ongoing studies of carbon-based nanomaterials, including single-walled carbon nanotubes, using a wide range of *in vitro* and *in vivo* model systems were also presented. The current review will provide some highlights and conclusions from this exciting meeting.

Keywords: *Nanotoxicology, carbon nanotubes, inflammation, pulmonary exposure, risk assessment*

Entering nanocosmos: Opportunities and uncertainties

Richard Feynman is credited as being the first person to see the potential of the manipulation of matter at the nano-scale. In his famous lecture at the California Institute of Technology entitled '*There's plenty of room at the bottom: An invitation to enter a new field of physics*' he postulated that the manipulation of atoms and molecules would open up new frontiers in science and technology (Feynman 1960). As a starting point for his seminal lecture, Feynman asked the question: 'why cannot we write the entire 24 volumes of the *Encyclopedia Brittanica* on the head of a pin?' and then proceeded to explain that there is no question that there is enough room on the head of the pin to put all of the information contained in the 24 volumes in such a manner that we could read it without difficulties. He also noted: 'In the year 2000, when they look back at this age, they will wonder why it was not until the year 1960 that anybody began seriously to move in this direction'. Of course, today, there is an ever-increasing 'movement' in this

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direction, and nanotechnologies are expected to have a profound impact on society and economy. However, in this golden era of nano-opportunities, one must not forget to assess any potential threats of nanotechnologies to human health (e.g., through occupational exposure) and the environment (through the disposal of nanodevices that have expired, or accidental release of nano-pollutants) (Nel et al. 2006). Indeed, the responsible pursuit and sustainable development of nanotechnologies represents a major challenge and should be tackled in a cooperative manner by the global research community (Maynard et al. 2006).

Feynman himself did not use the term 'nanotechnology'; this expression was coined in 1974 by the Japanese engineer, Norio Taniguchi. Furthermore, the tools were not yet available in the 1960s to realize the potential of nano-engineering. However, the development of the scanning tunneling microscope in the early 1980s, followed by the subsequent arrival of the atomic force microscope and other analytical techniques, enabled the essential structure of materials to be probed and explored, and new materials with nano-dependent properties to be developed, and were thus crucial in propelling the field of nanoscience (Gerber & Lang 2006). Feynman also predicted the emergence of nanomedicine in his lecture, insofar as he noted that 'it would be interesting in surgery if you could swallow the [mechanical] surgeon – it finds out which [heart] valve is the faulty one and takes out a little knife and slices it out'. While miniaturized nano-robots in the bloodstream are still far from becoming a reality, research into the delivery and targeting of nano-sized therapeutic and diagnostic agents for the treatment, diagnosis, and monitoring of human disease is at the forefront of the novel field of nanomedicine (Moghimi et al. 2005). Again, such intentional exposure of humans to engineered nanomaterials needs to be pursued in the context of careful assessment of safety issues (Kagan et al. 2005). Moreover, concerns have been raised with regard to the real or perceived threat of loss of privacy resulting from the development not only of nano-scaled sensors in medical diagnostics, but also of diminutive surveillance devices (Toumey 2007). These issues also need to be taken into consideration when nanotechnologies are developed and put to use.

Nanomaterials (often defined as objects with one or more dimensions in the range below 100 nm) (The Royal Society and The Royal Academy of Engineering 2004) can be on the same scale as elements of living cells, including proteins, nucleic acids, lipids and cellular organelles. Therefore, one must focus particular attention on how engineered nanoobjects can interact with or influence biological systems, which may be desirable for certain medical applications, such as targeted drug delivery in cancer or atherosclerosis (Caruthers et al. 2007), but may cause unanticipated hazardous effects in scenarios of occupational or environmental exposure to nanomaterials. Indeed, Feynman the physicist notes that the fact that enormous amounts of information can be stored in an exceedingly small space, is well-known to biologists: 'all of the information for the organization of a complex creature such as ourselves can be stored – in a very tiny fraction of the cell in the form of long-chain DNA molecules'. Therefore, nanotechnologies provide the tools and technological platforms required for the investigation and transformation of biological systems, and biology on the other hand offers inspiration to nanotechnologies in the form of ingenious information storage systems and models of bio-assembled 'nanomachines' operating at the cellular or subcellular level (Roco, 2003; Bath & Turberfield 2007; LeDuc et al. 2007).

The properties of materials are different on a nanoscale for two reasons. First, nanomaterials have, relatively, a larger surface area than the same mass of material produced in a larger form. This can make materials more chemically reactive, and affect their strength or electrical properties. Second, below 50 nm, the laws of classical physics give way to quantum effects, provoking optical, electrical, and magnetic behaviors different from those of the same material at a larger scale. However, the very same properties that make nanomaterials so uniquely useful, such as a high degree of chemical reactivity, may also be associated with unforeseen adverse effects on health and the environment. The emergence of novel materials with new and unanticipated properties has prompted the emergence of a new subcategory of toxicology (*nanotoxicology*) to address the potential threats that widespread use of new nanomaterials could bring (Donaldson et al. 2004). However, it is also important to consider this nascent discipline in the context of knowledge accumulated through previous toxicological studies of air pollution particles, asbestos fibers, metal fumes, etc (for an excellent overview, see Oberdörster et al. 2005; Oberdörster et al. 2007). Indeed, studies of the deposition of foreign particles ('*Fremdstoffpartikeln*') of various origins in the lungs of rats were published just prior to Feynman's famous lecture (Gieseking 1958). The current presentation will summarize recent discussions held at the Nobel Forum in Stockholm on the topics of standardization of materials and assays for nano-specific toxicity, as well as risk assessment and legislative issues. Illustrative examples of recent nanotoxicological research conducted in the United States and in Europe on single-walled carbon nanotubes using

various *in vitro* and *in vivo* model systems will also be provided, as well as a discussion of recent studies of inhalation exposure to human subjects of nano-sized carbon particles.

Materials and methods: The importance of standardization

Current definitions of nanotechnologies commonly include a minimum requirement with regard to the size of the material that is used or produced. Thus, they point out that in at least one dimension these materials must have a size smaller than 100 nm (The Royal Society and The Royal Academy of Engineering 2004; United States Congress Joint Economic Committee 2007). However, this is more or less an arbitrary suggestion because one can in principle use any other nanometer-sized dimension as well. Therefore, a working group of the European Academy for the Research on the Consequences of Scientific and Technological Advances has summarized all current and previous definitions in a recent publication and presented a new definition independent of an exact specification of size (Schmid et al., 2006): '*Nanotechnology comprises the emerging applications of Nanoscience. Nanoscience is dealing with functional systems based on the use of sub-units with specific size-dependent properties of the individual sub-units or of a system of those*'. This definition highlights the new properties that are strictly dependent on a *specific smallness* of the particles or materials. However, while this could serve as a useful definition for new technological processes or products, it may not be helpful in risk management as effects of nanomaterials on health or the environment will depend not only on size but also on other properties of the particles, including chemical composition, aerodynamic diameter, agglomeration behaviour, shape (spherical, cylindrical, fibres, tubes), surface properties (rough, smooth, circular, charge), surface area, and surface modifications/functionalizations (coatings, core-shell, etc) (Nel et al. 2006).

Standardization, in the context related to technologies and industries, is the process of establishing a technical standard among competing entities in a market, where this will bring benefits without hurting competition. International activities in the area of nanotechnologies are coordinated by the International Standards Organization (ISO) which established the ISO Technical Committee in 2005. Divided into three working groups this committee is dedicated to the following topics: (a) Terminology and nomenclature (convened by Canada); (b) Measurement and characterization (convened by Japan); (c) Health, safety and environment (convened by the United States). Standards are difficult to generate for nanotechnology-based products due to the different perceptions of nanomaterials that exist among the various scientific disciplines that are involved in the development of such materials and products. Furthermore, it becomes complicated to establish common standards that apply not only at the manufacturing stage but also during handling and use as well as later during disposal or recycling scenarios. While a unified procedure to classify all nanomaterials and their applications seems unlikely, there is nevertheless an urgent need for answering some outstanding questions especially in connection to the biological effects of novel nanomaterials and the possible health and environmental problems they may cause. The two most obvious requirements concern the comparability of the methods used for monitoring of adverse effects and the materials that are subject to such investigations. Therefore, there is a need for standardized toxicological assays as well as reference materials to classify the measured effects and compare them with those from other laboratories in other countries. A priority list of those topics that should be standardized or defined immediately in order to facilitate the development of the nanotechnologies was presented at the Nobel Forum mini-symposium (Table I).

Recent studies have demonstrated that not only nanomaterials themselves, but also contaminations within the produced fractions of nanomaterials may yield adverse biological effects and should therefore be evaluated as well. An interesting case in point is provided by preparations of carbon nanotubes (CNTs) that have been shown to include metals, amorphous carbon and other compounds (Donaldson et al. 2006). The most commonly used technologies in the manufacturing of CNTs rely heavily on the use of catalytically active metals, either iron or

Table I. Priority list of nano-related topics that need to be standardized or defined in the short-term.

1. *Systematic terminology for materials composition and features*
   - Composition
   - Morphology
   - Size
2. *General terminology for nanoscience and technology*
   - Definition of the term 'nano'
   - Consideration of impact on intellectual property/other issues
   - Sensitivity to existing conventions
3. *Metrology/methods of analysis/standard test methods*
   - Particle size and shape
   - Particle number and distribution
   - Particle mass
4. *Toxicity effects/environmental impact/risk assessment*
   - Environmental health and safety
   - Reference standards for testing and controls
   - Testing methods for toxicity

nickel. As a result, the final material usually contains significant amounts (up to 30–40 wt%) of these metals that may act as catalysts of oxidative stress. Indeed, a number of recent *in vitro* studies of CNTs appear to describe adverse effects of these contaminants and not of the nanotubes themselves (Kagan et al. 2006; Pulskamp et al. 2007). Similarly, recent studies have indicated that gold nanoparticles are not inherently toxic to human cells, despite being taken up into cells, whereas the presence of a variety of surface modifiers or contaminants induced significant toxicity (Connor et al. 2005). A further point of interest is that fine particles may adsorb environmental biomolecules such as lipopolysaccharide (LPS) and could thereby mimic the pro-inflammatory effects of bacteria on macrophages and other phagocytes (Ashwood et al. 2007). Indeed, recent investigations have shown that contamination of engineered gold nanoparticles with LPS can result in activation of antigen-presenting dendritic cells of the immune system, thereby interfering with the assessment of biological/medical effects of such nanomaterials (Vallhov et al. 2006). Therefore, careful characterization of the nanomaterial preparations being tested is mandatory for the assessment of toxic effects of these materials.

Finally, with regard to the need for standardization, not only the materials but also the methods should be standardized and validated. Recent studies demonstrate that results obtained with specific assays for toxicity may have been invalidated by the presence of investigated nanoparticles leading to false-positive outcomes (Monteiro-Riviere & Inman 2006; Wörle-Knirsch et al. 2006). For instance, the commonly used MTT assay for cell viability which is based on the reduction of a detectable product by specific enzymes in mitochondria may at times produce false results due to the strong interaction between CNTs and insoluble MTT-formazan crystals (Wörle-Knirsch et al. 2006). The latter findings strongly suggest verifying cytotoxicity data with at least two or more independent test systems for each new class of materials or newly synthesized nanoparticles.

Human studies of engineered nanoparticles: A comparison with air pollution

Numerous epidemiological studies have provided evidence that air pollution contributes to systemic as well as pulmonary diseases and reactive airway effects (Nel 2005; Gwinn & Vallyathan 2006). These findings pertain in particular to elderly people and susceptible persons with underlying diseases of various origins. Ultrafine particles (<100 nm) represent a substantial component of the particulate matter in ambient air, and these studies are therefore of relevance also for the emerging nanotechnologies. However, it is important to note that conventional health-related aerosol exposure measurements performed on the basis of the mass of particles per unit volume of air may not be applicable to airborne nanoparticles, since certain respirable nanometer-sized particles may be more toxic than larger particles with a similar composition (Maynard & Aitken 2007). New methodologies and instruments for exposure measurements are therefore needed in order to provide detailed information on the nature of airborne engineered nanoparticles to which humans are exposed.

Exposures to airborne ultrafine particles have increased dramatically because of anthropogenic sources such as internal combustion engines, power plants, and many other sources of thermodegradation. The rapidly developing nanotechnologies (a new industrial revolution) are likely to become yet another source for human inhalation exposures to nano-sized particles. Particles deposited in the airways are normally taken up and eliminated by professional alveolar macrophages. However, macrophages of the lung may not recognize ultrafine particles in the same way as they 'sense' larger particles; moreover, animal studies have shown that TiO_2 particles below about 50 nm elicit a more severe inflammation in the alveolar space as compared to larger-sized particles (Oberdörster et al. 1992). Ultrafine carbon particles may also impair phagocytosis of microorganisms by alveolar macrophages, which could contribute to an increased susceptibility to infections and/or exacerbations of asthma and chronic obstructive pulmonary disease (Lundborg et al. 2006). Several studies have found associations of ambient ultrafine particles with adverse respiratory and cardiovascular effects resulting in morbidity and mortality in susceptible parts of the population (Peters et al. 1997a; Peters et al. 1997b; Pekkanen et al. 2002).

The mechanisms underlying adverse health effects of air pollution are largely unknown, but autonomic regulation of the heartbeat, inflammation and systemic coagulation effects, and direct metal toxicity to the myocardium have been proposed (for a review, see Dockery 2001). The hypothesis that translocation of inhaled ultrafine particles to systemic circulation causes a direct effect on extrapulmonary organs has been tested in human subjects. In this study, five healthy volunteers were exposed to Technetium 99m (^{99m}Tc)-labelled ultrafine carbon particles (Technegas) and translocation of ^{99m}Tc into the blood compartment was observed (Nemmar et al. 2002). However, this finding has been refuted by other

investigators as an overestimation of particle translocation due to methodological problems (Brown et al. 2002). Indeed, a recent study showed conclusively that the majority of ^{99m}Tc-labeled carbon nanoparticles remained in the lung for up to 6 h after inhalation (Mills et al. 2006).

The Technegas generator is essentially a miniature high temperature furnace in which the heating element is also the source of graphite vapour which ultimately coats the Technetium metal. The 5 to 30 nm central crystal of metal is covered by a thin, but multi-layered graphitic shell. However, under standard operating conditions the Technegas generator does not provide ultrafine particles with a stable radiolabel. Leaching of the label may thus affect the results of inhalation/translocation studies due to the difficulty in differentiating between the solute label and labelled ultrafine particles in blood and extrapulmonary organs. Svartengren and associates have recently modified the production of particles in order to obtain leaching-free particles of a controlled size (Möller et al. 2006). Two studies using 35 and 100 nm particles have been conducted so far making use of the improved method for particle production. In the first experiment, 15 subjects, including non-smokers, smokers, and asthmatics, inhaled ^{99m}Tc-labelled 100 nm particles, and clearance of these ultrafine particles from the lungs and translocation to the circulation was investigated. The results showed that the 100 nm particles largely remained inside the lungs for up to 3 days, and there was no evidence of a significant ($> 1\%$) translocation of ultrafine particles to the systemic circulation (Wiebert et al. 2006a). In the second experiment, nine healthy subjects and four asthmatics inhaled 35 nm ^{99m}Tc-labeled carbonaceous particles. One additional subject was exposed to particles with unstable labelling. These studies demonstrated that over a 24 h period there was no significant translocation of inhaled 35 nm particles to the systemic circulation when the label was stable (Wiebert et al. 2006b). Overall, therefore, these findings challenge previous studies stipulating a rapid and substantial uptake of ultrafine particles; results of earlier studies may be a consequence of technical shortcomings resulting from leaching test particles (Figure 1). In conclusion, the hypothesis

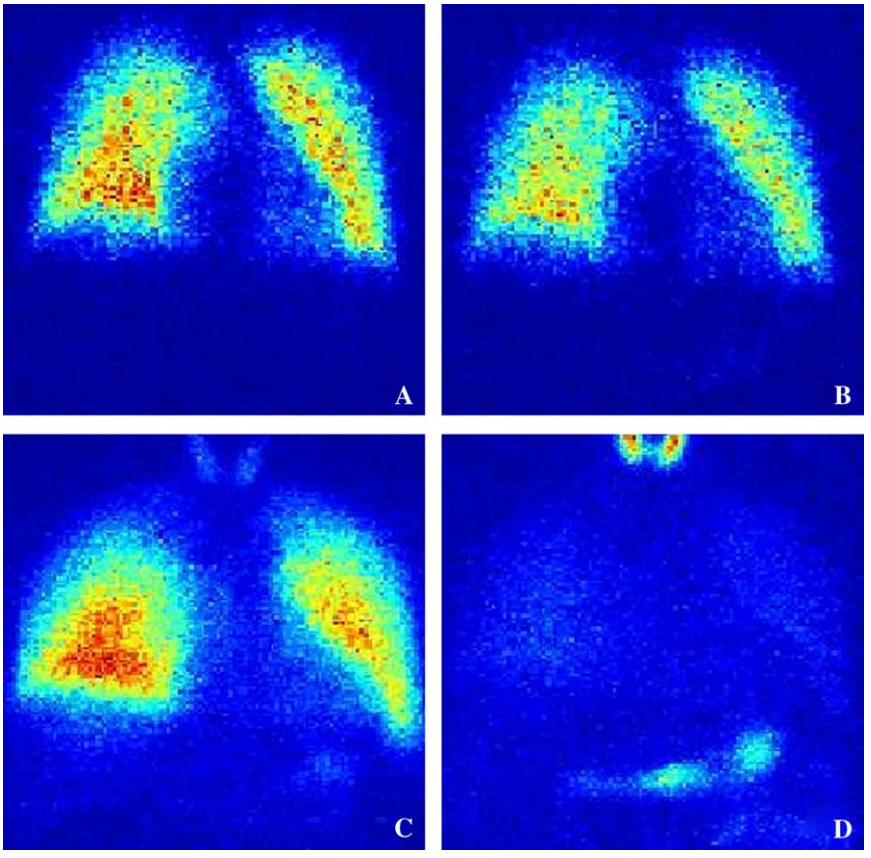

Figure 1. Lung deposition scans of human subjects exposed to leaching versus non-leaching aerosols of ultrafine particles. Radioactivity distribution in the thoracic region of two exposed subjects is depicted 10 min (A, C) and 100 min (B, D) after inhalation exposure to ^{99m}Tc-labeled ultrafine carbonaceous particles. Practically no radioactivity is visible outside the lungs in the subject exposed to non-leaching particles (A, B), indicating that there is no significant particle-bound radioactivity translocation from the lungs to the circulation. In contrast, when particles are leaching (C, D), the activity is visible in the thyroid and the gastrointestinal tract after a short period of time. Reprinted from Wiebert et al. (2006a), with permission from the Taylor & Francis Group.

that systemic access of ultrafine insoluble particles may induce adverse reactions in the cardiovascular system, and other organs, leading to the onset of cardiovascular disease in human subjects, requires careful consideration. Moreover, other, not generally recognized routes of exposure to engineered nanomaterials, including the putative uptake of inhaled nanoparticles into the brain via the olfactory nerve (Oberdörster et al. 2004; Elder et al. 2006) also need to be considered, although the relevance of such clearance pathways for human exposure remains to be established.

Single-walled carbon nanotubes: Understanding and controlling their toxicity

Carbon nanotubes (CNTs), and in particular single-walled carbon nanotubes (SWCNTs), have found numerous applications in different fields of technology and medicine. SWCNTs possess remarkable physical properties, and are reported to be stiffer than diamond and as much as 10 times stronger than steel (for a review, see Donaldson et al. 2006). Due to the increasing use of CNTs in industry, significant efforts have been directed towards elucidating their toxicity to human cells as well as to animals. Pulmonary effects of CNTs have attracted much attention in recent years due to the occupational and environmental significance of these materials (Lam et al. 2006). Studies of pulmonary toxicity of SWCNTs after pharyngeal aspiration in C57BL/6 mice have demonstrated that these nanomaterials caused a dose-dependent augmentation of biomarkers of cell injury quantified by cell counts, total protein, lactate dehydrogenase (LDH) and γ-glutamyltranspeptidase (GGT) activities, as well as oxidative stress as evidenced by reduced level of GSH and total antioxidant reserve along with the accumulation of lipid peroxidation products in bronchoalveolar lavage (BAL) fluid and in the lung (Shvedova et al. 2005). Moreover, markers of pulmonary cytotoxicity were associated with early development of acute inflammation, collagen accumulation, and progressive fibrosis and formation of granulomas (Figure 2). A subsequent study using C57BL/6 mice that were maintained on vitamin E-sufficient or vitamin E-deficient diets further emphasized the importance of oxidative stress and antioxidant depletion in the overall inflammatory response insofar as the toxicity of SWCNTs and the fibrotic responses (enhanced collagen deposition) were significantly higher in the latter group of mice, lacking the major the major lipid-soluble antioxidant, vitamin E (Shvedova et al. 2007). Overall, pharyngeal aspiration of SWCNTs elicits a robust acute inflammatory response with early onset of progressive pulmonary fibrosis whose expression and severity is associated with the intensity of oxidative stress in the lung of the exposed C57BL/6 mice.

An important question is whether *in vivo* studies in which SWCNTs are delivered at high doses and dose rates to the lower respiratory tract of mice through pharyngeal aspiration are relevant to inhalation scenarios with slow build-up of particles in the lungs. In this context, inhalation studies of SWCNTs conducted recently have confirmed the validity of the conclusions obtained in the above-mentioned studies with pharyngeal aspiration (Shvedova et al. unpublished results). A further question of interest is to what extent SWCNTs (nano-sized in diameter, and typically tens of microns in length) behave like asbestos; for a detailed discussion of the issue of nanoparticle-specific versus fiber-specific properties of CNTs, see a recent review in this journal (Oberdörster et al. 2007).

The distribution of CNTs in various tissues is dependent, to a large extent, on their specific interactions with cells of immune system, particularly phagocytes. Indeed, recent studies have indicated that disturbed macrophage functions in NADPH-oxidase deficient C57BL/6 mice challenged with SWCNTs were accompanied by drastic accumulation of unengulfed neutrophils (Shvedova et al. unpublished results). The sentinel or 'sensor' function of professional phagocytes, essential for the elimination of pathogens and particles, is also pivotal in the regulation of the adaptive immune response. Furthermore, macrophages are important regulators of innate immunity as they control the production of pro- and anti-inflammatory mediators upon exposure to foreign particles or cell debris. One of the signaling molecules involved in switching acute pro-inflammatory to chronic pro-fibrotic stages of inflammation is the phospholipid, phosphatidylserine (PS) appearing on the surface of apoptotic cell corpses, including the surface of dying neutrophils (Fadeel & Kagan 2003). It has been established that non-functionalized SWCNTs are not effectively taken up by macrophages (Shvedova et al. 2005; Kagan et al. 2006). In contrast, apoptotic cells with externalized PS and non-apoptotic cells enriched with PS on their surface are well recognized by macrophages *in vitro* and *in vivo* (Kagan et al. 2002; Huynh et al. 2002). One may thus hypothesize that coating of SWCNTs with PS could interface them with macrophages and stimulate their recognition and engulfment. Indeed, recent electron microscopic imaging has revealed engulfment of PS-coated SWCNTs by murine RAW264.7 macrophages and protrusions of SWCNT fibers through the surface into the interior of the cells (Kagan et al. unpublished results). In contrast, pristine or PC

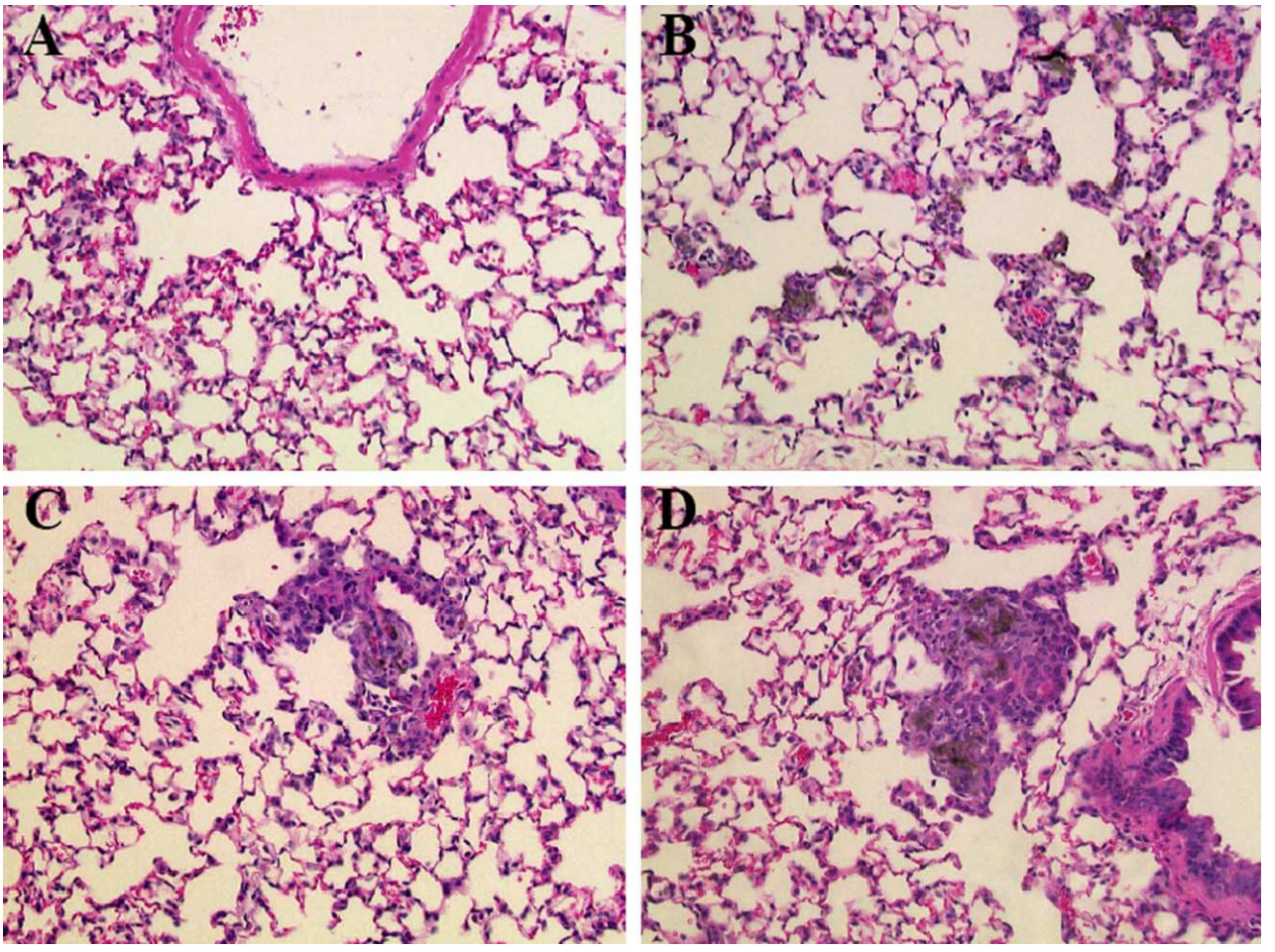

Figure 2. Rapid development of granulomatous pneumonia in mice after intratracheal aspiration of single-walled carbon nanotubes. (A) control; (B) 1 day post-exposure; (C) 3 days post-exposure; (D) 7 days post-exposure. These light micrographs of H & E-stained sections from lung tissue of C57BL/6 mice exposed to SWCNTs (10 μg/mouse) show that the inflammation is evolving with time from neutrophilic to granulomatous, with eosinophilic material consistent with fibrous connective tissue visible within the granulomas. Original magnification x200. Reprinted from Shvedova et al. (2005), with permission from the American Physiological Society.

(phosphatidylcholine)-coated SWCNTs were less attractive targets for these macrophages. In addition, macrophages co-incubated with PS-SWCNT had more endocytotic vesicles with entrapped nanotubes compared to macrophages exposed to PC-SWCNTs or non-coated SWCNTs. Moreover, PS-SWCNTs effectively suppressed LPS-induced production of TNF-α by macrophages. Furthermore, studies in mice demonstrated that PS-SWCNTs are preferably ingested by macrophages obtained from BAL as compared to PC-SWCNTs or non-coated SWCNTs (Shvedova et al. unpublished observations). Taken together, these findings suggest that specific coating of SWCNTs with the 'eat-me' signal PS could be utilized for the targeted delivery of nanotubes to professional phagocytes and the regulation of functions of these cells.

Other surface modifications of CNTs have also been utilized (for a review see, Lacerda et al. 2006), and two recent *in vivo* studies have provided encouraging results in terms of biodistribution and biocompatibility of functionalized CNTs since no acute toxicity or adverse reaction was seen, and rapid excretion of CNTs through the renal route was observed (Wang et al. 2004; Singh et al. 2006). Functionalization of CNTs may thus serve to minimize adverse biological effects related to unwanted tissue accumulation of this important class of nanomaterials.

Risk assessment of engineered nanomaterials: The importance of surface area

Particle toxicology, which encompasses the novel subcategory of *nanotoxicology*, can be broadly defined as the study of the adverse effects of internalized particles on the living organism and the mechanisms underlying these effects. It is clear that, for a full

assessment of these effects, one must have an adequate description of the particles' physico-chemical properties, and an appropriate measure of the effects, at the cellular or molecular level in relevant target organ systems. Paracelsus (1493–1541), widely regarded as the father of toxicology, formulated the expression, 'All things are poison and nothing is without poison', suggesting that 'dose determines the poison' (Orrenius & Zhivotovsky 2006). In the field of nanotoxicology, the critical question is which particle characteristics are central in initiating and perpetuating the adverse effects? For instance, will surface area serve as a better determinant of nanoparticle toxicity than mass, as suggested by pioneering animal studies of particle deposition and retention in the lung (Ferin et al. 1991; Oberdörster et al. 1994)? Indeed, as discussed in previous sections, the greater surface area per mass compared with larger-sized particles of the same composition renders nanomaterials more biologically reactive. The answers to these questions have profound implications for risk assessment of the hazardous effects of engineered nanomaterials.

Risk can be described as the product of *exposure* and *hazard*. An assessment of risk can only be realized if we know the level of exposure and the nature of the hazard. However, to minimize risk only an understanding of the latter can inform us on the correct choice of the former. To assess the specific risks posed by nanoparticles, a combination of *in vitro* and *in vivo* models, together with mathematical modeling to describe the chain of events, from exposure to responses, is needed. The essential steps for assessing the health risk from exposure to engineered nanoparticles, as outlined at the recent Nobel Forum mini-symposium are: (i) to determine the physico-chemical characteristics of the nanoparticles to be tested; (ii) to investigate the interaction of nanoparticles with the major target systems and the resulting adverse endpoints at the cellular and molecular level using *in vitro* models; (iii) to validate the *in vitro* findings with a small set of carefully chosen *in vivo* (animal) experiments; (iv) to construct mathematical models (Cullen et al. 2000; Tran et al. 2000) in order to extrapolate the exposure dose-response relationship from *in vitro* to *in vivo* models to humans; (v) to assess data gathered on the levels of exposure for the nanoparticles; (vi) to use Quantitative Structural Activities Relation (QSAR) models to identify the key nanoparticle characteristic(s) driving the adverse effects; and (vii) to combine *exposure* and *hazard* information, for risk assessment of the nanoparticles. One approach for extrapolating data from animal studies to human exposure scenarios was provided in a recent report by Kuempel et al. (2006), who concluded that established quantitative risk assessment methods can be used to estimate the risk of occupational exposure to fine and ultrafine particles.

There is a strong likelihood that the biological activity of nanoparticles will depend on physico-chemical parameters not routinely considered in toxicity screening studies (Oberdörster et al. 2005). Indeed, recent studies of so-called poorly-soluble particles of low toxicity (LSLTPs), such as TiO_2 and $BaSO_4$ in an *in vitro* model of human lung cancer cells have provided evidence that particle surface area is the dose metric which produces the most consistent trends (in the threshold and/or the dose response) in various biomarkers of inflammation, including interleukin (IL)-8 mRNA expression and IL-8 protein secretion (Monteiller et al. 2007). Furthermore, dose-response relationships observed in these *in vitro* assays were comparable to dose-response relationships obtained in previous *in vivo* studies (Duffin et al. 2001) when the doses were similarly standardized, and both sets of data suggested a threshold in dose measured as surface area of particles relative to the surface area of exposed cells, at around 1 to 10 cm^2/cm^2 (Monteiller et al. 2007; and Figure 3). These results thus indicate that a rational approach to extrapolation between *in vitro* and *in vivo* results is possible. Extension of this research should include a larger array of nanoparticles to determine their general conformity with the

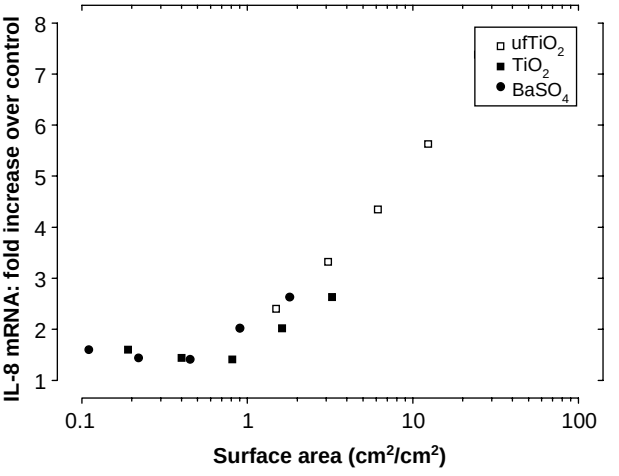

Figure 3. Effect of surface area-dose on IL-8 mRNA expression upon exposure of human lung cancer cells to TiO_2 and $BaSO_4$. The human A549 lung cancer cell line was exposed for 6 h to fine and ultrafine (uf) particles as indicated, and the level of expression of IL-8 mRNA was determined using reverse transcription (RT)-PCR, as a marker of inflammation. The data demonstrate that surface area-dose is related to the inflammogenic properties of the particles tested, and suggest a threshold in dose measured as surface area of particles relative to the surface area of exposed cells, at around 1 cm^2/cm^2. Adapted from Montellier et al. (2007), with permission from the BMJ Publishing Group.

'surface area' paradigm. Furthermore, the regulation of human occupational exposures, which is currently based on airborne mass concentration, should also be considered in light of these findings.

Regulation of the nanotechnologies: Identifying knowledge gaps

Through the emerging nanotechnologies it is possible to produce molecular structures and materials with new properties and behaviors. The step from research to industrial applications has been short, and the marketing of a broad range of nanotechnology-based consumer products is expected to rapidly increase. In June 2005, the European Commission adopted the Action Plan, '*Nanosciences and Nanotechnologies: Action Plan for Europe 2005-09*', in which a set of concrete actions covering a broad range of areas are proposed, including examination of relevant regulations and measures to fill the existing 'knowledge gaps'. Along similar lines, the US Environmental Protection Agency (EPA) has issued several documents which emphasize the urgent need for large-scale investigations of nanoparticles and their health effects and toxicological profiles. In the '*Nanotechnology White Paper*' from February 2007, the EPA states: 'Research is needed to inform the Agency's actions related to the benefits and impacts of nanomaterials. However, there are significant challenges to addressing research needs for nanotechnology and the environment. The sheer variety of nanomaterials and nanoproducts adds to the difficulty of developing research needs. Each stage in their lifecycle, from extraction to manufacturing to use and then to ultimate disposal, will present separate research challenges'. (Environmental Protection Agency 2007). Nanotoxicology, as highlighted throughout the current presentation, is the main element of risk and safety assessment of nanomaterials and is needed in order to provide adequate information for regulations and risk management measures.

Several reports have pointed out that appropriate legislation does exist; the challenge lies in the implementation of the legal frameworks in the face of insufficient scientific understanding of the toxic profiles of novel nanomaterials and a rapidly evolving market for products based on these materials (essentially, a 'moving target' from a legislative point of view). For instance, the European Group on Ethics in Science and New Technologies to the European Commission (EGE) stated in their Opinion in January 2007: 'As far as the legal implications [of nanomedicine] are concerned, the EGE does not propose any new regulatory structures specifically dealing with nanomedicine at this point, and argues that any changes should be made within existing structures (with focus on implementation of existing regulations). The Group proposes, however, that possible cases of nanomedicine applications where there might be overlap between regulations, which could create uncertainty as to which regulations should be applied, should be explored by the relevant authorities so that the existing regulations can be implemented in an unambiguous way'. In the same report, the EGE comments on public acceptance of nanotechnologies in general: 'As far as public participation is concerned, the EGE argues that transparency (including openness about uncertainties and knowledge gaps) is essential for public trust in nanotechnology' (European Group on Ethics in Science and New Technologies to the European Commission 2007).

Several important 'knowledge gaps' were identified and discussed at the Nobel Forum mini-symposium, and efforts to address these issues will be required to ensure science-based decision making and implementation of existing legislations: (i) nomenclature, definitions, and standards; (ii) hazard characterization; (iii) exposure and effects assessment; (iv) environmental fate, transport, and persistence; and (v) measurement, sampling, and monitoring of nanomaterials. The central challenge from a regulatory point of view is, of course, how to protect public interest (health, safety, privacy, environment, etc) without hampering technological progress. This can only be accomplished through a close dialogue between researchers and legislators to identify central issues to be tackled from both a scientific and a regulatory perspective.

Conclusions and perspectives: The princess and the pea

The Princess and the Pea is a fairytale about the search for a wife sensitive enough to feel a pea through 20 mattresses and 20 featherbeds (Andersen 1835). The original story is likely a reference to the importance of emotional sensitivity or intuition; a ruler should be sensitive to the needs of his people. However, we may also use this fairytale to describe the role of nanotoxicologists, who need to remain sensitive to the potential hazardous effects of nanoparticles (peas) even when these novel materials are embedded in the context of complex and evolving technologies, which are frequently hailed as the panacea or remedy for all our societal and medical needs.

There are considerable gaps in our knowledge concerning the potential hazardous effects of engineered nanoparticles on human health and the environment, as pointed out in a recent report from

the European Commission (Scientific Committee on Emerging and Newly Identified Health Risks 2005). However, in choosing the title '*There's plenty of room at the forum*' for the current discourse, we intended to emphasize the fact that there is plenty of opportunity not only for discussion, but also for actual collaboration in the emerging and important area of nanotoxicology; indeed, we need to recognize that it is essential for the international community of nanotoxicologists to coordinate its efforts in order to avoid duplication, and also to enable us to cover a wider area of research, which is important because the nanotechnologies are developing at a very rapid pace. While the 'grand' challenges proposed by Maynard and his associates in a recent commentary in *Nature* (Maynard et al. 2006) are an important step forward and serve to focus public attention on strategic risk management of nanomaterials, we as toxicologists and risk assessment experts need to discuss in detail and in the spirit of pre-competitive cooperation how to reach the goal of safe handling of the nanotechnologies.

Finally, one should consider that we are currently witnessing the first generation of engineered nanomaterials, while more sophisticated nanomaterials and nanodevices are waiting around the corner. Therefore, it is of utmost importance to develop strategies and methodologies that will allow us to predict and respond to the potential hazardous effects not only of existing (passive) nanomaterials but also of active nanostructures and integrated nanosystems that may gradually approach the dimensions and the enormous complexities of biological systems (Environmental Protection Agency 2007; United States Congress Joint Economic Committee 2007). While we cannot predict the future, we need to prepare for it. Towards that aim, we believe that a proportion of the global funding of nanotechnology-related activities should be devoted to nanotoxicological research and risk assessment, which should be conducted on an interdisciplinary and intercontinental level.

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