# 9

# NANOTOXICOLOGY

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## 9.1 INTRODUCTION

In 1895, Wilhelm Roentgen discovered that a strange beam coming from the cathode-ray tube he was studying allowed him to see the bones in his hand against a fluorescent screen. Roentgen's discovery of X-rays sparked such great public interest that photographers were offering X-ray "portraits" by early 1896. In February 1896, a physics professor at Vanderbilt University persuaded the dean of the medical school to be the subject of a skull radiograph. Three weeks later the dean's hair fell out.

X-rays were one of the research community's first experiences with public reaction to scientific innovation, but it certainly was not the last. Controversies over genetically modified organisms and stem-cell research already have had significant impacts on research and development. While researchers tout the promises of nanomaterials to produce revolutionary advancements in drug and gene delivery, medical imaging, and cancer treatment, media coverage has focused on questions about how well we understand the safety aspects of this emerging technology.

Nanomaterials can have optical, magnetic, electronic, and structural properties that are unrealizable in corresponding bulk materials. These unusual properties, however, complicate determining the safety of nanomaterials because nanomaterials often do not

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behave like their bulk counterparts. Our understanding of the bulk toxicity of particular elements and alloys may not be directly translatable to the nano versions of these materials. A gold nanoparticle, for example, may not have the same chemical reactivity as bulk gold. Most nanomaterials used in biological applications contain multiple components for targeting, increasing circulation time, or other functionalities, which further complicate matters. Ironically, the very feature that makes nanomaterials so promising is the same feature that raises very important questions about potentially toxic effect.

An additional, but equally important, issue is the public perception of nanotechnology. In 1986, Eric Drexler expressed fears that self-replicating nanotechnology could spread at exponential rates. His use of the term "grey goo" to describe the eventual takeover of society by nanotechnology became one of the most prevalent public images of nanotechnology. This dire vision of the future was the theme of Michael Crichton's book *Prey*, which featured swarming nanobots intent on destroying their creators.

The casting of nanotechnology as villain surprised even Drexler [1]. In 2004, Drexler noted surprise at how warnings had dominated the public perception of nanotechnology:

> I expected the contemplation of the broad societal impacts of nanotechnology to cause some discomfort, but did not expect that depictions of swarms of self-replicating nanobugs would dominate popular perceptions of advanced nanotechnology, nor did I envision that the term "nanotechnology" would come to describe a wide range of almost unrelated research fields, and that efforts to disassociate those fields from concerns about "grey goo" would spur false scientific denials of the original concepts.

Despite Drexler's recent statement that "I wish I had never used the term 'grey goo' " [2], the idea that nanotechnology represents a potential threat remains in the public awareness due to the popular press and governmental studies [3–8]. Governmental reports generally have been supportive of nanotechnology, but express concerns about rushing forward without reliable, consistent data on which to base decisions and regulatory activities. The continuing public interest emphasizes the need to accurately assess and communicate information about potentially negative effects of nanotechnology to the public. This brief summary of the literature regarding questions of toxicity is meant to provide nanomaterials researchers—especially those coming from the nonmedical sciences—an introduction to the critical issues surrounding this important topic.

## 9.2 NANOTOXICOLOGY

### 9.2.1 Parameters

The fact that nanomaterials behave in new—and sometimes unexpected—ways has important implications for the field of toxicology [9–18]. While nanomaterials enter the body in the usual ways (orally, through the respiratory system, through the blood circulation, and through the skin), they can interact with biological materials very differently than larger versions of the same material. Although size is important, it is far

from being the only relevant factor. Chemical composition, surface area, surface charge, crystal structure, chemical reactivity, solubility, shape, and degree of agglomeration are also important parameters for understanding toxicity. This produces a much more extensive list of parameters than those addressed in typical toxicity studies.

Nanotechnology has changed the research paradigm. Addressing complex biomedical issues requires truly interdisciplinary teams; however, the confluence of researchers from different disciplines underscores a need for common definitions and usage. For example, does the stated size of a nanoparticle include surfactant, coating, or functionalizing molecules, or does it specify only the core? Coating molecules, or even residual impurities from synthesis procedures, can change the chemical behavior of a nanoparticle, so that comparing two gold nanoparticles is not as straightforward as one might expect. For example, some evidence of toxicity in carbon nanotubes may be due to residual metal catalysts used in their fabrication [19]. The nature of nanomaterials is that we cannot say a particular material is "biocompatible": We must study specific combinations of materials and evaluate these on an individual basis.

## 9.2.2 Toxicity Mechanisms

There are numerous reasons why nanomaterials may have greater toxicity than their bulk counterparts. In general, nanomaterials can penetrate into smaller structures and move deeper into passageways and tissues than larger particles. Nanoparticles may have increased mobility within human cells [20–23]. Once inside cells, they can produce oxidative stress, impair phagocytosis, inhibit cell proliferation, and decrease cell viability. The mechanisms of transport, cohesion, and adhesion are highly dependent on nanoparticle size [24]. Smaller particles may evade the reticuloendothelial system more effectively, and thus the nanoparticles remain in the body for longer times. Nanomaterials may generate mobile complexes that can enter tissue sites normally inaccessible to the proteins. Unexpected chemical reactions due to enhanced reactivity can impair functional and structural cell properties. All of these possibilities contribute to the potential toxicity of nanomaterials.

***9.2.2.1 Production of Reactive Oxygen Species.*** One of the most important toxicity mechanisms is the production of reactive oxygen species (ROS). For example, electron capture of $O_2$ can lead to the formation of the superoxide radical $O_2^-$, which then generates additional ROS. Normally, glutathione and other antioxidant enzymes maintain equilibrium in the body. An increasing ROS generation rate causes oxidative stress and triggers inflammatory responses. High levels of oxidative stress perturb the mitrochondrial PT pore and disrupt electronic transfer, which can produce cellular apoptosis or necrosis [25]. Reactive oxygen species can damage cellular proteins, lipids, membranes, and DNA.

The mechanism by which oxidative species are produced is still being studied, but it appears that pro-oxidative organic hydrocarbons (such as polycyclic aromatic hydrocarbons and quinones) and transition metals such as copper, vanadium, chromium, nickel, cobalt, and iron are likely to participate in these reactions [9,25–34]. Many carbon

nanotube production techniques require a metal catalyst, and significant concentrations of metals such as nickel, yttrium, and iron can be found in nanotubes, which may contribute to higher toxicity. Some semiconductors, when photoexcited, can create electron–hole pairs that can produce ROSs [20].

***9.2.2.2 Effect of Physical Parameters.*** Ascribing toxicity solely to a nanomaterial's size, mass, or surface area is difficult because of the many variables that characterize a nanomaterial. A coated nanoparticle and a bare nanoparticle of the same material may have the same size, but distinctly different surface characteristics. Studies in which these variables can be isolated are difficult, but critical to understanding toxicity mechanisms [24, 35–37].

One can find in the literature examples in which smaller nanoparticles have greater toxicity than larger nanoparticles, especially in terms of inflammatory responses, and in which toxicity can be scaled to surface area or mass uptake [27, 38–45]. Other studies, however, show no or minimal surface area or size effect, and some show that smaller nanoparticles are less toxic than larger materials [18,36,46]. One possible contributor to these results is that aggregation and agglomeration significantly change the surface area of a material. If smaller nanoparticles aggregate to a greater degree than larger particles, the aggregates may not be taken up by cells as readily and thus show lower toxicity [24].

The size and shape of the nanomaterials may interfere with the efficiency of macrophage clearance. Fibrous shapes in which the length is larger than the diameter of the alveolar macrophage cannot be taken up and removed by macrophages [47]. In vitro studies focus primarily on the effect of nanomaterials in cell cultures, which is measured by cell viability and by the release of chemical mediators such as cytokines that are related to immunological and inflammatory reactions.

Structural properties also play a role in toxicity because of the role of structure in determining surface electronic states. Crystalline silica, for example, has a more deleterious effect on lung epithelial cells than similarly sized amorphous silica particles [48]. Materials such as $TiO_2$ that have multiple phases for the same chemical composition may exhibit very different toxicity in the different phases [49].

***9.2.2.3 Effect of Surface Chemistry.*** Other forms of toxicity may be produced by unanticipated chemical reactions, including protein denaturation, membrane damage, DNA damage, and immune reactivity. The requirement of putting nanomaterials in an aqueous suspension adds another level of complexity because toxicity may be due to functionalizing molecules [36] or unexpected reactions between the nanoparticles and the functionalizing molecules [50].

Surface treatment is one route to mitigating surface reactions. Direct toxicity of materials is a problem in semiconductor quantum dots, which are possible alternatives to organic dyes. Most cadmium-based materials, such as cadmium selenide (CdSe) and cadmium telluride (CdTe), are known to be toxic, due to the release of Cd and/or Se ions [51–54]. A zinc selenide (ZnS) coating was used on CdSe to prevent Cd ion release; however, DNA damage was produced by oxidation of the ZnS cap [55]. Exposure to UV light appears to increase the amount of damage, likely due to exciton-induced chemical reactions from the semiconductors. Although surface modifications may confer

biocompatibility, many coatings can be removed by chemical reactions within the body (such as oxidation), physical abrasion, or exposure to UV radiation [51,56].

***9.2.2.4 Effect of Surface Charge.*** Polycationic macromolecules interact strongly with cell membranes, and the multiple positive charges appear to be an important factor in the cytotoxicity for cells of all kinds [50,57]. The addition of polyanionic compounds appears to protect against polycationic cytotoxic effects [14]. The polycation effect also may explain why branched and flexible polymers, which can have more attachments on a cell surface than rigid or globular polymers, have higher cytotoxic effects. The configuration of the polymer on the cell surface produces a high charge density [58, 59]. Globular polymers appear to be less cytotoxic than linear polymers. Even 2-nm nanoparticles made of gold—a material known for its inertness—were toxic when made with a cationic surface charge [60]. The mechanism was proposed to be concentration-dependent lysis mediated by initial electrostatic binding. Surface charge also affects the degree of agglomeration, which can affect particle uptake [24].

***9.2.2.5 Carbon Nanostructures.*** Carbon nanostructures, such as fullerenes and nanotubes, deserve special attention because these materials receive the most popular press. Carbon nanostructures have many potential applications because they have a unique free radical chemistry and, due to their strong attraction for electrons, antioxidant properties. Some carbon-60 ($C_{60}$) fullerenes have been found to bind strongly to nucleotides and may hamper self-repair in double-strand DNA [61]. Carbon nanomaterials also have high electrical and thermal conductivity, high strength, and rigidity. The many potential medical and nonmedical applications suggest that these materials may be produced on an industrial scale, increasing concern about occupational or accidental exposure.

There are a wide variety of reports regarding toxicity of carbon fullerenes (closed cage structures), single-walled nanotubes (SWNT), and multi-walled nanotubes (MWNT). Some studies suggest not only that fullerenes are nontoxic, but that they may provide protection against pathologies such as acute or chronic neurodegenerative diseases and liver disease [62–71]. Other reports show that carbon nanostructures produce $O_2^-$ anions, lipid peroxidation, and cytotoxicity in plants and animals [72–78]. For example, uncoated fullerenes in largemouth bass show lipid peroxidation in brain tissue and glutathione depletion in gills [75]. Studies comparing carbon nanomaterials with oxide and metal nanomaterials suggest that, in inhalation studies at least, the toxicity mechanism may be quite different for carbon nanomaterials [29, 79, 80].

The disparity of results suggests that the problem is more complex than originally realized. For example, one of the first reports of toxicity in $C_{60}$ was samples in which the surface was modified with PVP [81]; however, later studies showed that $C_{60}$ can react with PVP to produce highly stable charge-transfer complexes [82]. A similar concern has been expressed that some fullerene samples are suspended in solution by dissolving $C_{60}$ in THF and that not all of the solvent was removed prior to toxicity testing [68]. Although $C_{60}$ may not cross the blood–brain barrier, the THF may. As mentioned, metal catalysts used in nanotube fabrication may be toxic, and sample preparation may exacerbate the

toxicity of these metals [18, 77, 80]. Accurate and detailed characterization of samples used for these studies is critical.

More derivatized $C_{60}$ species appear to be less toxic, in part due to a lower efficiency in generating ROS [72,83]. Derivatization originally was required because carbon nanomaterials are not water-soluble. Toxicity effects ranging over seven orders of magnitude for different functionalizing molecules have been observed [72]. Aggregation becomes a factor, because interior members of the aggregate may not be appropriately derivatized. The most recent studies suggest that sidewall functionalization is significantly better at reducing toxicity than surfactant coating [84].

## 9.2.3 In Vivo Studies

***9.2.3.1 Inhalation.*** The largest number of studies of *in vivo* toxicity deal with the respiratory route, and most focus on carbon black, $TiO_2$, and diesel particulates. There remains significant disagreement in the literature, much of which will likely be resolved by more detailed physical and chemical characterization of the materials being studied. The primary problems with respirated nanoparticles is that they can travel further into the lung, have a higher probability for chemical reactions that cause oxidative stress, and are more likely to persist in the body. Persistent nanoparticles may increase the risk of tumor formation.

Inhaled particulate matter can induce pulmonary and airway inflammations, interfere with the clearance and inactivation of bacteria in the lungs, and affect other organs by translocation. Oxidative stress and inflammation may lead to atherosclerotic plaques, failure to properly regulate heart rate, and decreased blood clotting ability.

Particles smaller than 2.5 $\mu$m can reach the alveoli, where clearance requires macrophage phagocytosis. Macrophage phagocytosis can produce sustained inflammation and some types of nanomaterials interfere with the efficiency of the clearance [47]. Smaller nanoparticles can take a significantly longer time to clear from the body and may promote translocation to interstitial sites and regional lymph nodes [85]. Dose-dependent formation of epithelioid granulomas (combinations of living and dead tissue that are signs of toxicity) observed with carbon nanostructures appear to originate in aggregates of nanotube-containing macrophages [79, 86].

Although the primary impact is in the pulmonary system, translocation of nanoparticles leads to circulatory access and allows distribution throughout the body [85, 87]. Translocation is very dependent on the specific properties of the nanoparticles. Many nanomaterials translocate to the liver, with secondary uptake by the spleen, bone marrow, heart, kidney, bladder, and brain [12]. Nanoparticles that migrate to the heart can affect arrhythmia and coagulation [88]. There is evidence that inhaled micron-sized particles affect the autonomic nervous system, so it is possible that similar or larger effects might be seen with nanoparticles [89–91]. Recent data suggest that some nanoparticles can move from olfactory nerve endings in the nose to the brain. Manganese appears to be translocated in this manner, but iron does not [92, 93].

The lung epithelial barrier is an example of how size can impact translocation. The epithelial barrier in a dog could be modeled by a three-pore system with pore radii of 1 nm, 40 nm, and 400 nm comprising 68%, 30%, and 2% of the pores, respectively. This

suggests that smaller nanoparticles could more easily pass through the lung epithelial barrier [94].

At high doses, toxicity has been observed due to physical blockage produced by agglomerates. Carbon nanotubes, for example, have a strong electrostatic attraction and easily form aggregates [79]. A potential advantage of this tendency to agglomerate is that they may form unrespirable masses and thus decrease exposure [95].

***9.2.3.2 Dermal Toxicology.*** Nanoparticle use in cosmetics (especially sunscreen) is widespread, with the primary materials being nanoscale $TiO_2$ and ZnO. The primary barrier to absorption through the skin is physical: the outer layer of the epidermis, which contains mostly dead skin cells. There is less information about dermal toxicity nanoparticles than about other types, and specific skin conditions may affect greatly the penetration of nanoparticles; however, the primary toxic mechanism appears to be generation of ROS. Whether the nanoparticles penetrate the epidermis depends on a number of factors. Movement of the skin or damaged skin can allow the penetration of microscale beads to the dermis and may allow movement to regional lymph nodes, where chemical reactions with proteins can affect the autoimmune system [96, 97]. There is evidence that submicron particles penetrate the skin shallowly and may penetrate more deeply via hair follicles [98, 99]. Smaller particles may penetrate far enough into the skin to interact with the immune system [100], or they may not penetrate deeply enough to be removed to the lymph nodes by macrophages and remain in the skin [101]. Even when nanoparticles remain in the dermis, molecules associated with the nanoparticle may detach and be transported through the skin.

An addition factor for dermal toxicity is the possibility of light-stimulated changes to the nanomaterials. Semiconducting anatase $TiO_2$ can create an electron–hole pair by absorbing a photon of light in the UVA or UVB region; interaction of this pair with water can produce ROS, including hydroxyl radicals, singlet oxygen, and superoxide [102]. Some researchers found that coating with inert oxides (silica, alumina, or zirconia) may reduce ROS generation [18]; however, photostimulated ROS production has been seen in commercial sunscreens [103].

***9.2.3.3 Other Pathways.*** Nanoparticles can enter the gastrointestinal tract by direct methods (eating and drinking), or as a result of clearance via the mucociliary escalator in the respiratory tract. Most nanoparticles are rapidly eliminated through the bowel [104, 105]. Particle size, surface charge, and attachment of ligands or surfactants affect particle uptake [106–109]. In general, smaller particles are taken up more than larger particles and can be transported to the liver, spleen, blood, and bone marrow [110–112].

Ingestion of water-soluble radiolabeled fullerenes in rats show a 98% clearance in feces, while 90% of radio-labeled fullerenes administered intravenously were retained for at least a week with more than 70% residing in the liver [113, 114]. Intravenously injected water-soluble-single-walled carbon nanotubes functionalized with chelating molecule diethyletriaminepentaacetic and a radiotracer were found to be rapidly cleared from systemic blood circulation in mice via renal excretion with a half-life of about three and a half hours. Both functionalized SWNT and MWNT were excreted as intact

nanotubes [115]. As in inhalation, aggregation plays a role in toxicity. Intestinal obstruction due to agglomerates of nanoscale zinc (Zn) powders produced mortality in some mice, while no impairments were observed with microscale Zn [116].

## 9.3 CONCLUSIONS

The field of nanotoxicity is in the very early stages of development, but it is clear from the available data that our knowledge of bulk material toxicity is not directly transferable to nanoscale counterparts. The toxicology community is developing methods and protocols to collect relevant and specific data on nanomaterial toxicity. Federal funding of studies specifically aimed at determining toxicity is increasing. Improved physical and chemical characterization of nanomaterials, combined with detailed reporting of these parameters, will help in comparing toxicity studies. In some respects, the question of whether carbon nanotubes are "safe" is academic because most biomedical nanosystems contain multiple materials. A definitive determination as to whether $C_{60}$ "is toxic" is not as important as determining the toxicity of specific formulations.

Despite the potential for greater toxicity, nanomaterials will be an important tool in toxicology because of the ability to tightly control size dispersion, surface characteristics, and purity compared to naturally occurring materials. Toxicologists have an opportunity to investigate in greater detail the mechanisms responsible for toxicity, especially in the interactions of nanomaterials with cells. Once toxicity mechanisms are determined, nanoparticles that do exhibit toxic effects may find use as antibiotics or anticancer agents.

Finally, nanomaterials researchers need to be aware of the high visibility of anything "nano" in the eyes of the public. Previous experiences with stem cell research, dioxins, and genetically modified organisms should serve as lessons. Reports of potentially toxic effects of fullerenes have received far more attention in the popular press than have reports of nontoxic formulations. Special interest groups already have called for a moratorium on nanomaterials research and development [117, 118]. One highly publicized incident, even if minor, could prove a serious setback to progress. It is in the best interests of nanomaterials researchers to educate the public so that they can make informed decisions about the role that nanomaterials will play in their lives.

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