



Invited Review Article

Mechanisms of carbon nanotube-induced toxicity: Focus on oxidative stress

Anna A. Shvedova^{a,b,*}, Antonio Pietroiusti^c, Bengt Fadeel^{d,e}, Valerian E. Kagan^e^a Pathology and Physiology Research Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, University of Rome "Tor Vergata", Rome, Italy^b Department of Physiology and Pharmacology, West Virginia University, Morgantown, WV, University of Rome "Tor Vergata", Rome, Italy^c Department of Biopathology, University of Rome "Tor Vergata", Rome, Italy^d Division of Molecular Toxicology, Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden^e Department of Environmental and Occupational Health, University of Pittsburgh, Pittsburgh, PA, USA

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ABSTRACT

Nanotechnologies are emerging as highly promising technologies in many sectors in the society. However, the increasing use of engineered nanomaterials also raises concerns about inadvertent exposure to these materials and the potential for adverse effects on human health and the environment. Despite several years of intensive investigations, a common paradigm for the understanding of nanoparticle-induced toxicity remains to be firmly established. Here, the so-called oxidative stress paradigm is scrutinized. Does oxidative stress represent a secondary event resulting inevitably from disruption of biochemical processes and the demise of the cell, or a specific, non-random event that plays a role in the induction of cellular damage e.g. apoptosis? The answer to this question will have important ramifications for the development of strategies for mitigation of adverse effects of nanoparticles. Recent examples of global lipidomics studies of nanoparticle-induced tissue damage are discussed along with proteomics and transcriptomics approaches to achieve a comprehensive understanding of the complex and interrelated molecular changes in cells and tissues exposed to nanoparticles. We also discuss instances of non-oxidative stress-mediated cellular damage resulting from direct physical interference of nanomaterials with cellular structures.

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Introduction

The trouble with the world is not that people know too little, but that they know so many things that ain't so

Mark Twain

* Corresponding author at: Health Effects Laboratory Division, NIOSH, Morgantown, WV 26505, USA. Fax: +1 304 285 5938.

E-mail address: ats1@cdc.gov (A.A. Shvedova).

Developments in nanotechnology have already yielded breakthrough discoveries in nanomedical devices, biosensors, drug delivery protocols and theranostics (Cabezon et al., 2011; Fazil et al., 2012; Leucuta, 2010; Motwani et al., 2011). A large diversity of nanomaterials have found their way into many consumer items, such as clothing and cosmetics, thus increasing the likelihood of human contact and exposure to nanomaterials. In addition, occupational, environmental and indoor exposures to nanoparticles remain a public concern and a potentially serious health hazard (Maynard et al., 2011). Extrapolations from the field of toxicology of particulate matter (PM), especially from extensive work on PM with less than 10 μm in diameter (PM₁₀), laid the background for the suspicions that nanoparticles are not innocuous but may yield a range of harmful effects (Oberdörster, 2012). Indeed, some animal studies of pulmonary toxicity of nanoparticles, particularly carbon nanotubes (CNTs), revealed robust, pro-inflammatory responses accompanied by oxidative stress (Ravichandran et al., 2011; Shvedova et al., 2005).

In 2009, the marked demand for single-walled CNTs (SWCNTs) was 90 million US\$ versus 120 million US\$ for multi-walled CNTs (MWCNTs). However, the projected market growth predicts that in year 2014 the demand for SWCNTs and MWCNTs will be 600 and 470 million US\$, respectively (<http://nextbigfuture.com/2008/06/carbon-nanotube-production.html>). Thus potential adverse health effects of both SWCNTs and MWCNTs, particularly in occupational settings, should be seriously considered along with the toxicity mechanisms of these types of CNTs.

There is evidence that ENMs, including CNTs, may be released in the work environment: many activities have been investigated such as workplace cleaning, packaging/bag filling, reactor cleanout, mixing, cutting composite, and in almost all cases evidence for some release of ENMs in the workplace has been detected (Bello et al., 2009; Han et al., 2008; Kuhlbusch and Fissan, 2006; Maynard et al., 2004; Peters et al., 2009; Tsai et al., 2009; Vosburgh et al., 2011). Some reports describing serious adverse effects of occupational exposure to ENMs have been published: These include several cases of rescue workers present at the World Trade Center in New York City after the tragic events of 9/11 (Aldrich et al., 2010; Gupta, 2011; NIOSH, 2011; Romano, 2011; Zeig-Owens et al., 2011), reports of occupational exposures to mixtures containing nanoparticles in factories in China and India (Jaakkola et al., 2011; Song et al., 2009), a case-report regarding a worker who inhaled an estimated gram of nickel nanoparticles over an approximate 90-minute period, and who died from adult respiratory distress syndrome (Phillips et al., 2010), the development of unusual pulmonary disease in workers exposed to silica nanoparticles (Song et al., 2011), and the development of toxic epidermal necrolysis-like dermatitis in a chemist exposed to high levels of intermediate or final products of dendrimers while performing dendrimer synthesis (Toyama et al., 2009). Finally, a recent occupational survey showed an association with mortality due to chronic obstructive pulmonary disease and to ischemic heart disease in a cohort of workers exposed to metalworking fluids containing a substantial amount of incidental nanoparticles (Eisen et al., 2011).

Is oxidative stress a uniform and sensible paradigm for nanoparticle-induced cytotoxicity?

The intrinsic toxicity of nanoparticles has been attributed to their unique physico-chemical characteristics — i.e. their smallness and the remarkably large surface area per unit mass and high surface reactivity (Auffan et al., 2009). This physico-chemical reactivity is frequently correlated with the ability of nanoparticles, especially metal-based nanoparticles, to trigger the formation of free radicals (i.e. reactive oxygen species (ROS) such as superoxide radical anions and hydroxyl radicals) directly or via activation of oxidative

enzymatic pathways leading to oxidative stress. It is believed that ROS generation by nanoparticles is realized through several mechanisms frequently including redox-cycling pathways. These may include the oxidant-generating properties of particles themselves as well as their ability to stimulate generation of ROS as a part of cellular response to nanoparticles (Knaapen et al., 2004). Among the former, the major sources of oxidative stress are (i) transition metal-based nanoparticles or transition metal contaminants used as catalysts during the production of non-metal nanoparticles, including SWCNT (ii) relatively stable free radical intermediates (detectable by electron paramagnetic resonance spectroscopy) present on “reactive” surfaces of particles such as quartz, carbonaceous particles, and (iii) redox-active groups resulting from functionalization of nanoparticles (e.g., quinones capable of generating ROS) (Knaapen et al., 2004; Kovacic and Somanathan, 2010; Li et al., 2010). In addition to direct prooxidant effects of nanoparticles, they may activate the redox-machinery of the cell especially in the lungs where inflammatory cells — neutrophils and macrophages — can act as potent generators of ROS. Mitochondria are the major source of ROS in the cell; indeed, the production of superoxide is an inevitable “side-effect” of respiration and, hence, of life itself, and cells balance this continuous production of ROS through expression of a plethora of anti-oxidant enzymes. However, in phagocytic cells of the immune system, NADPH oxidase-dependent generation of ROS is also a critical source of ROS in response to ingestion of offending agents e.g. bacteria, which are killed through a combination of oxidative and proteolytic activities within the phagocytic cell (more recent studies suggest that neutrophils may also capture extracellular bacteria through the generation of so-called neutrophil extracellular traps or NETs; however, this formation of NETs is also NADPH oxidase-dependent) (Fadeel, 2009; Fadeel and Kagan, 2003). Recent studies have shown that ROS-producing mitochondria can trigger inflammasome activation in phagocytic cells (Zhou et al., 2011), thus providing a link between mitochondria and inflammation, with ROS acting as the signal. In addition, the activation of lysosomes, cellular “suicide bags” with high content of digestive enzyme, may in turn lead to the triggering of mitochondrial apoptosis, with production of ROS (Torres Andon and Fadeel, *in press*). It is our firm belief that we may gain a much better appreciation for the biological/toxicological effects of nanoparticles if we consider the highly specialized (organelle-specific) pathways of ROS production and the ways in which nanoparticles may impinge upon these pathways. Nel and co-workers provided evidence for the differential induction of cytotoxicity by different nanoparticles depending on cell type-specific uptake mechanisms (Xia et al., 2008). In a recent study, we provided an example of nanoparticle-specific, ROS-dependent cytotoxicity insofar as overexpression of the anti-oxidant enzyme, microsomal glutathione transferase-1 (MGST1) protected cells from silica nanoparticle-induced cell death but not from ZnO nanoparticle-induced cell death; the protective effect was likely exerted through the prevention of nanoparticle-induced lipid peroxidation in critical cellular membranes. Both nanoparticles, however, triggered the production of cellular ROS (Shi et al., 2012). Moreover, active cellular internalization of nanoparticles or fibers through phagocytic pathways may, at least in professional phagocytic cells, result in activation of the NADPH oxidase with resultant massive generation of ROS (the so-called “oxidative burst”). Indeed, studies in mice lacking expression of a functional NADPH oxidase have shown that NADPH oxidase-derived ROS are critical in determining the course of pulmonary response to SWCNT (Shvedova et al., 2008a). The localization of nanoparticles to lysosomes may lead to activation of the lysosomal-mitochondrial axis with attendant cell death. Using rapid multicolor 3D live cell confocal fluorescence microscopy, combined with transient overexpression of small GTPases marking various endocytic membranes, Dawson and co-workers were able to reveal the kinetics of nanoparticle trafficking through early endosomes to late endosomes and lysosomes (Sandin et al., 2012).

CNT represents an illustrative case. Hence, several groups have shown that the presence of iron in SWCNT may be important in determining redox-dependent responses of macrophages. Hence, in zymosan-stimulated RAW 264.7 macrophages, non-purified iron-rich SWCNT were more effective in generating hydroxyl radicals (documented by EPR spin-trapping with 5,5-dimethyl-1-pyrroline-N-oxide, DMPO) than purified (iron-stripped) SWCNT (Kagan et al., 2006). Similarly, Pulskamp et al. (2007) reported a dose- and time-dependent increase of intracellular ROS and a decrease of the mitochondrial membrane potential with non-purified CNT in rat macrophages and human lung cells whereas incubation with the purified (acid-treated) SWCNT had no effect. Furthermore, others have suggested that, contrary to traditional toxicants, such as asbestos, CNT may quench rather than generate oxygen free radicals. This scavenging activity was suggested to be related to the presence of structural defects (Fenoglio et al., 2008). Notwithstanding, upon interaction with phagocytic cells of the immune system, CNT may induce ROS production through NADPH oxidase-dependent pathways, as we shall discuss in a following section. Notably, we have found that NADPH oxidase-deficient mice respond to SWCNT exposure with a marked accumulation of neutrophils and production of pro-inflammatory cytokines, decreased production of the anti-inflammatory and pro-fibrotic cytokine, TGF- β , and significantly lower levels of collagen deposition, as compared to wild-type control mice, thus supporting a prominent role for NADPH oxidase-ROS in determining course of pulmonary responses to SWCNT.

This emphasis and wide acceptance of “oxidative stress” pathways as one of the fundamental paradigms for toxicity necessitates a very careful analysis of specific mechanisms elicited in cells and tissues by nanoparticles and assessment of possible role and significance of oxidative reactions in responses elicited by exposures to nanoparticles.

Remarkable advancements in chemistry of liquid-phase oxidation of organic compounds in the mid 1960–70s had a huge impact on several biomedical disciplines and designated the emergence of a new field of knowledge and practice – free radical biology and medicine (Emanuel, 1976). Initially applied to explain unusual features of radiation induced damage, these frontier chemical ideas propagated into different areas of biomedicine, particularly after the discoveries of oxygen radicals and their regulating enzymes in cells and tissues (Fridovich, 1995). Based on the premise of the essentiality of low molecular weight sacrificial antioxidants – small water- and lipid-soluble molecules such as vitamins C and E – capable of scavenging reactive radicals and slowing down the overall oxidation rates of essential biomolecules in biofluids and tissues (Packer, 1992), the concept of “oxidative stress” was put forward and quickly gained in popularity (Sies, 1985). According to this concept the disturbances in the balance between pro- and antioxidants disrupt the equilibrium in the redox “powers” resulting in accumulation of oxidation products and, potentially, cell damage and death. While the two corner-stone doctrines: i) oxidative stress and ROS are essential mechanisms of injury and diseases and ii) antioxidants can be used as preventive/protective treatments – have received much attention, they have ignored, at least for some time, possible important metabolic functions of oxidative reactions. The latter re-emerged and became central in the recent studies, particularly as simplistic understanding of “oxidative stress” as an exclusive mechanism of injury has been sobered by the outcome of clinical intervention trials that did not reveal significant beneficial effects of antioxidants (Abner et al., 2011; Azzi, 2007; Niki, 2010). There is a mounting consensus that the meaning of oxidatively modified biomolecules in biomedicine and toxicology may need a serious re-evaluation with regards to the major pathways involved and their significance in the pathogenesis of injury and disease. An illuminating example in this respect may be to assess the importance of oxidative reactions for the toxicity of nanoparticles.

Lipid peroxidation in nanoparticle-induced cell death: oxidative lipidomics approaches

Lipid peroxidation products are among the most common types of oxygenated molecules implicated in oxidative stress responses. Overall, lipid peroxidation reactions are sub-categorized into two types: enzymatic and non-enzymatic. The latter proceed as typical “free radical” reactions whereby the oxidation rates are defined mainly by the number of double bonds in their polyunsaturated fatty acid (PUFA) residues. Consequently, this random process should affect all different (phospho)lipid classes with six, five and four double bonds. The dependence of this type of reactions on the chemical nature of phospholipid polar heads is usually minimal (Kagan, 1988). Enzymatic oxygenations of free PUFA, on the other hand, have been known for a long time as the major source of numerous extra- and intracellular biological regulators that include a large diversity of eicosanoids, prostanooids, docosapentanooids and docosahexanooids (Praticò, 2008; Serhan, 2010). Their major metabolic sources are polyunsaturated phospholipids that undergo an enzymatic attack by selective phospholipases with subsequent specific enzymatic oxygenation of free PUFA. Until recently, enzymatic peroxidation of PUFA esterified into phospholipids has not been recognized as one of the major metabolic pathways. This was mostly due to the fact that detailed analysis of diversified peroxidation products in different classes of phospholipids and identification of their individual molecular species was difficult. Current advancements in different types of mass-spectrometry (MS) of small molecules gave a remarkable boost to MS of phospholipids (Blanksby and Mitchell, 2010; Hsu and Turk, 2009; Ivanova et al., 2009; Tyurin et al., 2009) and their oxidation products thus heralding the appearance of the research field of (global) oxidative lipidomics (Kagan and Quinn, 2004; Kagan et al., 2004). As we shall discuss below, the application of oxidative lipidomics protocols to the study of nanomaterial-induced cell and tissue damage may yield novel and important information.

It is a common belief that nanomaterials cause non-specific oxidative damage that underlies, to a large extent, their cytotoxicity (Li et al., 2008; Nel et al., 2006). This may be true for some specific cases e.g. for nanoparticles capable of acting as pro-oxidants due to the chemical nature of their surface (see above). Indeed, Fenoglio et al. (2008) have suggested that, contrary to traditional toxicants, such as asbestos, CNT may quench rather than generate oxygenated free radicals. This scavenging activity, related to the presence of structural defects, appeared to be associated with the genotoxic and inflammatory potential of CNT (Muller et al., 2008).

However, this view may be an oversimplification of the complex interactions between a robust inflammatory response, cytotoxicity and oxidative reactions accompanying these processes. Notably, no detailed lipidomics/oxidative lipidomics studies have been performed to characterize “oxidative stress” elicited by nanoparticles *in vivo*. These considerations prompted us to undertake an MS-based oxidative lipidomics analysis of all major pulmonary phospholipid classes in mice exposed to CNT through inhalation (5 mg/m³, whole body inhalation for 4 consecutive days, 5 h/day) and euthanized 1, 7, and 28 days thereafter. Because transition metals, particularly iron, are frequently used as catalysts in single-walled CNT (SWCNT) synthesis (Tessonier, 2011), in these experiments, we intentionally utilized the preparations of CNT with a relatively high content of Fe (17.7% weight) as well as other transition metals capable of inducing non-enzymatic oxidative stress – Cu (0.16%), Cr (0.049%), and Ni (0.046%) (Valko et al., 2005). In spite of the presence of several potential transition metal catalysts of non-enzymatic lipid peroxidation, we found highly selective patterns of pulmonary phospholipid peroxidation after the inhalation exposure of mice to SWCNT. No oxidized molecular species were found in the two most abundant phospholipid classes: phosphatidylcholine (PC) and phosphatidylethanolamine (PE) – that were rich in

peroxidizable PUFA residues. Instead, peroxidation products were detected and identified in three relatively minor classes of anionic phospholipids, cardiolipin (CL), phosphatidylserine (PS) and phosphatidylinositol (PI), whereby oxygenation of polyunsaturated fatty acid residues showed unusual substrate specificity. Moreover, we demonstrated that this non-random profile of SWCNT-induced lipid peroxidation in the lungs of exposed mice was associated with an accumulation of significant amounts of apoptotic cells in the lung. We believe that the pattern of lipid peroxidation reflects the specific cytotoxic (pro-apoptotic) effects of SWCNT and therefore argues against non-specific oxidative damage.

It is intriguing to uncover the catalytic nature of the selectivity of SWCNT-triggered phospholipid peroxidation in spite of the presence of high doses of “non-specific” transition metal catalysts commonly associated with induction of “oxidative stress”. Indeed, our studies indicated that phospholipid peroxidation was secondary to the cytotoxic effects of inhaled SWCNT that caused massive apoptotic cell death. We previously identified an intermembrane hemoprotein of mitochondria, cytochrome c (cyt c), as a catalyst of selective peroxidation of CL and PS in mitochondrial and extramitochondrial compartments of apoptotic cells, respectively (Ardail et al., 1990; Bayir et al., 2006; Jiang et al., 2003, 2008; Kagan et al., 2002, 2003, 2005; Schlame and Haldar, 1993; Tyurina et al., 2004a, 2004b). Notably, a similar selective phospholipid peroxidation profile was detected upon incubation of a mixture of total lung lipids with H_2O_2 /cyt c known to catalyze CL and PS peroxidation in apoptotic cells (Fig. 1). Using similar oxidative lipidomics protocols, we have previously characterized the pattern of phospholipid peroxidation following several other insults, including hyperoxic acute lung injury, γ -radiation-induced lung injury, and traumatic brain injury (Bayir et al., 2007; Sparvero et al., 2010; Tyurin et al., 2010; Tyurina et al., 2008,

2009, 2010, 2011a). Strikingly, in all cases, a selective pattern of phospholipid peroxidation involving primarily CL and/or PS was observed (Tyurina et al., 2011a). The fact that inhalation exposure to SWCNT with high content of transition metals, particularly iron, known to effectively catalyze non-specific peroxidation of polyunsaturated phospholipids, also induces a similar, selective pattern of peroxidation of phospholipids (Fig. 1) suggests that common pathways of cellular and tissue damage are engaged. This is encouraging because it implies that common “anti-apoptotic” countermeasures against a diverse range of insults, including nanomaterials, may be envisioned. This is based on the essentiality of CL peroxidation for the execution of the apoptotic program (Tyurin et al., 2007); thus, suppression of this important function of cyt c/CL peroxidase complexes may be used as a target for the regulation of apoptosis (Hoye et al., 2008; Huang et al., 2008; Kagan et al., 2009a, 2009b). This protective/mitigative approach has very recently been proven successful in vitro (Belikova et al., 2009a; Jiang et al., 2009) and against total body irradiation-induced injury (Atkinson et al., 2011). On the other hand, treatment with antioxidant compounds should not be neglected. For example, in one recent study, the presence of vitamin E was reported to protect PC12 cells from the injury induced by SWCNT through the down-regulation of oxidative stress and prevention of mitochondria-mediated apoptosis (Wang et al., 2012). In line with these studies, lowered levels of antioxidants in vitamin E-deficient mice were associated with a higher sensitivity to SWCNT-induced acute inflammation and enhanced pro-fibrotic responses (elevation of TGF- β and collagen deposition) (Shvedova et al., 2007).

It will be important in the future to evaluate the extent to which the apoptosis-associated selectivity of phospholipid peroxidation detected after SWCNT exposure is characteristic of exposures to other engineered nanomaterials in the lung or in other tissues. Other forms of cell death including accidental necrosis, regulated necrosis

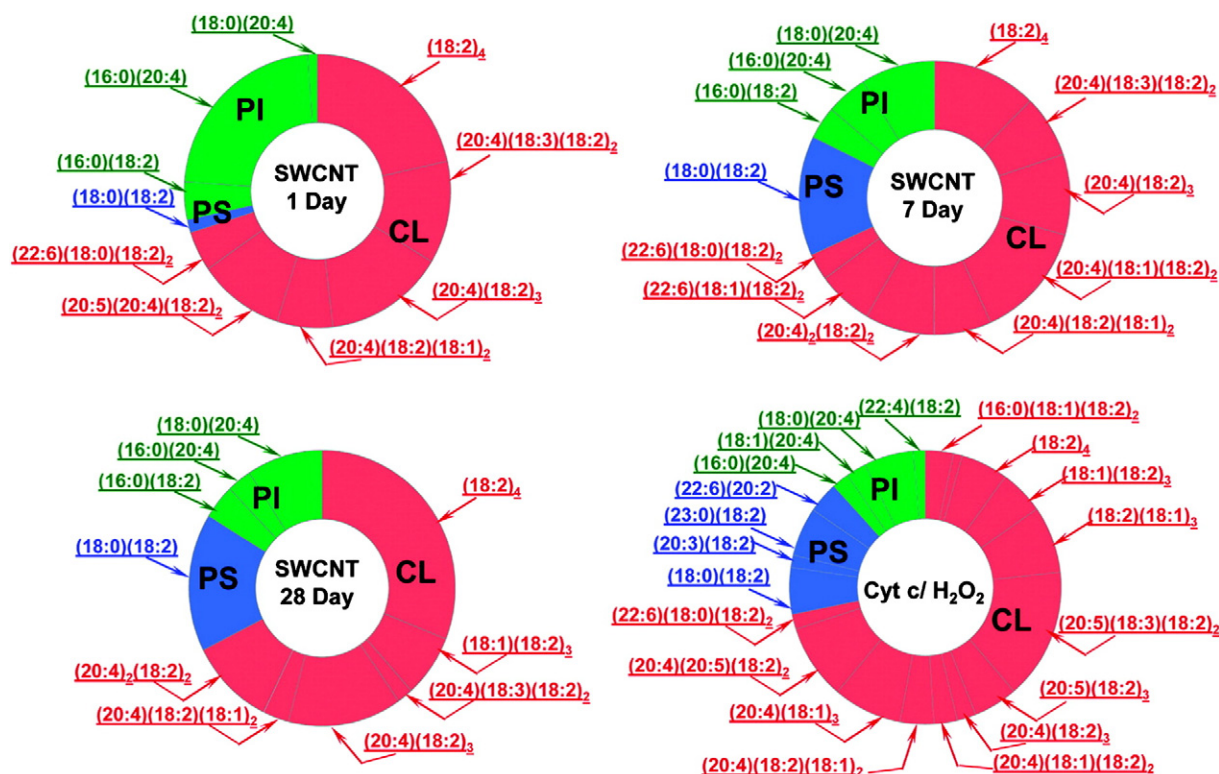


Fig. 1. Oxidative lipidomics reveals non-random phospholipid oxidation in the lungs of CNT-exposed mice. Profiles of phospholipid oxidation in the lung induced by the exposure of mice to SWCNT and by incubation of phospholipids extracted from the lung in a model system containing cyt c and H_2O_2 . Lipids from lungs of control mice and mice exposed to SWCNT were isolated and resolved by LC/MS. The data are presented as decreases in the amounts of oxidizable molecular species of phospholipids after exposure to SWCNT. CL = cardiolipin, PS = phosphatidylserine, PI = phosphatidylinositol.

Reprinted from Tyurina et al. (2011b) with permission from American Chemical Society.

(necroptosis), and cell death resulting from excessive autophagy may also be associated with the exposure to nanoparticles (Torres Andon and Fadeel, *in press*). It will be of considerable interest to establish whether these various cell death modalities are associated with specific patterns of phospholipid peroxidation catalyzed by specific enzymatic mechanisms.

Oxidative stress as a mechanism of biodegradation of carbon-based nanomaterials

The fact that specific rather than random oxidative reactions were elicited by exposure to nanomaterials raises a possibility that other responses, different from cell death mechanisms, may be critical as determinants of their fate and effects, including distribution and clearance from the tissues and body, degradation, etc. Indeed, the dimensions of nanoparticles are comparable to those of viruses or bacteria for which our immune system has elaborated sophisticated oxidative responses effectuated by cells of the innate immune system (Shvedova et al., 2010). Engulfment of bacteria provokes the “oxidative burst” in phagocytes with massive generation of ROS. Given that the oxidizing potentials of these reactive species are sufficient for breakage of C–H and C–C bonds it is likely that oxidative modification of nanoparticles will occur, if they are engulfed. Myeloperoxidase (MPO) – an abundant enzyme of neutrophils – is one of the most potent enzymatic sources of oxidants involved in host defense responses. We reported that, in simple biochemical model systems, MPO turned out to be effective in oxidative biodegradation of SWCNT via a plethora of intermediate aliphatic and aromatic products to ultimately yield CO₂ and H₂O by a combination of two pathways associated with: i) hypochlorite generated by the enzyme in the presence of chloride, and ii) reactive intermediates of the enzyme (Kagan et al., 2010). Molecular modeling showed that the carboxyls on the carbon nanotubes – through the interaction with the basic amino acids-rich domain of the enzyme – position the SWCNT in the proximity of the catalytic site. It is likely that oxidative biodegradation of pristine SWCNT is initiated by the hypochlorite-driven carboxylation that primes them for binding with the enzyme and subsequent degradation that also involves the reactive intermediates of the enzyme. Notably, the biodegradation products were ineffective – in contrast to SWCNT – at inducing pulmonary inflammatory responses in mice upon pharyngeal aspiration (Kagan et al., 2010). Importantly, this propensity of MPO to oxidatively biodegrade SWCNT was also realized in activated human neutrophils *in vitro* – suggesting that MPO-driven reactions may represent an important biodegradation pathway.

Indeed, our recent studies using MPO-deficient mice provided direct evidence for the effectiveness of this mechanism *in vivo* (Shvedova et al., 2012). We found that clearance of SWCNT from the lung of MPO knockout mice was markedly compromised compared to wild-type animals. It is likely that two major pathways – hypochlorite generated by the enzyme and MPO reactive intermediates – are both involved in the oxidative biodegradation process (Bianco et al., 2011; Shvedova et al., 2012; Vlasova et al., 2011). Overall, these experiments identified a previously unknown role of MPO in the body to oxidatively biodegrade SWCNT. It has been demonstrated that other carbonaceous nanoparticles – multi-walled carbon nanotubes, fullerenes, and graphene may also undergo MPO-catalyzed modifications and degradation (Bianco et al., 2011). Given that fate and distribution of CNT in the body are defined, to a large extent, by their high aspect ratios (Donaldson et al., 2011), oxidative shortening of CNT should inevitably affect their toxicity. This warrants further studies of specific interactions of different types of oxidative reactions involved in oxidative stress and oxidative biodegradation of nanoparticles by inflammatory responses of immune cells.

Unlike NADPH oxidase-deficient individuals who suffer from life-threatening infections, persons with MPO deficiency are relatively unaffected apart from an increased susceptibility to *Candida albicans*, a common fungal pathogen (Hampton et al., 1998) suggesting an auxiliary role for MPO in immunity against infections. One may speculate that the catalytic competence of MPO towards CNT is a reflection of a very ancient role of this enzyme towards carbonaceous particles generated during food cooking, high temperature processing of metals, forest fires, volcanic eruptions, etc. to which animals and humans have been exposed from primordial times.

Several other peroxidase – lactoperoxidase, eosinophil peroxidase, thyroid peroxidase, and prostaglandin synthase – are activated by H₂O₂ to form intermediates with the oxidation capacities high enough to degrade carbonaceous nanomaterials. In addition, several other hemo-proteins in animal and plant tissues (e.g., hemoglobin and myoglobin, cytochromes P450, horseradish peroxidase) are also known to generate highly oxidizing reactive intermediates. Indeed, several of these enzymes have been shown to effectively degrade CNT (Allen et al., 2008, 2009; Vlasova et al., 2011). The initiation and specific mechanisms of these oxidation reactions may differ in different oxidizing systems. For example, pristine SWCNT demonstrates no degradation with horseradish peroxidase (HRP) but displays significant degradation by either hemin or FeCl₃. This suggests that a heterolytic mechanism of cleavage of H₂O₂ (Belikova et al., 2009b) by HRP is not effective towards pristine nanotubes, whereas Fenton catalysis results in the homolytic cleavage of H₂O₂ producing free radicals that oxidize pristine SWCNTs.

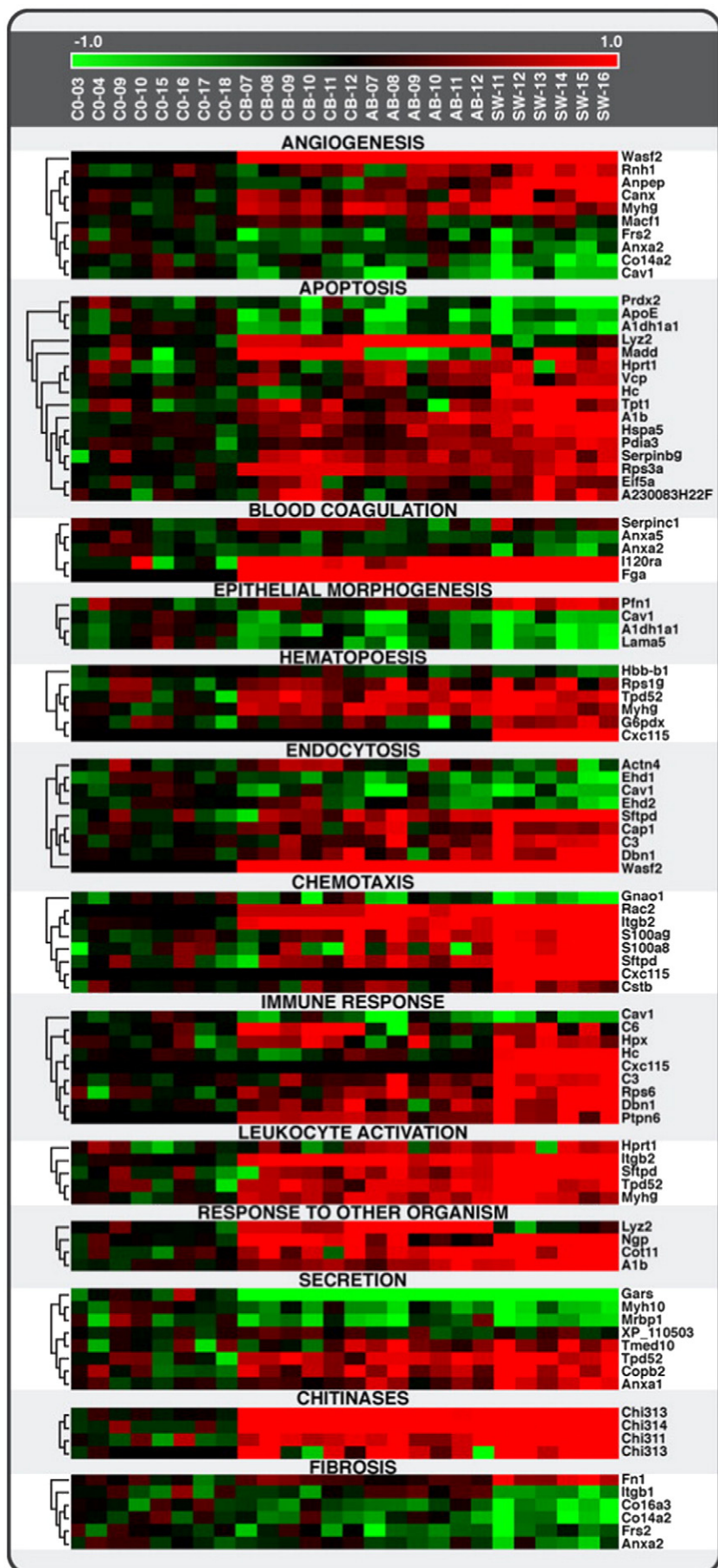
In a recent study, Donaldson and co-workers reported on the durability of four types of CNTs in simulated biological fluid (so-called Gambles solution) and their subsequent pathogenicity *in vivo* using a mouse model sensitive to inflammogenic effects of fibers (Osmond-McLeod et al., 2011). The results supported the view that CNTs are generally durable but may be subject to bio-modification in a sample-specific manner. This study also indicated that durable carbon nanotubes that are either short or form tightly bundled aggregates with no isolated long fibers are less inflammogenic in fiber-specific assays. In a related study, Liu et al. (2010) reported that SWCNTs with carboxylated surfaces are unique in their ability to undergo 90-day degradation *in vitro* in an artificial phagolysosomal simulant fluid leading to length reduction and accumulation of ultrafine solid carbonaceous debris. Unmodified, ozone-treated, and aryl-sulfonated tubes did not degrade under these conditions. The authors attributed the difference to the specific chemistry of acid carboxylation, which not only introduces COOH surface groups, but also causes collateral damage to the tubular graphenic backbone in the form of neighboring active sites that provide points of attack for further oxidative degradation.

Finally, not only SWCNTs but also other types of carbonaceous nanomaterials such as multi-walled CNT are sensitive to oxidants produced by peroxidases (Russier et al., 2011; Zhao et al., 2011).

A recent study demonstrated that two-dimensional graphitic carbon – a new material with many emerging applications – also undergoes enzymatic oxidation by HRP (Kotchey et al., 2011). Overall, the discovery of enzymatic biodegradation of carbonaceous nanomaterials by peroxidases emphasizes the general thesis of the current review that “oxidative stress” is not a uniform platform that is indiscriminately responsible for toxic effects of nanoparticles but should be viewed as an array of regulatable oxidative reactions. A better understanding of the nature and significance of such oxidative (signaling) responses will offer new opportunities to control the distribution, degradation and clearance of nanoparticles as well as their toxicity.

Oxidative stress as a signaling component in phagocytic cells: inflammasome activation

The dual role of ROS is particularly evident when considering the function of phagocytic cells of the innate immune system. Hence,



direct oxidative damage is involved in the killing of microorganisms and chronic granulomatous disease (CGD) patients lacking a functional NADPH oxidase, the major source of ROS in phagocytic cells, are at risk of life-threatening bacterial and fungal infections (van den Berg et al., 2009). However, phagocyte-derived ROS are also known to injure human tissues and to contribute to inflammation. Therefore, if one focuses on non-specific “oxidative stress” as a main culprit underlying toxicity of nanoparticles or other exogenous agents one may overlook other, vital signaling functions of reactive oxygen and nitrogen species, including the protective role of the so-called “oxidative burst” in phagocytic cells engaged in the engulfment and degradation of microorganisms. Of note, when mice were exposed to SWCNT and then challenged with *Listeria monocytogenes* this sequential exposure amplified lung inflammation (Shvedova et al., 2008b). However, despite the robust inflammatory response, SWCNT pre-exposure was associated with decreased phagocytosis of bacteria by macrophages in the lung and a decrease in nitric oxide production by these phagocytes. It is intriguing to speculate that the preoccupation with SWCNT leads to an inability of alveolar macrophages to handle subsequent exposure to microorganisms. The study suggests that exposure to CNT may exacerbate infections in exposed individuals.

One-half of the Nobel Prize in Physiology or Medicine in 2011 was awarded to Beutler and Hoffmann for their work on toll-like receptors (TLRs), cell surface proteins that provide a broad, first-line defense against microbial pathogens. Activation of the so-called inflammasome complex in the cytoplasm of phagocytic cells occurs via engagement of TLRs leading to subsequent assembly of the NLRP3 (NLR-related protein 3)-containing inflammasome complex and activation of caspase-1 with processing and secretion of the pro-inflammatory cytokine, interleukin (IL)-1 β (Dagenais et al., 2012). ROS production is thought to be an essential requirement for inflammasome activation. NLRP3 is also activated in response to host-derived particulate matter precipitates such as uric acid and cholesterol crystals and studies in recent years have shown that exogenous structures including asbestos fibers and crystalline silica also activate the inflammasome (Cassel et al., 2008; Dostert et al., 2008). Furthermore, very recent studies have shown that long and needle-like MWCNT also can activate the NLRP3 inflammasome, at least in vitro (Palomäki et al., 2011). How do CNT activate the inflammasome? One possibility is that phagocytic cells attempt to engulf the fiber-like structures leading to activation of the NADPH oxidase and production of ROS with or without internalization of the CNT. However, the role of NADPH oxidase-derived ROS for inflammasome activation has been called into question (van de Veerdonk et al., 2010) and an alternative hypothesis is therefore that nanotubes are recognized by phagocytes not as single fibers but as entangled particles leading to their internalization via endocytosis with subsequent fusion with lysosomes within the cell. The ensuing disruption of lysosomes may lead to the release of cathepsins and activation of the inflammasome. Notably, Zhou and colleagues reported recently that mitophagy/autophagy blockade leads to the accumulation of damaged, ROS-generating mitochondria, and this in turn activates the NLRP3 inflammasome (Zhou et al., 2011). One may thus speculate that autophagy blockade by nanoparticles (Ma et al., 2011) may provide an alternative pathway for inflammasome activation. The contribution of mitochondria-generated versus NADPH oxidase-generated ROS for nanomaterial-induced activation of the inflammasome merits further investigation.

The work cited above on the interaction of needle-like CNT with immune cells highlights a non-trivial point: all “carbon nanotubes” are not alike. Hence, these materials may be long or short, thin or wide, aggregated or well-dispersed, purified or raw (containing other contaminants), pristine or functionalized (Shvedova et al., 2009). Moreover, it remains possible that different properties of CNTs could underlie different toxicities, e.g. the length (high aspect ratio) may be an important determinant for frustrated phagocytosis/inflammation (Poland et al., 2008) whereas the diameter/rigidity may be critical for mesothelial cell damage and mesothelioma induction (Nagai et al., 2011). In the former case, toxicity may conceivably result from phagocytosis (or an attempt by macrophages to engulf the foreign material) leading to ROS production and inflammation. In the latter case, physical interference with cells i.e. the needle-like piercing of the plasma membrane may serve as the main driving mechanism of toxicity. In a very recent study, Fenoglio et al. (2012) provided evidence that the thickness of MWCNT plays a role in lung toxicity following intratracheal instillation in rats. They found that thin MWCNT appeared significantly more toxic than the thicker ones, both in vitro and in vivo, when compared on a mass-dose basis. Both forms of CNT were highly purified.

Other examples of non-oxidative stress mediated toxicities of nanomaterials are discussed below.

Non-oxidative stress-related mechanisms of nanoparticle-induced cellular damage

The data on selective phospholipid peroxidation induced upon exposure to CNT (Tyurina et al., 2011b) strongly suggest that oxidative damage is associated to induction of the apoptotic process. However, substantial cellular damage in the absence of any sign of oxidative stress or apoptosis has also been reported (Tabel et al., 2009). Interestingly, in the latter study, no evidence of cellular internalization of the CNT was presented. Research aimed at the elucidation of alternative, non-oxidative stress mediated pathways of cellular damage is therefore warranted. Some data are available and may be of help in orienting future studies. One potentially relevant mechanism of damage is represented by the physical interference of CNT with cellular and extracellular constituents, which may cause alterations of vital cellular processes, leading to various degrees of cellular injury, and in some cases even to cell death. Sargent et al. reported that the physical similarity of SWCNT to cellular microtubules forming the mitotic spindle was the cause of significant cytotoxicity in human airway epithelial cells exposed to this nanomaterial (Sargent et al., 2009). SWCNTs were in fact incorporated into the centrosome structure and induced DNA damage. Notably, these findings were observed at doses of SWCNT compatible with those expected in an occupational setting (Sargent et al., 2011). Another example of physical interference of carbon nanotubes with intracellular structures has been reported by Holt et al. who found that the intracellular presence of SWCNT caused the assembly of actin filaments into bundles with strong impairment in proliferative activity (Holt et al., 2010). This is not surprising, because the actin cytoskeleton is essential in maintaining cellular shape and in performing cellular functions (Heng and Koh, 2010). These findings are in keeping with previous experiments, in which profound alterations of the actin cytoskeleton of mesothelioma cells were seen after 5 day exposure to 15 $\mu\text{g}/\text{mL}$ SWCNT, whereas milder changes were observed in epithelial cells (Kaiser et al., 2008). The

Fig. 2. Comparative proteomics of instilled single-walled carbon nanotubes, crocidolite asbestos, and ultrafine carbon black in mice. Heat map showing protein levels for 13 cellular processes (GO functional categories) related to inflammation/immune response, fibrosis, and tissue remodeling in response to SWCNT, AB, and UFCB. SWCNT appeared to have the strongest effect on protein levels for most biological processes. Among the 109 proteins uniquely affected by SWCNT exposure were some related to the GO functional category “cell redox homeostasis”, suggesting a high level of oxidative stress, either directly from the SWCNT or indirectly from the resulting inflammation. Reprinted from Teeguarden et al. (2011) with permission from Oxford University Press.

outcome of these experiments was dependent on CNT quality (i.e. whether the materials were purified or not).

The cell may be viewed essentially as a collection of “nanomachines” as many enzymes possess nano-scale dimensions (van den Heuvel and Dekker, 2007). It is therefore perhaps not surprising that engineered nanomaterials may interfere directly with cellular functions. Indeed, Park et al. (2003) reported that SWCNTs of certain dimensions act as ion channel blockers. The authors speculated that nanotubes dampened receptor function by fitting into the pore and thus either hindering ion movement or alternatively preventing further conformational changes. Using large-scale molecular dynamics simulations, other investigators have shown that SWCNT can plug into the hydrophobic core of proteins to form stable complexes, leading to the disruption of ligand binding and loss of protein function (Zuo et al., 2010). Similarly, using a docking algorithm, Calvaresi and Zerbetto (2010) provided evidence for the interaction of C₆₀ fullerenes with a range of proteins. The authors proposed that the findings could be exploited for future biomedical applications of fullerenes, but the information may also shed light on the potential toxicity of nanomaterials. Overall, the above mentioned studies

suggest that nanomaterials may exert biological or toxicological effects through direct physical interaction.

SWCNT may also cause the appearance of unusual ultrastructural modifications in cells. Mangum et al. have shown in an in vivo study the presence of “carbon bridges” between alveolar macrophages in rats intratracheally instilled with SWCNT, in the absence of any sign of inflammation (Mangum et al., 2006). These peculiar structures were not observed after exposure with other carbonaceous material. It has been hypothesized that carbon bridges could affect the phagocytic function of macrophages or inhibit their ability to release or respond to cytokines. However, the process underlying this inter-cellular rearrangement remains elusive. Our studies have shown that primary human macrophages exposed to SWCNT exhibit a decreased ability to engulf apoptotic cells in the absence of overt cytotoxicity (Witaspl et al., 2009). This could potentially be explained by effects of the nanomaterials on cellular architecture especially on the actin cytoskeleton.

Adsorption of protein constituents of cellular plasma membrane onto the CNT surface, with subsequent loss of membrane integrity and cellular damage, is suggested by a recent study of mouse

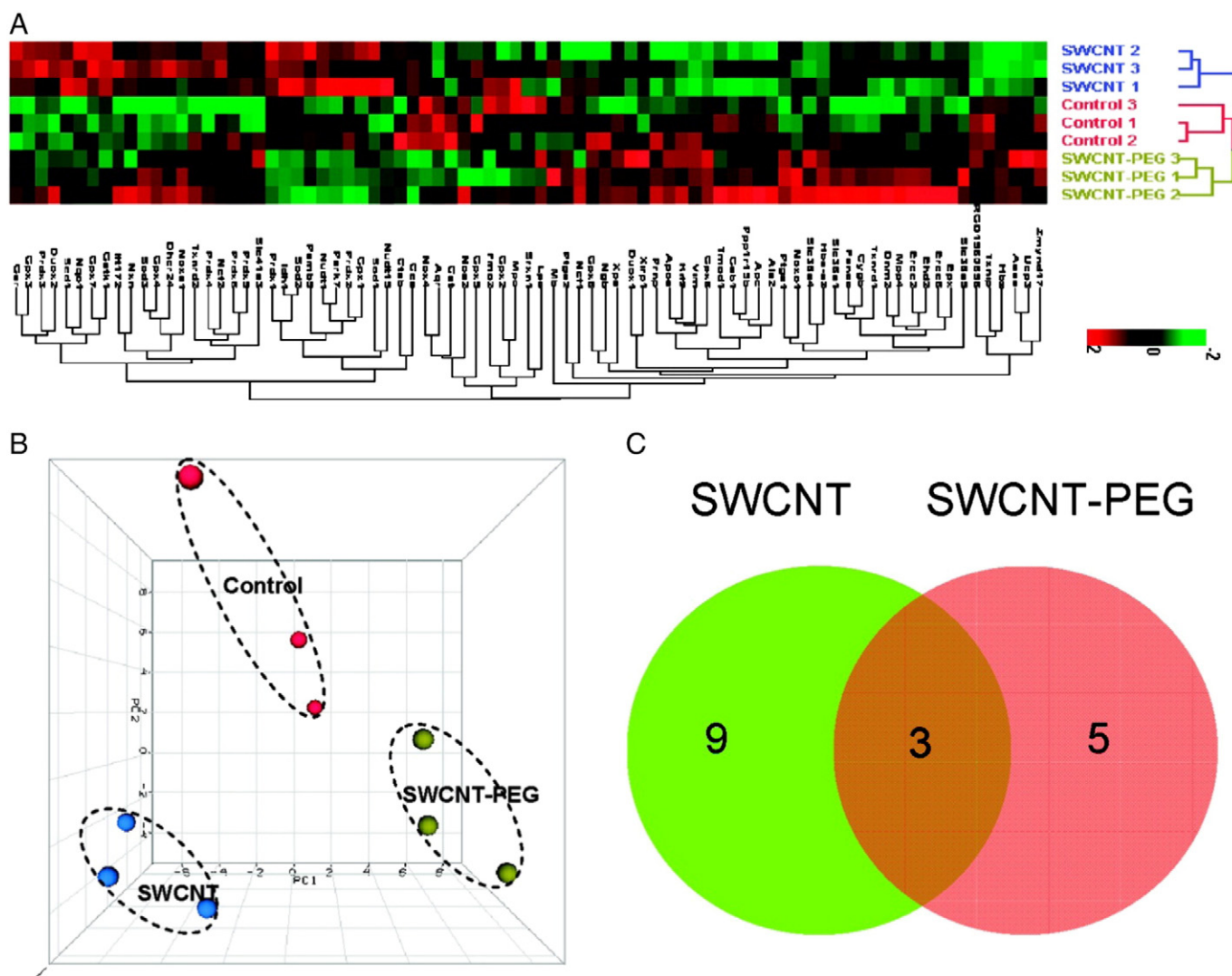


Fig. 3. Transcriptomic assessment of cytotoxic effects of SWCNT. The effect of surface modification of SWCNT on oxidative stress genes was determined using real-time PCR array to evaluate 84 genes related to oxidative stress. (A) Hierarchical cluster analysis (HCA) and (B) principal component analysis (PCA) of nine array data from neuronal PC12 cells treated with SWCNT (blue dots), SWCNT-PEG (green dots), and vehicle (red dots). (C) Venn diagram of the genes significantly changed by SWCNT or SWCNT-PEG treatment. Reprinted from Zhang et al. (2011) with permission from American Chemical Society.

macrophages exposed to MWCNT (Hirano et al., 2008). The relevance of this mechanism is questionable, however, since serum proteins are likely adsorbed onto nanomaterials in vivo so that cells are not directly exposed to the surface of nanoparticles. In fact, using both experimental and theoretical approaches Ge et al. show that binding of blood proteins to CNT strongly reduces their toxicity approaches (Ge et al., 2011). Further analysis of the protein “corona” on various nanoparticles, including MWCNT, was reported recently by Sund et al. (2011) who found that adsorbed proteins derived from plasma are involved in cellular internalization and seem to cover the nanomaterials independently of their surface properties while the binding efficiency to cell lysate proteins (derived from the cell) appears to depend on the characteristics of the nanomaterial surface. Overall, these studies elucidate the importance of understanding how cells “see” nanomaterials (Walczyk et al., 2010).

Other investigators have recently reported that pulmonary phospholipid coating may alter the plasma protein coating of functionalized MWCNT. This change in the pattern of adsorbed proteins is hypothesized to influence significantly the interaction and subsequent effects of the MWCNT on biological systems (Gasser et al., 2010; Schleh et al., 2011).

Notably, the corona of proteins and/or lipids may vary depending on the biological compartment, e.g. the blood stream versus lungs or the GI-tract.

Interactions of nanoparticles with cells of the innate immune system and the role of oxidants as signaling molecules were discussed in previous sections. Can nanomaterials also impact on cells of the adaptive immune system? Recent studies on SWCNT and MWCNT suggest that this is the case. Dendritic cells are professional antigen-presenting cells that play a key role in orchestrating immune responses and one-half of the Nobel Prize in Physiology or Medicine in 2011 was awarded to Steinman for his discovery of the dendritic cell (DC) and its role in adaptive immunity. Our recent work has shown that pulmonary exposure of mice to SWCNT leads to systemic immunosuppression as evidenced by decreased proliferative T cell responses in the spleen (Tkach et al., 2011). Notably, these effects were apparently mediated by a direct effect of SWCNT on DCs that

had infiltrated into the lung upon exposure to the nanomaterials. In contrast, other investigators have previously reported that exposure of mice to MWCNT via inhalation involved activation of cyclooxygenase enzymes in the spleen in response to a signal from the lungs, likely transforming growth factor (TGF)- β 1 (Mitchell et al., 2009). Spleen cells from exposed animals partially recovered their immune function when treated with ibuprofen, a drug that blocks the formation of cyclooxygenase enzymes. Hence, it appears that CNT may adversely affect systemic immune responses either directly (Tkach et al., 2011) or indirectly (Mitchell et al., 2009).

Omics approaches to understand nanoparticle-induced toxicity: the response of the Sybil

Ibis redibis non morieris in bello was the response (in its Latin translation) of the Sybil to a Roman soldier asking her about his fate in the war. However, the interpretation of the response depends on how it is written: if the comma is inserted before the word “non” it means: you will come back and will not die in war, but if the comma is after “non”, the meaning is the opposite: you will not come back and will die in war. Incidentally, the soldier was killed in war. In a similar manner, according to the proteomic response, two opposite outcomes may be predicted when challenging cells with CNT: apoptosis (i.e. death) or resistance to apoptosis (i.e. survival). In this case, it is an adjective and not a comma that determines the fate, as we shall explain below. However, before making explicit the adjective, it should be emphasized that the information provided by proteomic analysis of cells and tissues is far from being sybilline or cryptic; on the contrary, given the high level of complexity of the network of apoptosis regulation, the proteomic approach serves as an important aid in understanding the mechanisms behind the induction of susceptibility or resistance to cell death in response to exogenous substances. Global proteomic analysis allows the mapping of protein expression data into relevant molecular interaction networks and may thus provide additional insights into nanomaterial effects on apoptosis signaling and other cellular pathways.

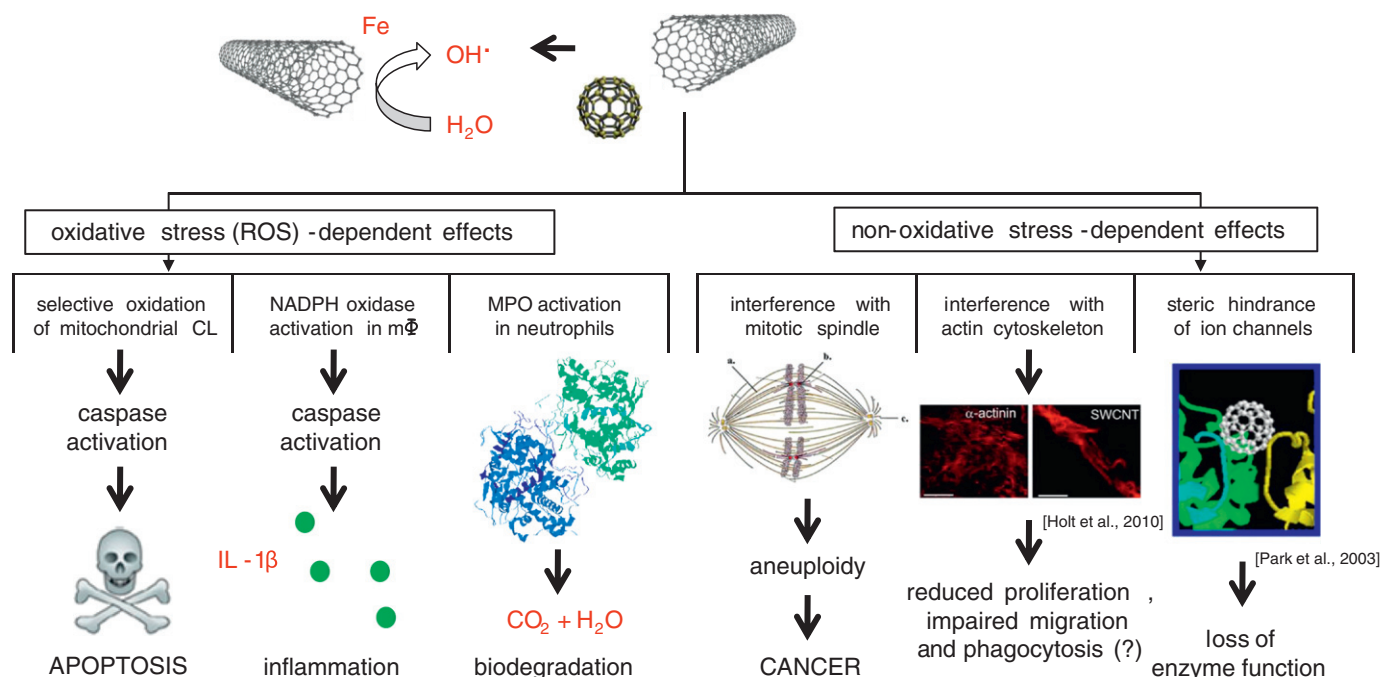


Fig. 4. Schematic figure depicting reactive oxygen species (ROS)-dependent and ROS-independent pathways of cellular toxicity induced by nanoparticles. Consult text for details.

The most extensively studied protein families in the evaluation of the interaction of CNT with mammalian cells are the caspase family and p53. Caspases are aspartate-specific proteases that play a critical role in apoptosis and inflammation (Fadeel et al., 2000). p53 is a pro-apoptotic protein and a tumor suppressor and is considered as the “guardian of the genome” (Rufini and Melino, 2011). Most studies of cellular protein profiles performed to date show over-expression of the p53 dependent and/or the caspase-dependent pro-apoptotic pathway after exposure to CNT (Cui et al., 2005; Pacurari et al., 2008; Ravichandran et al., 2010; Teeguarden et al., 2011; Yuan et al., 2011). This is generally associated with evidence of cellular oxidative stress and also with upregulation of genes involved in oxidative stress or with upregulation of oxidative stress sensitive proteins (Pacurari et al., 2008; Ravichandran et al., 2010; Teeguarden et al., 2011; Yuan et al., 2011). Thus, these proteomics data support the concept that apoptosis and oxidative stress are tightly interconnected events in response to CNT exposure (Fig. 2). It should be noted, however, that the experimental studies above refer to models of acute exposure, whereas in human populations, such as workers and consumers, a chronic low-dose pattern of exposure is expected. This gap is filled by a recent study in which an in vitro model of chronic exposure to SWCNT was applied (Wang et al., 2011). Interestingly, the findings are in sharp contrast to the acute exposure studies, showing not only that chronically exposed cells (i.e. 24 weeks of exposure, at a subcytotoxic dose of 0.02 µg/cm²) do not develop apoptosis, but also that these cells become apoptosis resistant. Protein array analysis of human lung epithelial cells BEAS-2B, showed a distinctive protein profile of apoptosis proteins, and in particular a striking decrease in the expression of phospho-p53 (Ser 15) protein, which is critical for p53-associated apoptosis (She et al., 2000). Testing SWCNT-exposed cells for sensitivity to known apoptotic inducers, such as etoposide and TNF-α, confirmed the acquired resistance to apoptosis. Thus, the critical adjective determining the cell's fate after exposure to CNT has perhaps been found: *acute* exposure causes increased apoptosis, *chronic* exposure apoptosis resistance. Of note, the aberrant survival of cells that have sustained DNA damage may produce adverse effects (cancer).

Transcriptomic analysis of mRNA expression has also been applied to understand cellular and tissue responses to nanomaterials. For instance, in an attempt to obtain a molecular characterization of the cytotoxic mechanism of MWCNT versus so-called multi-walled carbon nano-onions (MWCNO), Ding et al. (2005) performed whole genome expression analysis and high-content image analysis-based phenotypic measurements on exposed human fibroblasts. The microarray analysis indicated that multiple cellular pathways are perturbed after exposure to the nanomaterials, with material-specific toxigenomic profiles observed. Promoter analysis of the microarray results demonstrated that interferon and p38/ERK-MAPK cascades are critical pathway components contributing to the more adverse effects observed upon exposure to MWCNT as compared to MWCNO. Similarly, Zhang et al. (2006) performed genome-wide expression analysis of human fibroblasts exposed to PEG-coated silanized quantum dots and found that the quantum dots, unlike CNT, do not activate genes indicative of a strong immune and inflammatory response or heavy-metal-related toxicity. In a more recent study, the concentration-dependent cytotoxicity of SWCNT and SWCNT functionalized with polyethylene glycol (SWCNT-PEG) was compared in neuronal PC12 cells (Zhang et al., 2011). SWCNT elicited cytotoxicity in a concentration-dependent manner while SWCNT-PEG was less cytotoxic. ROS were generated after exposure to both nanomaterials. Gene expression analysis using a real-time PCR array showed that the genes involved in oxidoreductases and antioxidant activity, nucleic acid or lipid metabolism, and mitochondria dysfunction were highly represented (Fig. 3). The alterations of genes were surface coating-dependent. The findings suggest that surface functionalization of SWCNT decreases the oxidative stress-associated response. We have found that the exposure of primary human immune-competent cells

to cytotoxic ZnO nanoparticles results in upregulation of genes encoding metallothioneins, low-molecular weight proteins with metal-binding capacity and antioxidant properties (unpublished observations). The results are not unexpected in light of the fact that ZnO nanoparticles undergo dissolution when immersed in cell culture.

Conclusions

Nanoparticle toxicity may be understood in the context of the oxidative stress paradigm. However, this statement is only true insofar as we understand the meaning of “oxidative stress”. In the present review, we have emphasized that oxidative stress does not necessarily mean non-discriminative production of oxygen radicals with ensuing non-specific damage to cells and tissues. Instead, the induction of oxidative stress and related signaling pathways in response to nanoparticle exposure may also be viewed as a non-random event resulting in specific (programmed) cellular fates, e.g. apoptosis. Understanding the pathways leading to oxidative stress and the consequences thereof may yield novel approaches to mitigate the toxicity of engineered nanoparticles. To this end, it is important not only to emphasize the need for careful physico-chemical characterization of the nanomaterials, but also to consider cell type-specific differences in responses to nanomaterial exposure. We have also discussed examples of non-oxidative stress-mediated cellular damage resulting, for instance, from direct physical interference with the nano-scale machineries of cells. Fig. 4 illustrates oxidative stress dependent pathways and alternative, non-oxidative stress mediated pathways of nanoparticle toxicity.

We have also presented recent evidence for the enzymatic biodegradation of engineered nanoparticles and attempted to elucidate the importance of considering interactions of nanoparticles with the immune system, our principal defense system against foreign intrusion. Cells of the innate system are equipped with oxidative degradative machinery to dismantle microorganisms and several studies have shown that similar mechanisms may also be harnessed for the degradation of engineered nanomaterials.

Finally, we have highlighted the potential of global omics approaches (e.g. lipidomics, proteomics, transcriptomics) for the understanding of biological interactions of nanomaterials. The latter approaches may not only lead to the generation of new hypotheses regarding bio-nano-interactions but could also yield biomarkers of human exposure to engineered nanomaterials.

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.

Conflict of interest

None.

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