

Toxic Potential of Materials at the Nanolevel

Andre Nel,^{1,2*} Tian Xia,¹ Lutz Mädler,³ Ning Li¹

Nanomaterials are engineered structures with at least one dimension of 100 nanometers or less. These materials are increasingly being used for commercial purposes such as fillers, opacifiers, catalysts, semiconductors, cosmetics, microelectronics, and drug carriers. Materials in this size range may approach the length scale at which some specific physical or chemical interactions with their environment can occur. As a result, their properties differ substantially from those bulk materials of the same composition, allowing them to perform exceptional feats of conductivity, reactivity, and optical sensitivity. Possible undesirable results of these capabilities are harmful interactions with biological systems and the environment, with the potential to generate toxicity. The establishment of principles and test procedures to ensure safe manufacture and use of nanomaterials in the marketplace is urgently required and achievable.

By some estimates, nanotechnology promises to far exceed the impact of the Industrial Revolution and is projected to become a \$1 trillion market by 2015. Engineered nanomaterials (NM) are already being used in sporting goods, tires, stain-resistant clothing, sunscreens, cosmetics, and electronics and will also be increasingly utilized in medicine for purposes of diagnosis, imaging, and drug delivery. Mihail Roco of the U.S. National Nanotechnology Institute envisages four generations of nanotechnology. The current era is that of passive nanostructures, materials designed to perform one task. The second phase will introduce active nanostructures for multitasking, for example, actuators, drug delivery devices, and sensors. The third generation is expected to emerge around 2010 and feature nanosystems with thousands of interacting components. A few years after that, the first integrated nanosystems, functioning much like a mammalian cell with hierarchical systems within systems, are expected to evolve.

The unusual physicochemical properties of engineered NM are attributable to their small size (surface area and size distribution), chemical composition (purity, crystallinity, electronic properties, etc.), surface structure (surface reactivity, surface groups, inorganic or organic coatings, etc.), solubility, shape, and aggregation. Although impressive from a physicochemical viewpoint, the novel properties of NM raise concerns about adverse effects on biological systems, which at the cellular level include structural arrangements that resemble NM in terms of their function. Indeed, some studies suggest that NM

are not inherently benign and that they affect biological behaviors at the cellular, subcellular, and protein levels (1–5). Moreover, some nanoparticles readily travel throughout the body, deposit in target organs, penetrate cell membranes, lodge in mitochondria, and may trigger injurious responses.

There is almost unanimous opinion among proponents and skeptics alike that the full potential of nanotechnology requires attention to safety issues. Already there are outcries from environmental activists calling for a worldwide moratorium on NM research and marketing until protocols are in place to ensure worker safety. Science fiction novels and news media reports have also perpetuated a scary scenario in which self-replicating nanoscale robots consume all available materials, ultimately strangling the planet in a “gray goo.” Although this scenario is implausible from an energy as well as a structural assembly viewpoint, it points to the need to develop a rational, science-based approach to nanotoxicology. It is our opinion that such an approach is feasible and should be implemented to ensure the safe manufacturing and marketing of engineered nanoproducts.

Do Nanomaterials Properties Necessitate a New Toxicological Science?

The main characteristic of NM is their size, which falls in the transitional zone between individual atoms or molecules and the corresponding bulk materials. This can modify the physicochemical properties of the material as well as create the opportunity for increased uptake and interaction with biological tissues. This combination of effects can generate adverse biological effects in living cells that would not otherwise be possible with the same material in larger form. Although the extraordinary properties of NM may necessitate a novel investigative

approach to assess their hazard potential, particle toxicology is a mature science that addresses the mechanisms of lung injury by inhaled particles (4–6). Inhaled or instilled ambient ultrafine particles (particulate matter with an aerodynamic diameter < 100 nm) can induce pulmonary inflammation, oxidative stress, and distal organ involvement. In a similar fashion, occupational exposure to quartz, mineral dust particles (e.g., coal and silicates), and asbestos fibers induce oxidative injury, inflammation, fibrosis, cytotoxicity, and mediator release from lung target cells (4–8). The same holds true for experimental instillation of titanium dioxide (TiO₂) and carbon black nanoparticles in animal lungs. Tissue and cell culture analysis support the physiological response seen in animal models, pointing to the role of oxidative stress in the production of inflammatory cytokines and cytotoxic cellular responses. Taken together, these clinical and experimental studies indicate that a small size, a large surface area, and an ability to generate reactive oxygen species (ROS) play a role in the ability of nanoparticles to induce lung injury (4–8). Thus, as the particle size shrinks, there is a tendency for pulmonary toxicity to increase, even if the same material is relatively inert in bulkier form (e.g., carbon black and TiO₂). However, particle coating, surface treatments, surface excitation by ultraviolet (UV) radiation, and particle aggregation can modify the effects of particle size. It is possible, therefore, that some nanoparticles may exert their toxic effects as aggregates or through the release of toxic chemicals.

Particle size and surface area are important material characteristics from a toxicological perspective. As the size of a particle decreases, its surface area increases and also allows a greater proportion of its atoms or molecules to be displayed on the surface rather than the interior of the material. Figure 1 shows the inverse relationship between the particle size and the number of molecules expressed on the particle surface. Table 1 shows that, as the particle size for a group of airborne particles with fixed mass (10 µg/m³) and unitary density (1 g/cm³) decreases, their number increases exponentially along with the surface area. The increase in surface area determines the potential number of reactive groups on the particle surface.

The change in the physicochemical and structural properties of engineered NM with a decrease in size could be responsible for a number of material interactions that could lead to toxicological effects (4, 7). For instance, shrinkage in size may create discontinuous crystal planes that increase the number of structural defects as well as disrupt the well-structured electronic configuration of the material, so as to give rise to altered electronic

¹Department of Medicine, David Geffen School of Medicine, ²California NANOSystems Institute, ³Department of Chemical and Biomolecular Engineering, University of California, Los Angeles, CA 90095, USA.

*To whom correspondence should be addressed. E-mail: anel@mednet.ucla.edu

properties (Fig. 2) (4, 7). This could establish specific surface groups that could function as reactive sites (Fig. 2). The extent of these changes and their importance strongly depend on the chemical composition of the material. Surface groups can make NM hydrophilic or hydrophobic, lipophilic or lipophobic, or catalytically active or passive (Fig. 2). An example of how those surface properties can lead to toxicity is the interaction of electron donor or acceptor active sites (chemically or physically activated) with molecular dioxygen (O_2). Electron capture can lead to the formation of the superoxide radical ($O_2^{\cdot-}$), which through dismutation or Fenton chemistry can generate additional ROS. Single-component materials as well the presence of transition metals on the surface can participate in the formation of such active sites. For instance, ultrafine particles contain transition metals (e.g., Fe and vanadium) and are also coated with redox-cycling organic chemicals (e.g., quinones), whereas carbon nanotubes contain metal impurities that can amplify chemical changes in the NM environment (Fig. 2). Thus, several NM characteristics can culminate in ROS generation (9), which is currently the best-developed paradigm for nanoparticle toxicity (Table 2). Other NM properties such as shape, aggregation, surface coating, and solubility may also affect the addressed specific physicochemical and transport properties, with the possibility of negating or amplifying the size effects (Fig. 2).

The Biology of Particle-Induced Oxidative Stress as an Important Mechanistic Paradigm on Which to Base a Predictive Model for Studying NM Toxicity

There is a direct relationship between the surface area, ROS-generating capability, and pro-inflammatory effects of nanoparticles in the lung (4–8). From a mechanistic perspective, ROS generation and oxidative stress is the best-developed paradigm to explain the toxic effects of inhaled nanoparticles (3–10). Under normal coupling conditions in the mitochondrion, ROS are generated at low frequency and are easily neutralized by antioxidant defenses such as glutathione (GSH) and antioxidant enzymes (11). However, under conditions of excess ROS production, such as may occur in the lung and possibly the circulatory system during ambient or occupational nanoparticle exposures (8), the natural antioxidant defenses may be overwhelmed (11). Oxidative stress refers to a state in which GSH is depleted while oxidized glutathione (GSSG) accumulates (11). Cells respond to this drop in the GSH/GSSG ratio by mounting protective or injurious responses (8, 10–12). The oxidative stress resulting from real-life ambient and occupational particle exposures, as well as experimental challenge with ambient particulate matter (PM), quartz, carbon black, or TiO_2 nanoparticles, re-

sults in airway inflammation and interstitial fibrosis (3–8).

Mechanistic studies that use discovery tools such as proteomics and genomics have proven useful for substantiating mechanistic hypotheses (12) explaining the biology of oxidative stress, and developing biomarkers. According to the hierarchical oxidative stress hypothesis, the lowest level of oxidative stress is associated with the induction of antioxidant and detoxification enzymes (Fig. 3) (12). The genes that encode the phase II enzymes are under the control of the transcription factor Nrf-2. Nrf-2 activates the promoters of phase II genes via an antioxidant response element (ARE) (12). Defects or aberrancy of this protective response pathway may determine disease susceptibility during ambient particle exposure. At higher levels of oxidative stress, this protective response is overtaken by inflammation and cytotoxicity (Fig. 3). Inflammation is initiated through the activation of pro-inflammatory signaling cascades [e.g., mitogen-activated protein kinase (MAPK) and nuclear factor κ B (NF- κ B) cascades], whereas programmed cell death could result from mitochondrial perturbation and the release of pro-apoptotic factors (Fig. 3). It is noteworthy that several different types of nanoparticles, including ambient ultrafines, target mitochondria directly (4, 12).

A number of responses at each level of oxidative stress have now been successfully incorporated as screening assays for toxicological effects of ambient PM in vivo, for example, increased expression of antioxidant enzymes and cytokines in the lungs of exposed animals. Moreover, knockout or genetic polymorphisms of genes that encode for phase II enzymes establish a susceptibility mechanism that may determine why only some individuals develop PM-induced injury (13). Characterization of particle size and physical characteristics, together with in vitro assays for ROS and oxidative stress (phase II responses, inflammation, and mitochondrion-mediated apoptosis) plus in vivo markers of oxidative stress (e.g., lipid peroxidation and signature cytokines), is an example of a predictive paradigm for toxicity screening. Similar paradigms can be developed for engineered NM. These assays can be supplemented with nanosensor systems that are designed to interrogate the abilities of NM to generate ROS.

In addition to the paradigm of oxidative stress and inflammation, it is important to consider that some of the NM interactions depicted in Fig. 2 may also result in other forms of injury, such as protein denaturation, membrane damage, DNA damage, immune reactivity, and the formation of foreign body granulomas (Table 2). It is also possible that new NM properties may

emerge that can lead to novel mechanisms of toxicity.

Are Occupational and Inhalation Exposures to Ambient Nanoparticles Applicable to Engineered NM?

Uses of engineered NM in sunscreens [e.g., TiO_2 and zinc oxide (ZnO)] and cosmetics and as bio-imaging probes (e.g., superparamagnetic iron oxides) have not led to reports of clinical toxicity in humans. However, although inhalation of ultrafine ZnO particles at relatively high dose ($500 \mu\text{g}/\text{m}^3$) for 2 hours did not induce acute systemic effects in humans, inhalation of ZnO fumes in an occupational setting can cause metal fume fever (fatigue, chills, fever, myalgias, cough, dyspnea, leukocytosis, metallic taste, and salivation) (14). Only a limited number of NM have so far been shown to exert toxicity in tissue culture and animal experiments, usually at high doses (2, 4). For instance, intratracheal instillation of TiO_2 particles in rodents demonstrates that these nanoparticles induce bigger inflammatory responses than larger particles of an equivalent mass dose (4). However, when the instilled dose is expressed as particle surface area, the inflammatory response fits the same dose-response curve (3–7). This supports the concept that the surface area is the dose measurement that best predicts pulmonary toxicity (4–8). In addition to the generation of pro-inflammatory effects, nanoparticles of various sizes and chemical composition are able to lodge in mitochondria (4, 15). This can lead to disruption of the mitochondrial electron transduction chain, which leads to additional $O_2^{\cdot-}$ production (15). Further, ambient ultrafine particles perturb the mitochondrial permeability transition pore, which leads to the release of pro-apoptotic factors and programmed cell death (15).

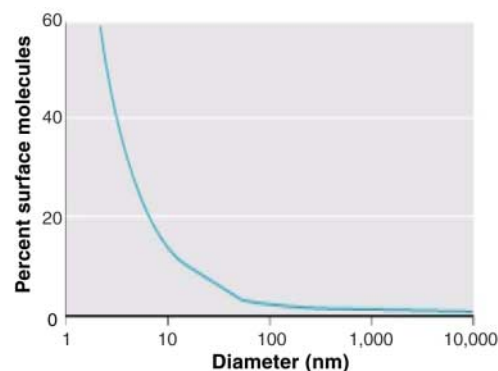


Fig. 1. Inverse relationship between particle size and number of surface expressed molecules. In the size range < 100 nm, the number of surface molecules (expressed as a % of the molecules in the particle) is inversely related to particle size. For instance, in a particle of 30 nm size, about 10% of its molecules are expressed on the surface, whereas at 10 and 3 nm size the ratios increase to 20% and 50%, respectively. Because the number of atoms or molecules on the surface of the particle may determine the material reactivity, this is key to defining the chemical and biological properties of nanoparticles. [Adapted from (4)]

In addition to the above materials, carbon nanostructures are one of the limited types of engineered nanostructures that have undergone some toxicity testing (2, 4). This includes testing of fullerenes, which in their basal state are composed of 60 carbon atoms (C60) in the shape of a sphere. Fullerenes have many potential applications based on their unique free radical chemistry and antioxidant properties. Specific surface treatments are required to disperse the fullerenes in suspensions for in vitro and in vivo testing (2). Their ability to induce toxicity may also require a light or ultraviolet (UV) source to excite the fullerene surface (2) (Fig. 2). Water-soluble, monodisperse, or colloidal fullerene aggregates induce $O_2^{\cdot-}$ anions, lipid peroxidation, as well as cytotoxicity. On the other hand, modification of the fullerene surface by attachment of malonyl groups yields nanoparticles with biologically useful antioxidant activity (16). Even at high doses, animal studies have demonstrated minimal dermal and oral toxicity (2). Fullerenes have to be systemically administered at relatively high dose to achieve acute toxicity; the median lethal dose for a water-soluble fullerene was 600 mg/kg (17). Although the exposure of largemouth bass to fullerenes leads to lipid peroxidation in the brain and glutathione depletion in their gills, it is unclear why these biological effects disappeared at higher concentrations (18).

Carbon nanotubes are long carbon-based tubes that can be either single- or multiwalled and have the potential to act as biopersistent fibers. Nanotubes have aspect ratios ≥ 100 , with lengths of several μm and diameters of 0.7 to 1.5 nm for single-walled nanotubes (SWNT) and 2 to 50 nm for multiwalled nanotubes (MWNT). In vitro incubation of keratinocytes and bronchial epithelial cells with high doses of SWNT results in ROS generation, lipid peroxidation, oxidative stress, mitochondrial dysfunction, and changes in cell morphology (19). MWNT also elicit proinflammatory effects in keratinocytes (20). Several studies using intratracheal instillation of high doses of nanotubes in rodents demonstrated chronic lung inflammation, including foreign-body granuloma formation and interstitial fibrosis (9, 21, 22). These studies also reveal the tendency of the nonphysiologic administration route and the unrealistic high doses to lead to asphyxiation through nanotube clumping in the airways (21). Although it has been suggested that the granulomatous inflammation could be a bio-

persistent fiber effect, similar to the effect of asbestos fibers, the high dose of the aggregated nanotubes and the presence of metal impurities (e.g., Fe) could account for artificial toxicity. There is also a question of whether the manufacture of nanotubes under closed gas-phase conditions will lead to substantial inhalation exposure of factory workers. It is possible that the release of nanotubes from an intended commercial use product such as car tires could become airborne.

All considered, the limited toxicity data for engineered NM confirm that ROS production may play a role under some experimental conditions (e.g., light or UV exposure) or when these materials contain metal impurities. Although at high dose the generation of ROS and oxidative stress could lead to NM toxicity, it is not clear that these experimental findings are directly related to clinical toxicity. This differs from the data on ambient ultrafine particles. Ultrafine particles are mostly derived from combustion sources, are heterogeneous in size, exist in single or aggregated form, and have a chemical structure consisting of a solid core made of either inorganic material (sulfuric acid and transition metals) or soot surrounded by a layer of adsorbed or condensed semi-volatile organic constituents, all of which can contribute to ROS generation (23). This is quite different from the homogeneous composition and size of engineered NM that occasionally contain transition metals. Even though these differences led some experts to question the relevance of ambient nanoparticle research to the study of NM,

it is important to recognize that PM research has established important principles of particle toxicity that may be applicable to NM. This includes the recognition that small particle size, chemical composition, and the presence of a large reactive surface area can catalyze ROS production.

What Are the Biological and Biokinetic Properties of NM that Need to Be Considered for Toxicity Testing?

The biological impacts of NM and the biokinetics of nanoparticles are dependent on size, chemical composition, surface structure, solubility, shape, and aggregation. These parameters can modify cellular uptake, protein binding, translocation from portal of entry to the target site, and the possibility of causing tissue injury (4). At the target site, NM may trigger tissue injury by one or more mechanisms (Table 2). Potential routes of NM exposure include gastrointestinal tract (GIT), skin, lung, and systemic administration for diagnostic and therapeutic purposes. NM interactions with cells, body fluids, and proteins play a role in their biological effects and ability to distribute throughout the body. NM binding to proteins may generate complexes that are more mobile and can enter tissue sites that are normally inaccessible. Accelerated protein denaturation or degradation on the nanoparticle surface may lead to functional and structural changes, including interference in enzyme function (24). This damage could result from splitting of intramolecular or intramolecular

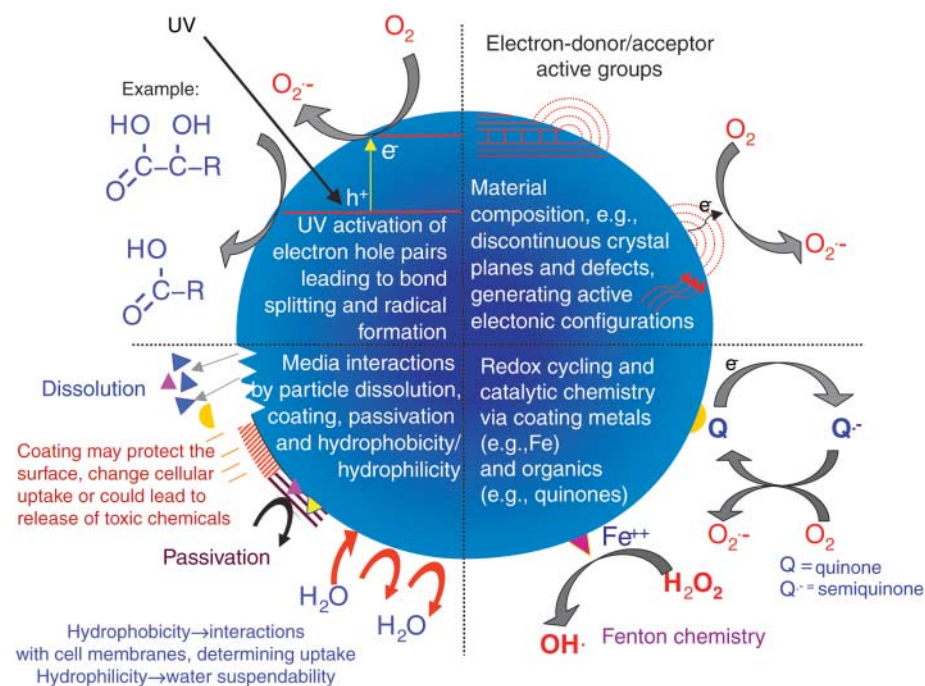


Fig. 2. Possible mechanisms by which nanomaterials interact with biological tissue. Examples illustrate the importance of material composition, electronic structure, bonded surface species (e.g., metal-containing), surface coatings (active or passive), and solubility, including the contribution of surface species and coatings and interactions with other environmental factors (e.g., UV activation).

Table 1. Particle number and particle surface area for 10 $\mu g/m^3$ airborne particles (5).

Particle diameter (μm)	Particles/ml of air	Particle surface area ($\mu m^2/ml$ of air)
2	2	30
0.5	153	120
0.02	2,390,000	3000

bonds by catalytic chemistry on the material surface (Fig. 2). Throughout their uptake and transport through the body, NM will encounter a number of defenses that can eliminate, sequester, or dissolve nanoparticles. In addition, cells and tissues have effective antioxidant defenses that deal with ROS generation (Fig. 3).

Although inhalation is a less likely route for engineered NM exposure compared with ambient or mineral dust particles, this can happen during bulk manufacture and handling of freely dispersible nanoparticles. Inhaled nanoparticles are efficiently deposited by diffusional mechanisms in all regions of the lung (4). Several defense mechanisms, including mucociliary escalator transport and phagocytosis by macrophages, keep the mucosal surfaces free from deposited particles. It has been proposed that Radio-labeled ultrafine carbon black may translocate through the respiratory epithelial layer to reach the lung interstitium or the blood and lymph circulations, but this finding has been refuted by others (25, 26). Theoretically this could involve alveolo-capillary translocation via endocytosis, transcytosis, or unidentified cellular mechanisms (27). In nonphagocytic cells, these endocytic events are regulated by clathrin-coated pits and caveolae, as well as scavenger receptors (e.g., scavenger receptor SR-A) (27, 28). Caveolae are indentations of the plasma membrane decorated with caveolin-1 and are abundantly expressed on lung capillaries and type I alveolar cells. It has been hypothesized that inspiratory expansion and expiratory contraction of lung alveoli may lead to the opening and closing of the caveolae to assist macromolecular transport across the alveolar membrane (29). Although it has been suggested that caveolae and coated pits preferentially transport small and large particles, respectively, it is unclear whether this specificity exists *in vivo* (27). Surface coating of nanoparticles also needs to be considered in particle uptake. Albumin, lecithin, polyethylene glycol, polysorbital 80 or peptide attachments can enhance nanoparticle uptake into cells, whereas polyethylene glycol interferes in nanoparticle uptake in the liver (30). Particle coating may be of particular importance in the lung, where adsorption of epithelial lining fluid components and surfactant can influence particle interactions with epithelial cells (Fig. 2). Similarly, the state of particle aggregation or dispersion is important in cellular interactions as exemplified by the finding that, if nanoparticles are coated with lung surfactant before cellular incubation, the cellular fate differs from that of uncoated particles. The assessment of nanoma-

terial inorganic and organic coatings and state of aggregation are therefore important considerations in evaluating NM toxicity.

Animal and human studies suggest that alveolar translocation of nanoparticles leads to circulatory access and allows nanoparticles to distribute themselves throughout the body, including the vasculature, heart, liver, spleen, and bone marrow. However, the extent of extrapulmonary translocation is highly variable and depends on particle size, surface characteristics, and chemical composition. Particle access to the blood circulation and effects on the endothelium and vasculature could explain why exposure to ambient ultrafine particles has been associated with cardiovascular events such as heart attacks and cardiac rhythm disturbances (8, 25, 31).

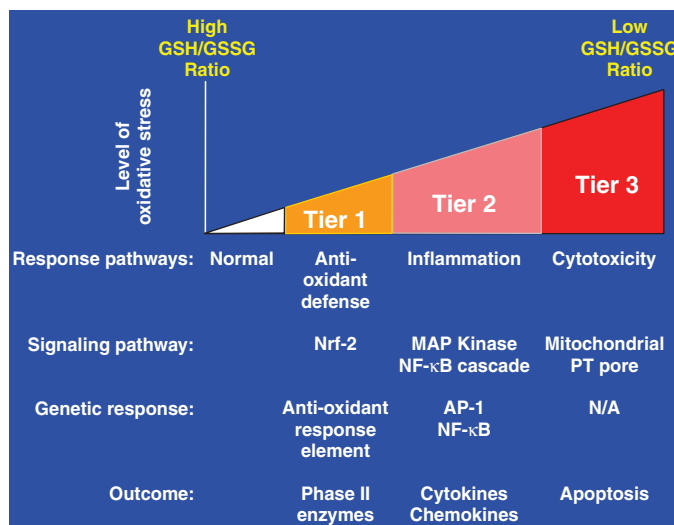


Fig. 3. The hierarchical oxidative stress model. At a lower amount of oxidative stress (tier 1), phase II antioxidant enzymes are induced via transcriptional activation of the antioxidant response element by Nrf-2 to restore cellular redox homeostasis. At an intermediate amount of oxidative stress (tier 2), activation of the MAPK and NF-κB cascades induces pro-inflammatory responses. At a high amount of oxidative stress (tier 3), perturbation of the mitochondrial PT pore and disruption of electron transfer results in cellular apoptosis or necrosis. [Adapted from (11)] N/A means not applicable.

Dermal exposure to NM occurs regularly during the use of sunscreen products, for example, TiO₂ and ZnO nanoparticles, that are often coated for minimizing their reactivity while maintaining their UV absorption properties. In healthy skin, the epidermis provides excellent protection against particle spread to the dermis. However, flexing of normal skin facilitates the penetration of micrometer-size fluorescent beads to the dermis (32). Damaged skin also allows micrometer-size particles access to the dermis and regional lymph nodes. *In vivo* imaging using intradermally injected quantum dots has been used to confirm particle trafficking to regional lymph nodes in animals (33). Such trafficking could deliver the par-

ticles to paracortical areas in the lymph nodes where macrophages and dendritic cells (DC) specialize in the uptake of particulate matter. This could lead to effects on the immune system.

The likelihood of immune perturbation by nanoproductions is unknown. Although the reticulo-endothelial system, which is composed of phagocytic cells in the liver, spleen, and lymph nodes, clears or sequesters nanoparticles, self-protein interactions with particles may change their antigenicity and initiate autoimmune responses. Nanoparticle-protein complexes are also more mobile and may facilitate antigen uptake by DC. This can lead to boosting of primary and secondary immune responses by changing the antigen presentation function of DC. For instance, diesel exhaust and other ambient particles act as adjuvants that, through their impact on DC function, lead to an exaggerated immune response to common environmental allergens (34). Lastly, there is the possibility that the immune system directly recognizes NM, as exemplified by the antibody response to C60 in mice injected with albumin-conjugated fullerenes (35).

NM can be ingested directly in food, water, cosmetics, or drugs. Alternatively, nanoparticles cleared via the mucociliary escalator in the respiratory tract can end up in the GIT. Although nanoparticles in food are infrequently taken up into gut lymphatics and distributed to other organs, most nanoparticles are rapidly eliminated via feces. For instance, radioactive iridium nanoparticles do not show substantial GIT uptake, whereas ingestion of water-soluble radio-labeled C60 fullerenes in rats show a 98% clearance in the feces (36, 37). In contrast, 90% of intravenously administered radio-labeled fullerenes are retained for at least a week, with >70% lodging in the liver.

The potential for nanoparticle translocation to the brain via olfactory nerve endings in the nose has recently been reported (38). The close proximity of the nasal olfactory mucosa to the olfactory bulb may facilitate neuronal uptake. Earlier studies showed that the olfactory nerve and olfactory bulb are indeed portals of entry into the primate brain by viral or metal nanoparticles instilled in the nose. Whether nanoparticles in the brain have any pathological or clinical significance is uncertain.

Although no clinically relevant toxicity has yet been reported, it is too early to draw meaningful conclusions about the inherent dangers of engineered NM. It remains to be determined whether the unique physicochemical properties of NM will introduce new mechanisms of injury and whether these will result in new pathology. Generally speaking, biological systems are able to integrate

multiple pathways of injury into a limited number of pathological outcomes, such as inflammation, apoptosis, necrosis, fibrosis, hypertrophy, metaplasia, and carcinogenesis (Table 2). However, even if NM do not introduce new pathology, there could be novel mechanisms of injury that require special tools, assays, and approaches to assess their toxicity (Table 2).

As NM Are Being Introduced as Commercial Products, What Should Be Done to Ensure That They Are Safe?

In contrast to the debates on nuclear power and genetically altered food, the public does not yet view nanotechnology as a noteworthy hazard. However, this position could change rapidly with media interest in this topic. Now is an opportune time to inform the public and to establish the principles and procedures that will ensure the safety of this technology for workers, consumers, and the environment. Because of the wide range of nanoproducts in use or under development, it is important to establish which materials should be tested first and how to perform this testing. NM that are near commercialization and are produced in large quantities as freely dispersible nanoparticles, with the potential of substantial exposures in humans and the environment, should probably be given preference. It is also important for regulatory agencies to develop positive and negative benchmarks that can be used as reference controls. On the basis of current understanding, the traditional study methods

for testing chemical toxicity are a good starting point for NM testing. However, given the unique characteristics of NM, this will necessitate new test strategies to delineate the novel mechanisms of injury that may arise from these materials (Table 2). More refined approaches for NM characterization and toxicological evaluations will emerge with time, for example, use of nanosensors to detect ROS generation by nanoparticles. This could make these evaluations cost effective, facilitating new product development.

What type of NM testing should be performed? The National Toxicology Program (NTP) in the United States has been established as an interagency program to evaluate chemical agents that are of public health concern by implementing modern toxicology tools. [Other governmental agencies, such as the Environmental Protection Agency (EPA) and the National Institute of Occupational Safety and Health (NIOSH) also have important roles in assessing nanomaterial safety in the United States, which will not be discussed here]. Although it is still questionable whether NM should be treated as commercial or industrial chemicals, the preferred NTP approach to chemical toxicity is a predictive scientific model that focuses on target-specific, mechanism-based biological observations, rather than a descriptive approach (39). Briefly, this strategy makes use of existing data, if available, to attempt to classify a material at the outset as hazardous or not. If a potentially hazardous chemical is nominated for study, a

specific test strategy for that chemical is developed that takes into consideration the existing data at the time of nomination. For instance, if the evidence is suggestive of pulmonary toxicity, a test strategy is used to study those effects on tissue culture cells of lung origin *in vitro* and in the lungs of live animals. *In vitro* assays allow specific biological and mechanistic pathways to be isolated and tested under controlled conditions in ways that are not feasible by using *in vivo* studies. Ideally, the studies are conducted in combination with *in vivo* studies that reveal a link of the mechanism of injury to the pathophysiological outcome in the target organ (Table 2). *In vivo* studies make use of animal models, including different time-length exposures (39).

While the same approach can work for NM testing, it is important that the design be pragmatic and mechanism-based. The demand for a predictive and pragmatic approach becomes clear when we consider that, among the 80,000 chemicals that are currently registered for commercial use in the United States, only 530 have undergone long-term and 70 short-term testing by the NTP. Moreover, the resource-intensive nature of these studies puts the cost of each bioassay at \$2 to \$4 million and takes over 3 years to complete. Thus, although it is optimal to collect data at different tiers of toxicity, some flexibility is required to develop decision matrixes for *in vitro* and *in vivo* testing. Ultimately, the goal of the predictive approach would be to develop a series of toxicity assays that can limit the demand for *in vivo* studies, both from a cost as well as an animal-use perspective. Much can be learned from research into the adverse health effects of ambient PM, where progress was slow until major mechanistic hypotheses were introduced. Armed with the knowledge that particle size, surface area, and chemical composition are important for ROS generation as a key toxicity principal, it has become easier to design *in vivo* studies in at-risk populations (8). The extent to which this or other paradigms of injury (Table 2) apply to a wide range of NM needs to be determined.

Although it is not possible to provide detailed protocols for nanotoxicity testing here, it will suffice to mention that the three key elements of a toxicity screening strategy should include physicochemical characterization of NM, *in vitro* assays (cellular and noncellular), and *in vivo* studies (40). There is a strong likelihood that biological activity will depend on physicochemical characteristics that are not usually considered in toxicity screening studies. Thus, any test paradigm must attempt to characterize the test material with respect to size (surface area, size distribution), chemical composition (purity, crystallinity, electronic properties, etc.), surface structure (surface reactivity, surface groups, inorganic/organic coatings, etc.), solubility, shape and aggregation. This should be done at the time of NM administration as well as at the conclusion, if possible. It is beyond the scope of this paper to discuss the scientific methods for NM character-

Table 2. NM effects as the basis for pathophysiology and toxicity. Effects supported by limited experimental evidence are marked with asterisks; effects supported by limited clinical evidence are marked with daggers.

Experimental NM effects	Possible pathophysiological outcomes
ROS generation*	Protein, DNA and membrane injury,* oxidative stress†
Oxidative stress*	Phase II enzyme induction, inflammation,† mitochondrial perturbation*
Mitochondrial perturbation*	Inner membrane damage,* permeability transition (PT) pore opening,* energy failure,* apoptosis,* apo-necrosis, cytotoxicity
Inflammation*	Tissue infiltration with inflammatory cells,† fibrosis,† granulomas,† atherogenesis,† acute phase protein expression (e.g., C-reactive protein)
Uptake by reticulo-endothelial system*	Asymptomatic sequestration and storage in liver,* spleen, lymph nodes,† possible organ enlargement and dysfunction
Protein denaturation, degradation*	Loss of enzyme activity,* auto-antigenicity
Nuclear uptake*	DNA damage, nucleoprotein clumping,* autoantigens
Uptake in neuronal tissue*	Brain and peripheral nervous system injury
Perturbation of phagocytic function,* "particle overload," mediator release*	Chronic inflammation,† fibrosis,† granulomas,† interference in clearance of infectious agents†
Endothelial dysfunction, effects on blood clotting*	Atherogenesis,* thrombosis,* stroke, myocardial infarction
Generation of neoantigens, breakdown in immune tolerance	Autoimmunity, adjuvant effects
Altered cell cycle regulation	Proliferation, cell cycle arrest, senescence
DNA damage	Mutagenesis, metaplasia, carcinogenesis

ization except to comment that standard reference materials (e.g., TiO₂, carbon black, quartz) are essential to compare material behavior. Cellular assays should reflect portal-of-entry toxicity in lungs, skin, and mucus membranes as well as noxious effects on target tissue such as endothelium, blood cell elements, spleen, liver, nervous system, heart, and kidney. Noncellular assays could include protein interactions and pro-oxidant activity. The *in vivo* studies can make use of disease-specific animal models that assess portal of entry and target organ injury, as well as animal models in which live imaging can be used to show the activation of oxidative stress and redox signaling pathways that are involved in particle-induced tissue injury (Fig. 3). When *in vivo* toxicity is observed, it may also be appropriate to proceed with studies that formerly assess the absorption, distribution, metabolism, and elimination of NM. Because NM have the potential to spread beyond the portal of entry, it is important to assess systemic responses. Examples include assays for oxidative stress (e.g., lipid peroxidation), C-reactive protein, immune and inflammatory responses, and cytotoxicity (e.g., release of liver enzymes and glial fibrillary acidic protein). The biological studies can be strengthened by the use of discovery tools such as proteomics and genomics to develop biomarkers for toxicity screening (12).

As testing proceeds, it will be important to incorporate these data into a knowledge base that allows investigators to classify NM as safe or possibly hazardous. Negative data should be reported to show which materials are devoid of toxic effects. This could represent the majority of NM.

Potential difficulties may be encountered in conducting *in vitro* and *in vivo* studies with engineered NM. These include problems with dosimetry, state of agglomeration (singlets versus aggregates), impact of material coating, and lack of knowledge of real-world exposures to NM. Detection methods need to be developed for exposure assessment and dosimetry calculation. Current state-of-the-art methods to detect airborne nanoparticles should enable personal monitoring devices to be developed to assess these exposures. The position is more complicated for nanoparticles that are spread via water and, even more so, via soil. Major questions also remain how to detect nanostructures in biological tissues. To evaluate exposure-dose-response relationships, it is unclear whether the NM dose should be calculated as mass concentration, number concentration, or surface area. Where possible, it is advisable to use all three parameters. In evaluating oxidative stress injury, the most appropriate dose measure appears to be surface area, which likely reflects the number of active sites at which ROS production can take place. Successful evaluation of dose-response relationships requires an understanding of NM biokinetics, as well as developing models that reconcile experimental with *in vivo* dose amounts.

While engineered NM clearly represent a unique class of materials with many novel and unique physicochemical properties that could impact biological systems, it is still too early to define what hazards and risks these materials may pose. For noxious chemical substances, hazard is directly related to toxicological effects in humans and the environment. For NM it is important to consider that their unique biological properties may differ from the base materials or chemical compounds from which they are manufactured. It is still being debated whether engineered NM should receive unique identifiers for toxicological and regulatory purposes. Risk assessment takes into consideration hazard as well as exposure and is accomplished through epidemiological studies and performance of exposure modeling. Risk assessment is of key importance to the insurance industry as well as the regulatory agencies that are responsible for formulating exposure and safety guidelines. It is important for law- and policymakers to keep in mind that when scientific research or new technologies raise concern, there is often the tendency to overreact with new rules and regulations. When considering regulation of engineered NM due to concerns about adverse health and environmental effects, it is recommended that the decision be based on scientific evidence of toxicity, which preferably should consider specific products or product lines and the likelihood of an exposure risk. It is recommended that lawmakers make these decisions in consultation with the evaluating scientists, regulatory agencies, academia and industry. This could involve the establishment of a special international working committee or coalition to achieve this goal.

Issues regarding safe handling of potentially toxic NM, including questions of whether personal protective equipment is effective for protection against NM exposures, have not been solved. This problem relates to the uncertainty about the real-life NM hazards and how to demonstrate that a protective measure is effective. In the absence of quantitative information, a good approach is to start with standard hygiene procedures, including gloving, protective clothing, and highly efficient respirators capable of removing nanoparticles, and to move ahead as new information becomes available. As toxicological data become available, these should be used to develop material safety data sheets that inform workers and consumers of possible NM hazards, including safe handling procedures. Other important questions relate to disposal of NM and spill remediation. Although very little is currently known about this area, it is probably wise to regard NM waste as potentially hazardous until proven otherwise.

Summary

Although it is possible that engineered NM may create toxic effects, there are currently no conclusive data or scenarios that indicate that these effects will become a major problem or that

they cannot be addressed by a rational scientific approach. At the same time, we can no longer postpone safety evaluations of NM. A proactive approach is required, and the regulatory decisions should follow from there. In addition to facilitating the safe manufacture and implementation of engineered nanoproducts, an understanding of nanotoxicity could also have a positive sequel. For instance, the propensity of some nanoparticles to target mitochondria and initiate programmed cell death could be used as a new cancer chemotherapy principle.

References and Notes

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41. We apologize for the many seminal contributions that have not been cited for lack of space. This work was supported by U.S. Public Health Service grants RO-1 ES10553, RO1 ES12053, RO1 ES013432, and PO1 AI50495 (UCLA Asthma and Immunology Disease Center), and by the U.S. EPA (STAR grant to the Southern California Particle Center and Supersite). This manuscript has not been subjected to the EPA's peer and policy review. L.M. was supported by the Parson's chair at UCLA.

10.1126/science.1114397