Close Encounters of the Small Kind: Adverse Effects of Man-Made Materials Interfacing with the Nano-Cosmos of Biological Systems

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Abstract

Engineered nanomaterials have unique physico-chemical properties that make them promising for many technological and biomedical applications, including tissue regeneration, drug and gene delivery, and in vivo monitoring of disease processes. However, with the burgeoning capabilities to manipulate structures at the nano-scale, intentional as well as unintentional human exposures to engineered nanomaterials are set to increase. Nanotoxicology is an emerging discipline focused on understanding the properties of engineered nanomaterials and their interactions with biological systems, and may be viewed as the study of the undesirable interference between man-made nanomaterials and cellular nanostructures or nanomachines. In this review, we discuss recognition of engineered nanomaterials by the immune system, our primary defense system against foreign invasion. Moreover, as oxidative stress is believed to be one of the major deleterious consequences of exposure to nanomaterials, we explore triggering of pro- and antioxidant pathways as well as biomarkers of oxidative stress. Finally, we highlight in vivo studies of the toxicological outcomes of engineered nanomaterials, including carbon nanotubes, with an emphasis on inflammation and genotoxic responses.

INTRODUCTION

Nanomachine: a nanoscale device that performs a specific task

Nanotoxicology: interdisciplinary study of toxicity induced by nanomaterials

Nanomaterials: materials ranging from 1 to 100 nm in at least one dimension

Oxidative stress: a shift in the redox balance of cells, tissues, and biofluids with a depletion of antioxidant protective mechanisms and accumulation of oxidized biomarkers

In his famous speech half a century ago, Richard Feynman (1) fantasized about the creation of miniaturized robots fulfilling different tissue repair functions in our bodies, acting as nanosurgeons in the bloodstream. Today, these fantastic ideas are being explored and are coming to fruition in a number of laboratories. Man-made nanomachines are thus close to becoming a reality. Joseph Wang (2) has recently summarized some of the pertinent questions that are raised by researchers working in the field of artificial nanomachines. First, can one transform the basic principles of biomotors for designing powerful man-made (biomimetic) nanomachines? Second, can synthetic nanomotors be powerful, versatile, and smart enough to perform demanding tasks and complex self-regulating operations? Third, can one integrate nanoengines with more complex architectures, performing multiple functions? Another burning question to add to this list is whether or not and to what extent artificial nanomachines can interfere with the highly tuned and structurally coordinated function of intracellular or extracellular nano-assemblies in living organisms. This question represents the core content of a new discipline—nanotoxicology—as discussed in this review.

How are we to define nanomaterials? Most formal definitions revolve around the manipulation of phenomena and materials at length scales below 100 nm (3). However, an alternative way of defining nanotechnology is to focus on the novel properties that arise owing to the specific smallness of the particles or materials independent of an exact specification of size (4). Mauro Ferrari has proposed that an operational definition of nanotechnology be based on the following three components: (*a*) the nano-scale size of the device or its crucial components, (*b*) its artificial (man-made) nature, and (*c*) properties that only arise as a result of the nano-sized dimensions (5). Furthermore, it may be important to realize that the nanotechnology of today, which deals largely with the study and manipulation of individual classes of nanoparticles or nanostructured surfaces, may soon—in a not too distant future—evolve into the study of much more complex nanosystems or nanomachines analogous to endogenous (cellular) nanomachines such as the ribosome or the cargo-towing kinesin and dynein motors. Nanotechnology, then, is a toolbox that provides nano-sized building blocks for new materials, devices, and systems, with the potential to provide a major force powering economic growth. Nanotoxicology, consequently, is the science of investigating and understanding how such man-made nanoparticles or complex nanostructures may interfere with biological systems.

A cursory examination of the scientific literature reveals an exponential growth in the number of publications on engineered nanomaterials (6). However, only ~1 in 50 (2%) of the total number of published articles on nanoparticles during the past 10 years were devoted to the toxicity of nanoparticles. We propose that a stronger emphasis should be placed on the study of the potential hazardous effects of man-made nanomaterials. Moreover, a conceptual framework and a validated experimental paradigm need to be developed to allow for the evolution of nanotoxicology into a predictive science, as opposed to being merely a descriptive exercise. This review focuses on the toxicology of engineered nanomaterials following unintentional or deliberate (medical) exposure with emphasis on nanomaterial recognition by the immune system, the induction of oxidative stress and the ensuing cellular dysfunction, and in vivo responses to nanomaterials.

NANOTOXICOLOGY: AN EMERGING DISCIPLINE

The term nanotoxicology was coined in 2004–2005 (7–10), although the question as to whether nanotechnology is dangerous was voiced at least five years earlier (11). Whereas substantial experience was accumulated through the study of particles of different sizes and sources, including

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ultrafine particles, prior to the introduction of the term nanotoxicology, the uniqueness of the physico-chemical properties of nanoparticles suggests that their interactions with cells and tissues may be unpredictable. Furthermore, because cellular molecular machines and man-made nanoparticles have similar dimensions, the latter may directly interact and interfere with vital cellular processes. However, unique features may be relevant, for instance, to electron transfer reactions and oxidative stress induction, again emphasizing the potentially poor predictive power of classical toxicology for biological effects of novel nanomaterials.

In our view, nanotoxicology represents a departure from the traditional toxicology of fine and ultrafine particles and fibers. Instead, nanotoxicology should be defined as a discipline studying the interference of engineered nanomaterials with the functions of cellular and extracellular nanomachineries. This definition places emphasis on the specific responses that are directly related to the scaling and dimensions of nanomaterials. In addition to size, other physical and chemical properties of nanomaterials may induce toxicological outcomes in unanticipated ways. Norman Augustine quipped that "One should expect that the expected can be prevented, but the unexpected should have been expected" (11a). This rule of management is also applicable in some ways to nanotoxicology: We should expect nano-sized objects to behave in unanticipated ways that are not readily predictable from historical studies of larger particles or materials of the same chemical composition in their bulk form. Moreover, we should be prepared for the onslaught of new and even more complex nano-sized structures in the years to come. The nanotechnologies are a moving target, and nanotoxicologists should hasten to board the train if adverse health effects are to be understood and prevented.

How should we experimentally and conceptually approach and develop this new field? Aggressive interactions and foreign intrusion are constant features of our relationship with the surrounding world. This is clearly demonstrable through the interactions of a multitude of microorganisms, including viruses, with host cells. Significantly, these interactions—which may be hostile and detrimental and may sometimes result in parasitic coexistence and a temporary truce between the belligerents—are realized through the engagement of two types of biological nanosystems: that of the parasite and that of the host. In each of the numerous examples of bacterial, viral, or parasitic invasion that we may encounter, there are general principles of engagement as well as unique and specific features defining the course and outcomes for both the invader and the host. Considerable progress in microbiology and immunology during the past century has provided a clear understanding that the specific mechanisms of each parasite-host interaction have to be investigated in great detail. However, despite the remarkable diversity and complexity of the microorganism kingdom, some common principles have been derived. For instance, general principles of recognition of nonself (microbes) have been elucidated; we now know that a relatively small number of immune receptors operate to detect signature molecules that herald infection, and one or more of these signature molecules are displayed by almost all microbes (12). Thus, there is also optimism that general principles of interactions between other types of man-made nanoparticles and biological systems can be deduced as well.

Therefore, to understand the hazardous effects of engineered nanomaterials, we need to study the specific interactions of each novel nanomaterial with the relevant biological system. However, at the same time, we should aim to derive common principles for the understanding of nanomaterial interactions with cells and tissues and for elucidation of the biological and toxicological outcomes of these interactions. In this review, we consider two important paradigms: (*a*) recognition versus nonrecognition of nanomaterials by the immune system, which not only determines the distribution of nanomaterials in the body but may also dictate their toxic potential, and (*b*) oxidative stress, an underlying mechanism that drives the toxicities of engineered nanomaterials in vitro as well as in vivo.

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TOWARD A BIOLOGY OF NANOSYSTEMS

This review is devoted to understanding the hazardous effects of engineered nanomaterials. However, it may be instructive to compare lessons learned from the study of three different classes of nano-scale systems: (*a*) endogenous (cellular and extracellular) nanostructures or nanomachines, (*b*) parasites (viruses, bacteria, etc.), and (*c*) man-made or engineered nanomaterials. We propose that the study of these three classes of nano-objects constitutes the basis for a science that we refer to here as nanosystems biology. In this paradigm, nanotoxicology is defined as the study of the interference of man-made nanomaterials with endogenous (cellular) nanostructures.

Every student of cell biology is aware of many examples of extremely effective endogenous nano-based mechanisms: DNA repair is accomplished by cellular nano-machines that perform intracellular gene therapy in the most original sense of the word, the cytoplasmic proteasomes perform vital cleaning functions to rid the cell of damaged proteins, SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) complexes are involved in the export and import of various proteins, and molecular walking machines such as the kinesin motor complex organize the transportation of important cargo in cells by hauling packets of components directionally along microtubule highways. Richard Jones (13) recently considered the lessons that Mother Nature can teach us: Is it possible to develop biomimetic nanotechnologies in which the design philosophy of cell biology is applied to the creation of entirely synthetic components? Moreover, could a synthetic nanotechnology match or exceed the performance of cell biology?

Bruce Alberts has pointed out that the living cell can be viewed as a miniature factory that contains a large collection of dedicated protein machines of nano-scale dimensions, optimized by billions of years of evolution (14). Indeed, cells use biological nanomotors to build essential molecules (polymerases make RNA and DNA from nucleic acids, and ribosomes construct proteins from amino acids) as well as motor proteins/nanomachines to transport cargo along filamentous structures (15). The realization that the cell is a collection of biological nanomachines raises the question of whether scientists can employ these nanomachines in artificial environments to perform specified tasks, or whether an intimate understanding of the principles that underlie the operation of these nanomachines can inspire biomimetic, man-made nanomachines or nanosystems [for an excellent overview, see (16)]. The latter authors concluded their visionary discussion with the statement that the exploration of biotechnological motors or machines will remain an "interdisciplinary playground" for many years to come. However, despite the optimism of such nano-enthusiasts, the proverbial fly in the ointment is that we also must consider potential interferences of man-made nanodevices with biological nanomachines.

Generally, the sizes of nanoparticles are comparable to those of viruses, DNA, and proteins, whereas microparticles are comparable to organelles and cells (**Figure 1**). For instance, a C_{60} fullerene nanoparticle is 1 nm in diameter, whereas the diameter of a DNA helix is 2 nm (17). Viruses are, in fact, nanoorganisms ranging from 10 to 400 nm in diameter. The smallest viruses are thus on the order of tens of nanometers in size, whereas a human immunodeficiency virus particle is 100 nm in diameter. Bacteria are typically bigger (micron-sized), although so-called nanobacteria also exist.

Interestingly, whereas viruses have been traditionally considered as hostile enemies and agents of disease, from the common cold to acquired immunodeficiency syndrome and cancer, a new view is emerging that viruses may, in fact, be explored as platforms for synthetic manipulation with a range of applications including biomedical applications (18). In particular, considerable interest has focused on adenoviruses (60–90 nm in diameter), as these entities are already being extensively evaluated for clinical applications in the context of gene therapy trials (19). Using viruses for drug or gene delivery has obvious disadvantages, as interactions between protein capsids and the immune

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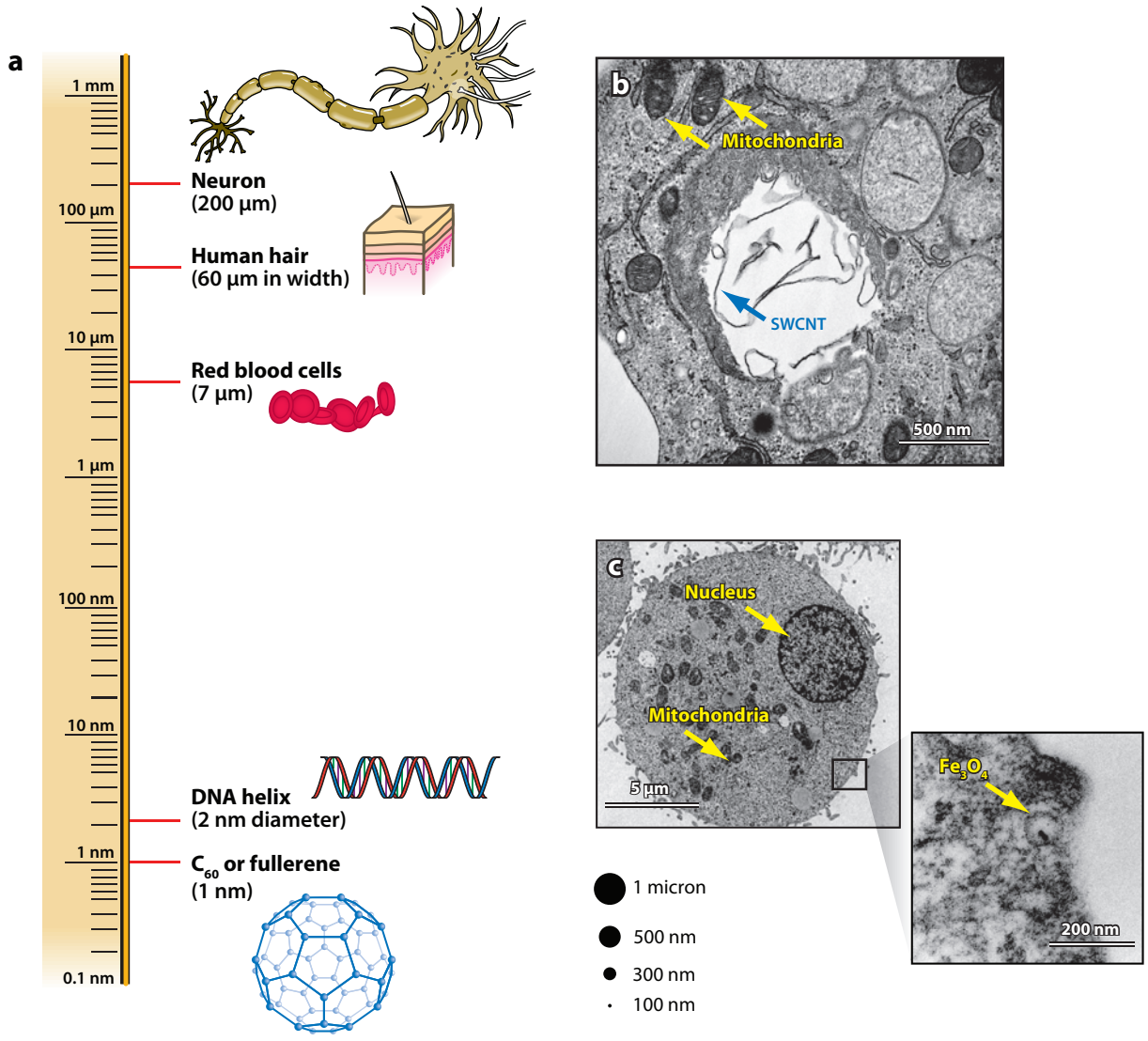

Figure 1

Toward a nanosystems biology. This review considers three classes of nano-scale systems: endogenous (cellular and extracellular) nanostructures or nanomachines, parasites (viruses, bacteria, etc.), and man-made or engineered nanomaterials. The schematic figure (*a*) depicts a logarithmic-length scale showing the size of a classical nanomaterial (C_{60} fullerene) compared with various biological components (adapted from 17). Particles of various sizes are drawn to scale. (*b*) Rat macrophage cells with internalized rope-like bundles of single-walled carbon nanotubes (SWCNT). For comparison, mitochondria are marked with arrows. Human macrophages are up to two times larger than their rat counterparts. (*c*) Human lung carcinoma cells with evidence of internalization of iron oxide (Fe_3O_4) nanoparticles of ~20 nm in diameter. Transmission electron microscopy images were recorded at the University of Pittsburgh/National Institute for Occupational Safety and Health (NIOSH) and Swiss Federal Laboratories for Materials Testing and Research (EMPA), respectively.

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system will occur that may result in strong cellular and humoral (antibody-mediated) immune responses and, potentially, a toxic inflammatory response. In addition, adenoviruses display a marked hepatic tropism upon systemic administration and appear to be taken up by resident macrophages in the liver, which precludes the targeted delivery of these vectors to other organs. However, synthetic modifications of adenoviruses could potentially overcome such problems, and so-called "designer adenoviruses" are thus being envisioned as nanoplatforms for drug and gene delivery (19). As pointed out in a recent review by Oberdörster et al. (20), it stands to reason that the knowledge accumulated from studies of different viruses and their interactions with the immune system may also serve as a valuable foundation for current research on the interactions between man-made (engineered) nanomaterials and biological systems. Moreover, the emerging synthetic virology research focusing on the exploitation of Mother Nature's own design for biomedical applications is a prime example of the new discipline of nanosystems biology.

Furthermore, so-called vaults, naturally occurring nanocapsules abundantly expressed in nearly all eukaryotic cells, have been considered as potential, biocompatible nano-carriers to target cancer cells (21). Vault particles are large ribonucleoprotein particles comprised of 96 copies of the major vault protein and have dimensions of ~70–40 nm, with a hollow interior large enough to encapsulate hundreds of proteins (22). Vaults, ovoid structures so named because they appear to represent the vaulted ceilings of medieval cathedrals, have been implicated in various cellular processes including nucleo-cytoplasmic transport, multidrug resistance, and innate immune responses, but their precise function remains unclear. Nevertheless, the utilization of recombinant, functionalized vault nanoparticles to target disease processes (21) represents a bona fide example of the science that we refer to here as nanosystems biology. However, major vault protein has recently been demonstrated to play a major role in innate immune responses in vivo (23), indicating that the administration of synthetically modified vault particles for therapeutic purposes could potentially interfere with the natural functions of these particles or their constituents. Nanosystems biology dictates that close attention be paid to the potential interference between man-made (or modified) materials and corresponding endogenous nanostructures.

In addition to the possibility that man-made nanomaterials or devices may interact with numerous biological nanostructures within the cell, there are also several examples of extracellular nano-sized structures, some of which have been implicated in intercellular communication (24). No studies to date suggest that engineered nanomaterials could interfere with or mimic these endogenous structures, but there is a possibility that such events may transpire, owing to the similarities in size:

1. Exosomes: 30–100-nm lipid bilayer vesicles that are released extracellularly after fusion of multivesicular endosomes with the cell membrane. Exosomes can be viewed as natural Trojan horses for intercellular communication and exchange of (genetic) material between cells (24). Indeed, recent studies have revealed that exosomes contain mRNA as well as microRNA, which can be delivered to other cells in a novel process of genetic (horizontal) exchange between cells (25).
2. Neutrophil extracellular traps: Complex extracellular structures that are extruded from dying neutrophils. These are composed of smooth threads, approximately 15 nm in diameter, which are likely to represent a chain of nucleosomes from unfolded chromatin. Several threads can be wound into cable-like structures that can be up to 100 nm in diameter (26) and are thought to be involved in the capture and extracellular killing of bacteria (27).
3. Tunneling nanotubes: Continuous membrane between connecting cells, with a diameter of 50—to 200 nm that can stretch over several cell diameters in length. They may represent a conserved means of cell-to-cell communication (28); Gousett et al. (29) have recently reported that tunneling nanotubes allow transfer of exogenous and endogenous prions

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(abnormal isoforms of host proteins that are the infectious agents in certain mammalian neurodegenerative diseases) between infected and naive neuronal cells.

NANOMATERIAL INTERFERENCE WITH CELLULAR MACHINERIES

Material scientists and engineers are actively pursuing the development of self-powered nano-scale transport and delivery systems. Such devices may incorporate more than one class of engineered nanomaterial (30), which in turn, poses specific challenges in terms of evaluating potential hazardous effects of the devices. At present, most efforts are focused on the utilization of artificial nanomachines or nanomotors outside the body, for instance, for enhancement of the analytical capabilities of laboratory-on-a-chip devices (2). However, material scientists are also entertaining the notion of in vivo applications of sophisticated nano-scale devices including self-regulating nanomachines for drug delivery as well as "nanorobots cleaning out clogged arteries" (2). Although the latter applications, which are reminiscent of the miniaturized submarine (1 μm) injected into the bloodstream of a comatose scientist in the 1960s movie *Fantastic Voyage* (the screenplay was later adapted into a novel by Isaac Asimov), may not be realized in the near future, these speculations nevertheless raise the issue of safety assessment of miniaturized objects envisioned for in vivo applications. For comparison, LeDuc et al. (31) have recently asked the question whether, instead of passively employing nanotechnology to deliver a drug, one could deliver the entire biochemical machinery to sense the need and then produce the drug on site. Indeed, these investigators proposed that it might be possible to design pseudocell nanofactories that work with molecules already in the body to fight disease.

The development of nanotechnologies is closely linked to the development of tools for imaging at the atomic level (32). Thus, the invention of the scanning tunneling microscope (STM) almost 30 years ago, followed by the arrival of the atomic force microscope (AFM) 5 years later, were crucial events in the history of nanotechnology. Indeed, as pointed out by Vives & Tour (33), "Although engineering challenges such as movement over long distance and nonatomically flat surfaces remain, the greatest current research challenge is imaging. The detailed study of nanocars [i.e., molecular machines that resemble macroscopic vehicles] requires complementary single molecule imaging techniques such as STM, AFM, TEM, or single-molecule fluorescence microscopy." This statement reminds us of another important lesson: The assessment of hazardous effects of engineered nanomaterials and nanodevices also requires an intimate understanding of the procedures for material synthesis and methods for material characterization, in addition to a detailed appreciation of the impact of nanomaterials on biological and physiological processes. Hence, nanotoxicology is, by its very definition, an interdisciplinary enterprise. Material properties that may be relevant to the toxicological outcomes of nanomaterial exposure are discussed below.

Numerous studies have shown that the addition of different types of nanoparticles to various primary cell cultures or transformed cell lines may result in cell death or other toxicological outcomes, depending on the dose of the nanomaterial (34, 35). However, one may ask whether these studies inform us on the relationship between the toxic responses and the specific smallness of the nanomaterials. Indeed, do the artificial nanomaterials interfere with the nanomachineries of the living cell? Thus, to paraphrase Steven Spielberg's classic science fiction movie, *Close Encounters of the Third Kind*, are there any examples of close encounters of the small kind, in cells exposed to engineered nanomaterials? The following nonexhaustive enumeration of recent publications suggests that the answer is yes:

1. Tsoli et al. (36) reported that the gold nanocluster compound, Au_{55}, with a distinct particle size of 1.4 nm, intercalates with the major groove of DNA and is a potent inducer of cell

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death in cancer cells (more potent than the commonly used chemotherapeutic agent cisplatin). Importantly, gold nanoparticles that are only marginally smaller or larger showed significantly reduced toxicity, whereas considerably larger gold nanoparticles were completely nontoxic in the various cell lines tested (37). The latter findings suggest that such gold clusters with perfectly completed geometries could be envisioned for cancer treatment, if toxicity to normal cell types can be controlled. In other words, in the context of cancer treatment, nanomedicine and nanotoxicology can be viewed as two sides of the same coin (38).

2. Volkov and colleagues have reported in a series of publications that quantum dots display a size-dependent localization to different cellular compartments such that smaller quantum dots (2.1 nm in diameter) localized to the nuclear compartment, whereas larger quantum dots (3.4 nm) were retained in the cytoplasm. Interestingly, the smaller quantum dots specifically targeted histones in the cell nucleus and nucleoli by a multistep process involving endocytosis, active cytoplasmic transport, and entry into the nucleus via nuclear pore complexes (39, 40). The authors suggested that the strong tropism of quantum dots for nuclei and nucleoli of living cells could be mediated by charge-related properties of the macromolecules (histones) present in the nuclear compartment.
3. Porter et al. (41) reported that single-walled carbon nanotubes (SWCNTs) interact with structural elements of the cell, with apparent binding to the cytoskeleton. More recently, Sargent et al. (42) have provided evidence that SWCNTs may mimic or interfere with the cellular microtubule system and thereby disrupt the mitotic spindle apparatus, leading to aberrant cell division. In a recent review, Pampaloni & Florin (43) presented evidence of numerous analogies between microtubuli and carbon nanotubes, supporting the idea of a unified nanosystems biology approach, as suggested in this review. In brief, both structures are similar in their mechanical behavior, having a tubular structure with exceptional resilience (that is, they can be bent to a small radius of curvature and are able to restore their original shape without permanent damage). Moreover, they have the ability to form large bundles with improved stiffness and resilience. A significant difference, however, is that microtubuli are capable of self-assembly, whereas carbon nanotubes are fabricated by spinning, layering, and other shaping procedures. However, the realization of self-assembly of carbon nanotubes in fluid environments could pave the way for novel, biomimetic nanomaterials. Obviously, close attention to safety issues will be required, in light of the potential interference between engineered nanotubes and their cellular counterparts.
4. Several studies have provided support for the notion that interactions of engineered nanomaterials with living systems may be understood in the context of biological nanoorganisms (viruses) and microorganisms (bacteria) and their interactions with the host. For instance, in a study of a series of cationic poly(ethylene glycol)-based nanoparticles with varying sizes and shapes at a constant chemical composition, Gratton et al. (44) found that rod-shaped nanoparticles enjoyed an appreciable advantage when it comes to cell internalization rates, and suggested that this is reminiscent of the behavior of many rod-shaped bacteria. Furthermore, Oberdörster et al. (45) have reported on the translocation of inhaled ultrafine particles (<100 nm) to the olfactory bulb of the central nervous system in a rat model. This novel route of entry, which may allow inhaled nano-sized particles to circumvent the so-called blood-brain barrier, is not predicted from previous studies of larger particles, but is reminiscent, instead, of the classical observations of retrograde neuronal transport of poliovirus (30 nm) from the nose to the brain via the olfactory nerve (20).

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NANOMATERIAL PROPERTIES DRIVING ADVERSE EFFECTS

Nanotoxicological studies conducted to date have suggested correlations between different physico-chemical properties and the biological and toxicological outcomes of exposure of cells and tissues to nanomaterials. However, there is as yet no consensus as to which is the most important dose metric in deciding the toxicity of engineered nanomaterials: particle mass, particle number, surface area, surface chemistry, or all of these parameters together (17). In this section, we discuss some examples of studies that shed light on specific properties of nanomaterials, including size, surface area, structure, and so on. It should be noted, however, that these studies merely represent a smorgasbord of current nanotoxicological research; there are significant differences in the choice of nanomaterial under investigation in each of the studies, and in the design of the studies in terms of whether cell lines or primary cell cultures are employed, and whether or not serum is present in the cell culture medium, as well as the choice of assays to measure cellular responses (some commonly used assays are fraught with technical limitations owing to the frequent interference of engineered nanoparticles with absorbance- or fluorescence-based read-out systems) (34, 46). Harmonization of protocols for material characterization and for cytototoxicity testing of nanomaterials is thus needed. Internationally standardized reference materials are also desirable. In addition, parallel profiling of several classes of nanomaterials, combined with detailed characterization of their physico-chemical properties, could provide a model for safety assessment of novel nanomaterials (47).

Dose-Dependent Toxicity

Generally, negative health effects of nanoparticles do not appear to correlate with particle mass dose (8). Indeed, paradoxically, a high concentration of nanoparticles may promote particle aggregation (48, 49) and could therefore reduce toxic responses compared to lower concentrations of the same particles (17).

Size-Dependent Toxicity

Examples of size-dependent toxicity were discussed in a previous section. Gold nanoclusters (1.4 nm) were shown to be toxic to cells owing to their specific interaction with major grooves of DNA, whereas smaller or larger gold particles did not behave in this way (37). Furthermore, quantum dots were reported to localize to different cellular compartments in relation to their size (39). Others have suggested that silica nanoparticles of 40–80 nm in diameter can enter the cell nucleus and localize to distinct subnuclear domains in the nucleoplasm, but do not colocalize with nucleoli. Moreover, these nanoparticles induced the formation of nucleoplasmic protein aggregates. In contrast, fine and course (0.5–2 μm) silica particles located exclusively in the cytoplasm (50).

Surface-Area-Dependent Toxicity

The relative fraction of surface atoms to bulk atoms is dramatically different in nano-sized versus microsized particles. Hence, while less than 1% of atoms of a microparticle occupy surface positions, more than 10% of the atoms in a 10-nm particle reside on its surface; this thus contributes to changes in surface physical and chemical properties as materials are reduced in size (46). For instance, following inhalation exposure of rats to 20-nm or 250-nm titanium dioxide particles, the half-times for alveolar clearance of polystyrene test particles were proportional to

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the titanium dioxide particle surface area per million macrophages (51). In a more recent study, Monteiller et al. (52) suggested that surface area is a more appropriate dose metric than mass for the proinflammatory effects of low-solubility, low-toxicity particles.

Reactive oxygen species: oxygen radicals such as superoxide and hydroxyl radicals

Phagocytosis: engulfment and ingestion of microorganisms or other foreign bodies or particles

Crystalline-Structure-Dependent Toxicity

Titania exists in a variety of crystal structures, the most actively researched of which are its rutile, anatase, and brookite forms (53). Of note, the cytotoxic properties of titanium dioxide nanoparticles appear to correlate with their phase composition. Hence, in a comprehensive study of titanium dioxide nanoparticles (3–10 nm), Sayes et al. (54) demonstrated that anatase titanium dioxide was 100 times more toxic than an equivalent sample of rutile titanium dioxide. The generation of reactive oxygen species under UV illumination correlated well with the observed biological responses. The same authors (55) also reported that the pulmonary toxicities of fine and ultrafine (nano-sized) quartz particles appeared to correlate better with surface activity than with particle size and surface area. Additionally, the crystal structure of titanium dioxide also dictates the mode of cell death: Anatase titanium dioxide nanoparticles, regardless of size, were reported to induce necrosis, whereas rutile titanium dioxide nanoparticles triggered apoptosis through the formation of reactive oxygen species (56).

Surface-Coating-Dependent Toxicity

Surfaces of nanomaterials make contact with cells, and a detailed understanding of surface composition is therefore paramount to understanding the interactions of nanomaterials with biological systems (46). Surface contamination may result from adventitious air- or water-borne contaminants or arise from synthetic modifications of the nanomaterial. Adsorption of the ubiquitous bacterial endotoxin, lipopolysaccharide, is one of the most common problems for all biomaterials, and could also contribute to the cellular responses evoked by nanoparticles, in particular immunological responses (57). It is also important to distinguish between adverse cellular responses to nanoparticles themselves and residual materials associated with the nanoparticles—such as surfactants (58) or transition metals (59)—as a consequence of the synthetic process.

In addition, as pointed out in a recent review (56), the state of agglomeration of nanoparticles must also be taken into account when considering the toxicity of these materials. Specifically, in an agglomerated state, some nanoparticles may behave like larger particles, and toxicity may depend on the size of the agglomerate and not on the original nanoparticle size itself (60). An important point, therefore, is that material characterization before and after exposure to cells or tissues may be necessary to understand the toxicological behavior of these materials.

CELLULAR RECOGNITION OF NANOMATERIALS

One century ago, in 1908, Ilya Ilich Mechnikov (1845–1916) received the Nobel Prize in Physiology or Medicine together with Paul Erlich (1854–1915) in recognition of their work on immunity. Their discoveries formed the foundation for our modern understanding of the fundamental principles of innate and adaptive immunity and of cellular versus humoral immune responses (61). Mechnikov discovered the physiological process of phagocytosis by which blood cells take up and digest bacteria. In fact, most cells have some phagocytic function; however, higher organisms also have professional phagocytes (monocytes and macrophages and polymorphonuclear granulocytes or neutrophils) equipped with dedicated receptors that are able to recognize foreign invaders (62). In addition to the recognition and clearance of microbes and particles and

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foreign debris, professional phagocytes (macrophages) also play an important role in the recognition and clearance of apoptotic cell corpses (63).

The degree of recognition and internalization of nanomaterials by professional phagocytes (macrophages) likely influences their biodistribution. Hence, Sadauskas et al. (64) reported that gold nanoparticles (40 nm) injected into mice were taken up primarily by macrophages resident in the liver and secondarily by macrophages in other organs. In contrast, inhaled titanium dioxide nanoparticles (20 nm) were shown to escape from clearance by alveolar macrophages in peripheral lung of exposed mice, and this phenomenon could potentially explain the translocation of such particles into circulation (65). Furthermore, Manolova et al. (66) reported that fluorescent polystyrene particles traffic to draining lymph nodes in mice in a size-dependent manner. Large particles (500 nm–2 μm) were thus associated with antigen-presenting dendritic cells at the injection site, whereas small particles (20–200 nm) were also found in lymph node–resident dendritic cells and macrophages. Understanding the targeting of nanoparticles and their cargo to antigen-presenting cells is an important challenge, especially for immuno-modulatory applications (67).

Inflammation: a localized protective reaction of tissue to microbial infection or chemical or other injury with recruitment and accumulation of immune-competent cells, sometimes with formation of granulomas (encapsulations of offending organisms or particles)

In addition, we posit that the degree of internalization of nanoparticles may determine not only their distribution in the body but also their toxic potential. Indeed, Chang et al. (68) reported that the number of internalized quantum dots, that is, the intracellular dose of the nanomaterial, correlates with in vitro toxicity in a human breast cancer cell line. It is important to emphasize that nanoparticles may aggregate, and this may be an essential feature determining their toxicity, potentially related to their clearance by macrophages, as this process is more effective for larger particles than for smaller ones.

How, then, are nanoparticles taken up by cells? Geiser et al. (69) reported that uptake of fluorescent polystyrene beads (78 nm) in vitro by porcine lung macrophages occurs through a passive (nonphagocytic) mechanism. In contrast, uptake of carboxylated polystyrene spheres (50 nm) in vivo was suggested to occur through particle opsonization with specific serum proteins, resulting in recognition by scavenger receptors on hepatic macrophages (70). Other investigators have reported that opsonization of nanoparticles with serum proteins may inhibit cellular uptake (71). These differences may be related to the specific physico-chemical properties of the nanoparticles studied and to the cell-type-specific cellular internalization pathways involved. Indeed, it may be relevant to consider the differences in phagocytic potential and repertoire of cell surface receptors between professional phagocytic cells (macrophages) and nonprofessional cell types including various cancer cells lines that abound in toxicological research laboratories. In addition, it may be relevant to study whether exposure to engineered nanomaterials impairs subsequent macrophage engulfment of other phagocytic prey, including microorganisms (72) and apoptotic cells (73). Tissue homeostasis could be compromised if nanomaterials were to interfere with the normal clearance (waste-disposal) processes of macrophages.

Recent studies have reported that certain engineered nanomaterials including fullerenes and quantum dots may induce autophagic responses (74–76). Autophagy (self-eating) is a conserved response to cellular starvation and serves as a major route for lysosomal protein degradation. Autophagic responses to toxic stimuli, including particles, may be viewed as a protective cell stress response, but could also account for the observed loss of cell viability in situations with induction of so-called autophagic cell death. Moreover, the autophagy pathway and the phagocytosis pathway, two ancient and highly conserved cellular processes, were shown recently to be linked through toll-like receptor signaling at the cell surface of macrophages (77), suggesting an important role for autophagic responses in microbial killing. Indeed, emerging evidence points to a fundamental role of autophagy genes in diverse aspects of innate and adaptive immunity, including elimination of intracellular microbes, antigen presentation, and regulation of cytokine signaling and inflammation (78). Delgado et al. (79) have recently proposed that autophagy may have emerged as the

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initial and primordial defense of eukaryotic cells against microbes. It is therefore perhaps not surprising that immune-competent cells may respond in a similar manner to viruses and bacteria on the one hand and to engineered nanomaterials of the same size and shape as natural nanoorganisms on the other. Elucidation of how engineered nanomaterials impact the conserved mechanism of autophagy and/or phagocytosis promises to be an interesting and fruitful area of research in the years to come, and could have important implications for nanotoxicological research.

Targeted delivery of nanomaterials is an important goal in various biomedical applications. Numerous studies have demonstrated that undesired removal of particles in organs that contain large numbers of macrophages, such as the liver, spleen, and lung, can be delayed by coating the particles with shielding molecules such as poly(ethylene glycol), but such strategies appear to negatively impact the targeting specificity (53). Other approaches to actively direct nanoparticles to the desired site of action are also being adopted, including surface functionalization of nanoparticles with various ligands (recognition motifs) that bind to specific receptors on tumor cells or other target cell populations (80, 81). Pristine (nonfunctionalized) carbon nanotubes are not readily taken up by macrophages (59). However, we recently demonstrated that functionalization of SWCNTs with an anionic phospholipid, phosphatidylserine (PS), a known recognition signal for macrophages, targeted the nanotubes to several classes of professional phagocytes, including monocyte-derived macrophages and dendritic cells as well as microglia, the resident macrophages of the brain (82).

In 1904 Wright & Douglas (83) suggested the concept of particle opsonization by serum factors (proteins). Moreover, Leo Vroman reported in a seminal publication almost 50 years ago that proteins adsorb to surfaces through a series of attachment and displacement steps (this phenomenon is now referred to as the Vroman effect) (84). Recent studies have brought these fundamental considerations to the forefront of nanotoxicological and nanotechnological research. In particular, there is an emerging understanding that the protein corona, that is, the specific proteins coating the nanoparticle surface, is one of the key factors determining the outcomes of nanomaterial interactions with biological systems (85, 86).

Several recent studies have thus shown—perhaps not surprisingly—that the uptake as well as the toxicity of engineered nanomaterials correlate with protein opsonization (87, 88). Using a set of tailored copolymer nanoparticles that allowed the systematic investigation of the influence of size and composition (hydrophobicity) of the particles on their interaction with proteins, Cedervall et al. (88) observed a clear dependency of the binding and dissociation parameters on protein identity and on particle surface characteristics. Subsequent studies revealed a specific pattern of binding to the nanoparticle surface insofar as albumin, the most abundant serum protein, was found to be successively replaced by the higher-affinity and slower-exchanging apolipoproteins AI, AII, AIV, and E (89)—the Vroman effect. In a more recent study, Ehrenberg et al. (90) showed that nanoparticle surface chemistry, as it pertains to the protein-adsorbing capacity of particles, determines the cellular binding of nanoparticles, and furthermore, that cellular association is not dependent on the specific identity of adsorbed proteins, at least in studies of polystyrene nanoparticles and cultured human umbilical vein endothelial cells. Finally, Dobrovolskaia et al. (91) identified 69 different plasma proteins bound to colloidal gold nanoparticles (30 and 50 nm) and studied the effect of protein binding on the hydrodynamic size of the particles. Overall, one may argue that the boundaries between man-made nanomaterials and biological nanoorganisms (viruses) or even cellular debris (apoptotic bodies) begin to blur in light of these observations, as the interaction with host cells is likely to depend, in all of these cases, on the nature of the protein and/or lipid or carbohydrate signals that are presented to cells.

Degradability of the material is also an important factor in the assessment of the toxicity of nanomaterials. Nondegradable nanomaterials can accumulate in organs and intracellularly, where

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they can exert detrimental effects on the cell. For instance, long-term accumulation of medicinal gold salts (nanoparticles) in the body may result in adverse or toxic effects in patients (92). Single-walled carbon nanotubes are known to be biopersistent and may remain inside macrophages in spleen and liver for prolonged periods of time following parenteral administration (93); carbon nanotubes have been observed in the lungs of exposed mice up to 1 year after pharyngeal administration (A. A. Shvedova et al., unpublished observation). On the other hand, biodegradable nanomaterials could also yield unpredictable toxic responses due to toxic degradation products (94). For instance, leaching of toxic core components such as cadmium from quantum dots with induction of oxidative stress has been suggested as a mechanism of in vivo toxicity of these nanomaterials (94).

Nanomedicine: application of nanotechnology for diagnostic purposes or for the treatment or prevention of disease

Controlled biodegradation of nanomaterials thus represents one of the important challenges not only in the field of nanotoxicology but also in nanomedicine, as the safe implementation of nanomaterials for biomedical purposes is contingent on the controlled degradation and/or clearance of the exogenous nanomaterials. In a recent proof-of-concept study, Park et al. (95) reported that multifunctional porous silicon nanoparticles self-destructed in a mouse model into renally cleared components—likely orthosilicic acid—in a matter of weeks, with no evidence of toxicity in animal tissues. Moreover, we and others have recently demonstrated the enzymatic degradation of SWCNTs by incubating the nanotubes in a cell-free system with horseradish peroxidase and low amounts of H_2O_2 (96). These results mark a promising possibility for carbon nanotubes to be degraded by horseradish peroxidase in environmentally relevant settings. More recently, we have achieved biodegradation of carbon nanotubes in a physiologically relevant environment based on the action of myeloperoxidase, a potent oxidant-generating enzyme in phagocytic cells (neutrophils) (V. E. Kagan et al., submitted for publication).

NANOMATERIALS AND OXIDATIVE STRESS

Cell death has been universally associated with the initiation and propagation of free radical oxidation reactions and excessive accumulation of their products (97). However, the question as to whether these effects cause cellular destruction or rather stem from the injury triggered by other mechanisms remains, in most cases, unanswered. Excessive oxidative stress has been proposed as a common paradigm for the toxicities of engineered nanoparticles (98–100). Although it is widely accepted, not all studies comply with this general notion (101), thus pointing to the need for careful scrutiny of this pervasive concept.

Two major factors should be addressed in any claim of the potential involvement of oxidative reactions in pathogenic mechanisms: (*a*) catalysts and (*b*) sources of oxidizing equivalents. Interestingly, not only redox biochemists but also artists have appreciated the importance of transition metals as potent catalysts of oxidation reactions. In 1978, Andy Warhol and friends executed a series of elegant abstractions called *Oxidation Paintings* in which they utilized iridescent, copper-containing metallic pigments and took advantage of their ability to interact with organic components of urine, creating canvases with an attractively shimmering patina. While this particular method for achieving the rich painterly abstractions was not unanimously accepted by the public, the importance of transition metals such as copper and iron in the catalysis of redox oxidations remains undisputable. A case in point, manufacturing of carbon nanotubes frequently involves the use of significant amounts of metals—Fe, Ni, Cr (59, 102). One may assume that these metals could act as potent oxidation catalysts. However, the metals in SWCNT samples are present almost exclusively in elemental rather than ionic form and so are not readily available for catalytic purposes.

Of course, uptake and concentration of these metals in the acidic environment of lysophagosomes of phagocytosing cells may stimulate their conversion into ions and resultant

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pro-oxidant effects of nanotube-associated metals. Closely associated with this is the propensity of metals to participate in one-electron reduction of oxygen and production of so-called reactive oxygen species such as superoxide radicals, hydrogen peroxide, and hydroxyl radicals (103, 104). The latter can act as an immediate and direct oxidizing entity causing oxidation of biomolecules in cells and tissues. Whereas carbon-nanotube-induced production of oxygen radicals relevant to the presence of adventitious metals has been well documented in simple model systems, the carbon-nanotube-dependent formation of oxygen radicals in realistic physiological conditions is more equivocal because of the potent capacities of cells and biofluids to bind transition metals, yielding redox-inactive complexes (105). Nonetheless, accumulation of oxidation products in proteins, DNA, and lipids in cells and tissues of animals exposed to carbon nanotubes has been reported by several independent laboratories (106, 107). Notably, dietary manipulations of the anti- and pro-oxidant status of animals achieved by maintaining them on vitamin E–deficient diets has been reported to exacerbate the inflammatory pulmonary response to aspirated SWCNTs (108). The major point, however, is the origin of these oxidation products: When and how have they been accumulated, and what is their role in pathogenic events in exposed tissues?

Aside from methodological difficulties associated with assessments of oxidative stress biomarkers in the presence of carbonaceous nanomaterials due to their interferences in light absorbance and fluorescence protocols (109), the association of such biomarkers with the direct induction of oxidative stress by carbon nanotubes versus the indirect impact on oxidative pathways triggered by carbon nanotubes should be carefully discriminated. For comparison, one may consider the initiation and execution of the apoptotic program, in which specific oxidation events are key to the execution process, as described below, while at the same time the disruption of electron transport during apoptosis massively generates superoxide radicals and hydrogen peroxide as a consequence of cell death (110, 111). Simultaneous with the generation of reactive oxygen species, the transmembrane migration of a mitochondria-specific anionic phospholipid, cardiolipin (CL) from the inner to the outer mitochondrial membrane facilitates binding of this phospholipid with an intermembrane hemoprotein, cytochrome c (cyt c), resulting in the assembly of cyt c/CL complexes with peroxidase activity (112). In this way, both of the critical components needed for the induction of mitochondrial oxidative stress are in place: cyt c/CL catalytic complexes and a source of oxidizing equivalents emanating from the disrupted electron transport chain. Consequently, selective accumulation of phospholipid oxidation products takes place (113). Involvement of CL oxidation products in mitochondrial permeability transition and release of cyt c into the cytosol is also linked to the catalytic role of cyt c in oxidation of yet another anionic phospholipid, phosphatidylserine (PS), in extramitochondrial locations.

Oxidation and externalization of PS in the plasma membrane of apoptotic cells has been associated with the recognition and uptake of apoptotic cells by professional phagocytes (114, 115). This PS-dependent pathway of cell clearance is thought to be essential for the resolution of acute inflammatory responses and to preclude chronic inflammation and fibrosis (63). Notably, we have observed that early onset of fibrosis is characteristic of pulmonary inflammatory reactions induced by exposures of mice to SWCNTs (116, 117). The arrival of macrophages with their NADPH oxidase-driven production of superoxide radicals and hydrogen peroxide creates conditions for further progression of oxidative and nitrosative stress (118). Our recent studies have shown that NADPH oxidase-deficient mice respond to SWCNT exposure with a marked accumulation of neutrophils and elevated levels of unengulfed apoptotic cells in the lungs, production of proinflammatory cytokines, decreased production of the profibrotic cytokine TGF-β, and significantly lower levels of collagen deposition, as compared with control mice (119). The latter studies thus demonstrate a role for NADPH oxidase-derived reactive oxygen species in determining the course of pulmonary responses to SWCNTs. It is worth noting that recent studies point to a role for a

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functional NADPH oxidase both for activation of the inflammasome, an intracellular molecular complex required for processing and secretion of the proinflammatory cytokine IL-1β (120), and for the regulation of antibacterial autophagy during phagocytosis (121). Future studies are warranted to address whether these NADPH oxidase-dependent pathways in professional phagocytes are also activated or perturbed by SWCNTs.

Whereas the source of oxidizing equivalents—the activated NADPH oxidase of recruited phagocytes—is readily identifiable in the context of inflammation, specific catalysts translating accumulated H_2O_2 into peroxidized biomolecules including lipids remain to be elucidated. However, metalloproteins with peroxidase function can be potentially important contributors (122). At this stage of the inflammatory response, it is also likely that lipid peroxidation displays a specific profile in which phospholipids in the outer layer of the plasma membrane will be preferred substrates for oxidative attack (123). Overall, we posit that oxidative stress induced by carbon nanotubes in physiologically relevant environments is unlikely to be a random chain reaction; it is more likely that the appearance of biomarkers of lipid peroxidation reflects specific oxidation mechanisms characteristic of different stages of the inflammatory response. In support of this notion, we have recently identified several specific oxidation products of PS in the lungs of mice following inhalation exposure to SWCNTs (**Figure 2**). The emerging field of oxidative lipidomics—based on mass spectrometry–based profiling of lipid profiles of exposed cells and tissues—thus opens new opportunities for studies of selective phospholipid oxidation after exposure to carbon nanotubes as well as other engineered nanomaterials. This may, in turn, lead to the elucidation of signaling roles of specific oxidized lipids in the induction or resolution of inflammatory responses.

Oxidative stress commonly accompanies cytotoxic effects originating from exposure of cells and animals to engineered nanomaterials, including carbon nanotubes. However, whether this is a correlation or a causative effect remains to be elucidated. Specific mechanisms underlying the propensity of carbon nanotubes to induce oxidative stress are not well understood. Oxidative stress responses to engineered nanomaterials are detectable as selective patterns of lipid peroxidation that can be utilized as time-dependent, albeit not uniquely selective, markers of oxidative stress and proinflammatory responses.

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NANOMATERIALS: LESSONS FROM IN VIVO STUDIES

Safety assessment of engineered nanomaterials is a key to the efficient development of a number of novel nano-applications essential not only for pharmaceuticals, biomimetics, and tissue engineering, but also to promote the well-being of humans during manufacturing and use. Although numerous data are available on the in vitro toxicity of nanomaterials, there is an emerging understanding that in vivo assessments of nanomaterial toxicity are essential. In the absence of predictive and validated in vitro assays, in vivo testing of potentially toxic materials appears to be indispensable for human safety (124).

Evaluation of human exposure to engineered nanomaterials requires knowledge of the likelihood of exposure, changes in particle concentration over time, and identification and characterization of exposure directly prior to uptake. The respiratory system is a unique target that encounters nanomaterials via inhalation. In addition, dermal and gastrointestinal exposure, eye absorption, and direct parenteral administration are also potential routes of entry and may be associated with adverse effects. Significant interest in the respiratory system as a target for both beneficial and adverse effects of engineered nanomaterials is reflected by a growing number of relevant scientific publications during the past decade (125).

Previous epidemiological studies have documented a strong association between so-called ultrafine air pollution particles, including particles in the nano-size range, and respiratory and

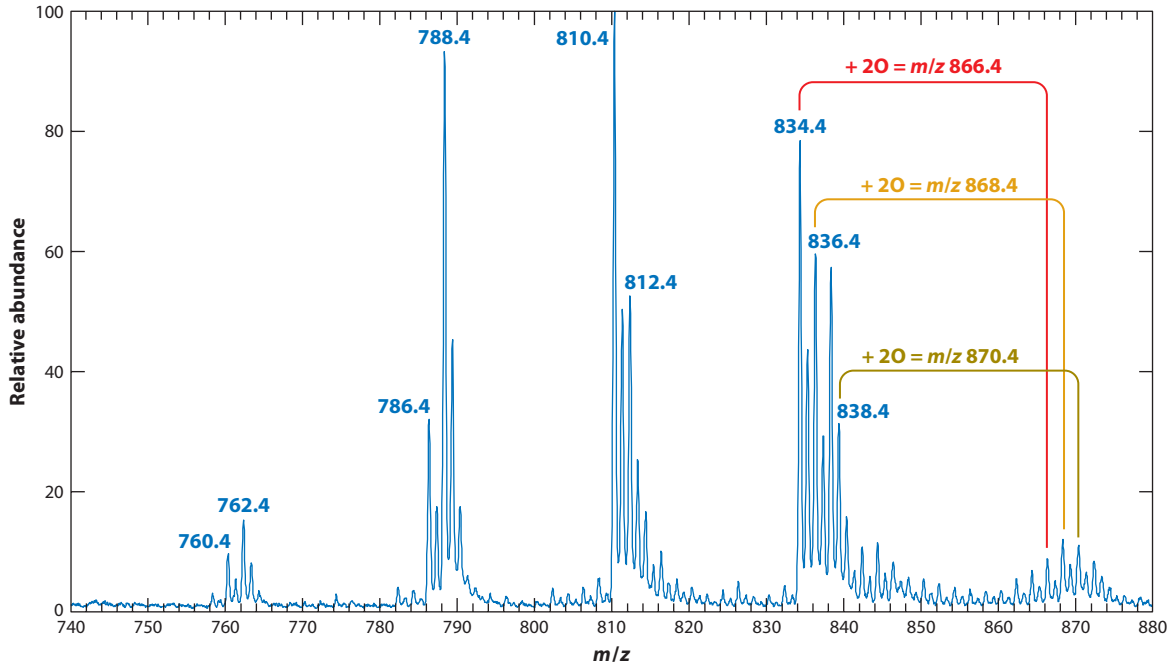

Figure 2

Oxidative lipidomics studies of nanotoxic responses. Typical negative electrospray ionization and mass spectrometry (MS) spectrum of molecular species of phosphatidylserine (PS) obtained from the lung of a mouse 7 days after inhalation exposure to SWCNTs. Inhalation exposure was performed as described by Shvedova et al. (117). For an outline of oxidative lipidomics protocols, the reader is referred to Tyurin et al. (138). Three major molecular clusters of PS with mass-to-charge ratios (m/z) 786.4:788.4, 810.4:812.4, and 836.4:838.4 were observed. A molecular ion with m/z 786.4 contained two molecular species, $C_{18:0}/C_{18:1}$ and $C_{18:0}/C_{18:2}$, whereas a molecular ion with m/z 788.4 corresponds to molecular species $C_{18:0}/C_{18:1}$. Ions with m/z 810.4 and 812.4 were identified as molecular species containing $C_{18:0}/C_{20:4}$ and $C_{18:0}/C_{20:3}$, respectively. Ions with m/z 834.4, 836.4, and 838.4 originated from molecular species of PS containing highly unsaturated docosahexaenoic ($C_{18:0}/C_{22:6}$), docosapentaenoic ($C_{18:0}/C_{22:5}$), and docosatetraenoic acids ($C_{18:0}/C_{22:4}$), respectively. Molecular ions with m/z 760.4 and 762.4 ($C_{16:0}/C_{18:1}$ and $C_{16:0}/C_{18:0}$) were also detectable in the MS spectrum, but their abundance was significantly lower than that of major molecular species of PS. Importantly, molecular ions of PS with m/z 866.4, 868.4, and 870.4 were observed in the MS spectrum. These molecular ions correspond to oxidized molecular species of PS $C_{18:0}/C_{22:6\text{-OOH}}$, $C_{18:0}/C_{22:5\text{-OOH}}$, and $C_{18:0}/C_{22:2\text{-OOH}}$, respectively, which partially overlap with ions of nonoxidized PS species $C_{18:0}/C_{24:4}$, $C_{18:0}/C_{24:3}$, and $C_{18:0}/C_{24:2}$. Overall, the experimental data reported here suggest that specific patterns of lipid peroxidation are detected following inhalation exposure to SWCNTs.

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cardiovascular morbidity and mortality (20). Moreover, several epidemiological observations support the notion that ultrafine and fine particles cause a higher rate of adverse respiratory outcomes than coarse particles (126, 127). Some, but not all, of these effects may be related to indirect actions of particles on components of the immune system, for instance, through modulation of inflammatory cytokine secretion. Indeed, Dobrovolskaia & McNeil (67) emphasized in a recent review that engineered nanomaterials can either stimulate or suppress immune responses, thus pointing to the fact that one of the fundamental questions in the field of nanotoxicology concerns the mechanisms through which the immune system senses nanoparticles. In the following sections, we focus on single-walled and multiwalled carbon nanotubes, two important types of engineered nanomaterials with numerous applications and hence with a potential for human exposure. Owing to a multitude of different nanomaterials with differences in physico-chemical properties, nanoparticles cannot be summarized in one homogenous group but instead should be studied on

a case-by-case basis. For a recent overview of in vivo studies of the toxicity of other representative engineered nanomaterials, including dendritic polymers, quantum dots, and gold nanoparticles, the reader is referred to Aillon et al. (94).

We have reported that exposure of C57BL/6 mice to SWCNTs through pharyngeal aspiration causes dose-dependent granulomatous pneumonia, oxidative stress, and acute inflammatory and cytokine responses, with fibrosis and decrease in pulmonary function (116). To avoid potential artifactual effects due to instillation and agglomeration associated with SWCNTs, we also conducted inhalation exposures using stable and uniform SWCNT dispersions obtained by a newly developed aerosolization technique (128). SWCNT inhalation was more effective than aspiration in causing inflammatory responses, oxidative stress, collagen deposition, and fibrosis (117). Broncheo-alveolar lavage (BAL) fluid cytology indicated a robust accumulation of neutrophils and lymphocytes after inhalation exposure to SWCNTs. Even 28 days postinhalation, the numbers of neutrophils and lymphocytes in BAL fluid of exposed animals remained significantly elevated compared with the control group. The principal histopathologic alterations in mice inhaling SWCNTs were pulmonary inflammation, bronchiolar epithelial cell hypertrophy, and the presence of foreign material in the interstitium, intracellularly within individual macrophages or free of lung tissue and most frequently aggregated near bronchoalveolar junctions, often with juxtaposed alveolar macrophages (**Figure 3**). Throughout the postexposure time course, macrophages were the principal inflammatory cells. In addition, anuclear macrophages were seen, suggesting the occurrence of abnormal mitoses or an apoptotic process involving dissolution of the cell nucleus (karyolysis). By 28 days postexposure, the granulomatous lesions were often well-organized. Overall, our studies (116, 117) demonstrate that SWCNTs cause unusual and robust pulmonary inflammation and fibrosis in mice, and suggest that the chain of pathological events is realized through interactions of inflammatory responses and oxidative stress culminating in the development of multifocal granulomatous pneumonia, interstitial fibrosis, and mutagenesis.

Do carbon nanotubes exert adverse immunological effects? Schipper et al. (93) concluded that SWCNTs were nontoxic when injected into the bloodstream of mice. However, this pilot study used nude (immunodeficient) mice as a model, which means that immunological responses could not be evaluated. In contrast, several recent in vivo studies have reported adverse immunological responses to carbon nanotubes. Mitchell et al. (129) reported that inhalation of multiwalled carbon nanotubes (MWCNTs) at particle concentrations ranging from 0.3 to 5 mg/m^3 did not result in

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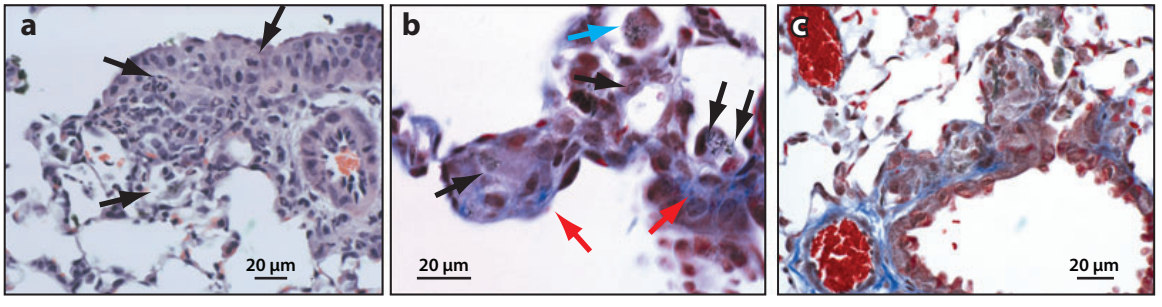

Figure 3

Typical pulmonary histopathology upon exposure to carbon nanotubes. (*a*) Neutrophilic pneumonia and bronchiolar epithelial hypertrophy in response to SWCNTs observed in the lungs of C57BL/6 mice on day 1 postinhalation (117). (*b*) Fibrosis (*black arrows*), alveolar macrophage infiltrates (*red arrows*), and anucleated macrophages (*blue arrow*) seen in lungs of C57BL6 mice 7 days postinhalation. (*c*) Granulomatous lesions and fibrosis evidently present in the lungs of C57BL/6 mice 28 days postexposure with SWCNTs.

significant lung inflammation or tissue damage, but caused systemic immune function alterations in C57BL/6 male mice. Immunosuppression was characterized by reduced T cell–dependent antibody response to sheep erythrocytes as well as T cell proliferative ability in the presence of mitogen. Furthermore, animals exposed to 1 mg/m^3 of MWCNTs had decreased natural killer cell function. Ryman-Rasmussen et al. (130) demonstrated pulmonary inflammatory responses in ovalbumin (OVA)-sensitized mice with allergic asthma after inhalation of MWCNTs. The latter studies suggested that preexisting allergic inflammation may increase the susceptibility for airway fibrosis. In a more recent study, exposure of mice to MWCNTs delivered by intratracheal instillation was shown to cause pulmonary and systemic immune responses. Total numbers of immune cells in BAL fluid were significantly increased following exposure, with increased numbers of neutrophils recovered by lavage. Pro-inflammatory cytokines were also increased in a dose-dependent manner, and B cell distributions in spleen and blood were increased. The authors concluded that MWCNTs may induce allergic responses in mice through B cell activation and production of IgE (131).

One of the characteristic features of immune respiratory responses is airway hyper-responsiveness. Airway hyper-responsiveness is an important factor in the pathogenesis of bronchial asthma. Our studies in mice exposed to SWCNTs have revealed a dose-dependent increase of airway hyper-responsiveness observed 1 day posttreatment (A. A. Shvedova et al., unpublished observations), indicating that exposure to these nanomaterials could potentially cause exacerbations of allergic asthma. We have also shown previously that exposure to SWCNTs is associated with decreased bacterial clearance in vitro and in vivo, which may lead to increased susceptibility to lung infection in exposed populations (132). Finally, the adjuvant capacity of carbonaceous nanomaterials was documented in a recent study employing Balb/c mice given OVA along with SWCNTs, MWCNTs, and ultrafine carbon black particles (UfCB) (133). Both OVA-SWCNT and OVA-MWCNT exposure strongly increased the level of OVA-specific IgE in serum, the number of eosinophils in BAL, and the secretion of Th2-associated cytokines. On the other hand, influx of neutrophils and increased levels of proinflammatory TNF-α and the chemoattractant protein MCP-1 were observed only in BAL fluids of mice treated with MWCNTs and UfCBs, not in mice exposed to SWCNTs. Based on these findings, it appears that carbon nanotubes can promote allergic responses in mice and may be even more potent than spherical particles such as UfCBs (133). Further studies are warranted to determine how the size and shape of nanomaterials may influence the ways in which these materials are sensed by immune-competent cells.

Do carbon nanotubes cause cancer? Poland et al. (134) recently reported on the so-called asbestos-like pathogenicity of MWCNTs following the introduction of these materials into the abdominal cavity of mice. However, no cancer development was reported in this pilot study. Instead, encapsulation of the carbon nanotubes by multinucleated cells of the immune system was evidenced, likely as a consequence of the inability of phagocytes to handle very long nanotubes. The authors suggested that the "frustrated phagocytosis" and granuloma formation seen in this study correlated with the length of the carbon nanotubes (134), but the stiffness of the materials, as well as other material properties and/or presence of contaminants in the materials included in the study, could also conceivably play a role in the pathological outcomes. Notwithstanding the different potential interpretations of the experimental data, this study is important because it suggests the need for further investigations of the in vivo biological responses to these nanomaterials.

In contrast, Takagi et al. (135) reported the induction of mesotheliomia upon intraperitoneal application of MWCNTs in p53 heterozygous mice that have been reported to be sensitive to asbestos. However, it should be noted that the authors injected 3 mg (3000 μg) of carbon nanotubes per mouse, a dose that appears to be unrealistically high. For comparison, we found that pharyngeal aspiration of nonpurified SWCNTs (iron content of 17.7% by weight) at doses varying from

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5 to 20 μg per animal or inhalation at 5 mg/m^3, 5 h/day, for 4 days, of the same SWCNTs resulted in pulmonary inflammation and oxidative stress in C57BL/6 mice; administration of carbon nanotubes via inhalation was more effective than aspiration in causing inflammation (117). Notably, an increased rate of SWCNT-induced mutations of the K-*ras* gene locus in the lung of mice took place very early after SWCNT inhalation (on days 1–7) and persisted through day 28. The mutagenic effect was coincident with the time of maximal inflammatory response, suggesting that inflammation and resultant oxidative injury could be a cause of mutagenicity. The potential role of this gene mutation in carcinogenesis awaits further investigation. However, taken together these studies suggest the need for considerable caution if long-term harm of carbon nanotubes is to be avoided.

CONCLUSIONS

The Greek philosopher Homer wrote, "Once harm has been done, even a fool understands it" (135a). In reference to engineered nanoparticles, the first question that comes to mind is, has harm been done? In other words, are there any examples of clinical toxicities related to exposure to engineered nanomaterials? To date, no such examples have been reported (136). However, as discussed, experimental studies indicate that nanomaterials could induce adverse responses in cell culture and in animal models. Moreover, adverse health effects have been documented in the past for other types of fine or ultrafine particles and fibers, such as diesel exhaust particles and asbestos, and it is possible that engineered nanomaterials may pose similar hazards, especially because human exposure to engineered nanomaterials is set to increase in the future as more and more applications are developed.

Furthermore, in anticipation of the harm that could be done by engineered nanomaterials, do we understand it? In other words, do we have an understanding of the physico-chemical properties of nano-sized materials that drive the biological and toxicological responses? Furthermore, are there unique (in other words, unanticipated or unpredictable) toxicities associated with the smallness of engineered nanomaterials or with any other properties of these materials? Clearly, this represents one of the major challenges in the field of nanotoxicology at present, and there is an urgent need for validation of appropriate test methods with which to assess nanomaterial toxicity, both in cell culture and in animal models (137).

Finally, we suggest that the specific properties of engineered nanomaterials should be considered in relation to the realm of biological systems. In other words, how do nanoparticles or devices interact with biological structures in the same size range? In considering this, we may learn more about the biological and toxicological responses to man-made nanomaterials, but we may also ultimately gain new insight into the biological systems themselves. This, therefore, represents the true benefit of the careful assessment of nanomaterial interactions with biological systems: Not only will such studies pave the way for the production of modified and improved nanomaterials, with mitigation of adverse responses, but we may also learn valuable lessons about the functions of cellular nanomachines.

SUMMARY POINTS

1. Engineered nanomaterials are novel materials that are manufactured in the same size range as cellular nanomachines and biomolecules, prompting the question of to what extent such artificial nanomachines can interfere with the highly orchestrated and structurally coordinated function of their intracellular counterparts.

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2. Nanotoxicology is an interdisciplinary science that builds on previous understanding of the toxic effects of particles and fibers; in addition, a more detailed comparison between man-made nanomaterials and biological nanoparticles, including viruses, may also yield important information.
3. Exposure of cells and tissues to engineered nanomaterials is commonly associated with oxidative stress, which in turn may trigger cytotoxic and proinflammatory responses to some nanomaterials or could represent an epiphenomenon that follows from the induction of cell death and disruption of cellular homeostasis for some other nanomaterials.
4. Engineered nanomaterials, as exemplified by carbon nanotubes, can cause robust and unusual inflammatory responses in vivo, with a rapid transition from the acute inflammatory phase to a chronic fibrotic phase; genotoxic effects have also been documented, but the realization of these events into a carcinogenic process remains to be demonstrated.

FUTURE ISSUES

1. The physico-chemical properties driving the adverse cellular responses to engineered nanomaterials need to be identified and investigated.
2. The mechanisms of cellular internalization of engineered nanomaterials by immune-competent cells and other, nonprofessional phagocytic cell types need to be characterized.
3. Future development may incorporate several nano-sized components into complex assemblies of nanomaterials. Nanotoxicologists therefore need to acquire and adapt suitable methodologies with which to assess the adverse effects of such composite structures.
4. Engineered nanomaterials pose a potential hazard to human health and the environment, and the development of strategies to promote efficient enzymatic or nonenzymatic biodegradation of these materials in the environmental and occupational settings but also in the context of biomedical applications is an important priority for future research.

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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.

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