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Research Strategies for Safety Evaluation of Nanomaterials, Part II: Toxicological and Safety Evaluation of Nanomaterials, Current Challenges and Data Needs

Michael P. Holsapple,* William H. Farland,† Timothy D. Landry,‡ Nancy A. Monteiro-Riviere,§ Janet M. Carter,¶ Nigel J. Walker,|| and Karluss V. Thomas|||,[1]

*ILSI Health and Environmental Sciences Institute, One Thomas Circle, NW, 9th Floor, Washington, District of Columbia 20005; †Office of Research and Development, US Environmental Protection Agency, 1200 Pennsylvania Ave, NW, Washington, District of Columbia 20460; ‡Toxicology and Environmental Research and Consulting, The Dow Chemical Company, Building 1803, Midland, Michigan 48674; §Center for Chemical Toxicology Research and Pharmacokinetics, North Carolina State University, 4700 Hillsborough Street, Raleigh, North Carolina 27606; ¶Central Product Safety, Procter & Gamble Co., Miami Valley Labs, PO Box 538707, Cincinnati, Ohio 45253; ||National Institute of Environmental Health Sciences, 11 Alexander Drive, Research Triangle Park, North Carolina 27709; and |||ILSI Health and Environmental Sciences Institute, One Thomas Circle, NW, 9th Floor, Washington, District of Columbia 20005

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This article summarizes a roundtable discussion held at the 2005 Society of Toxicology Annual Meeting in New Orleans, LA. The purpose of the roundtable was to review the current challenges and data needs for conducting toxicological and safety evaluations for nanomaterials, with the goals of presenting the current state-of-the science on the safety of nanomaterials and bringing together scientists representing government, academia, and industry to identify priorities for developing data to facilitate risk assessments for these materials. In this summary, the unique physicochemical properties associated with nanomaterials are reviewed in the context of the difficulties associated with measuring and characterizing them. In addition, the development of appropriate hazard data, the collection of accurate human and environmental exposure information, and the development of a better fundamental understanding of the modes of action for nanomaterials are discussed as factors that will impact the development of comprehensive toxicological and safety evaluations.

Key Words: **nanomaterials; nanoscale materials; nanotechnology; risk assessment; toxicology.**

While many definitions exist for nanotechnology, the National Nanotechnology Initiative (NNI) defines it as including all of the following: (1) research and technology development at the atomic, molecular, or macromolecular levels in the length scale of approximately the 1- to 100-nanometer range; (2) creating and using structures, devices, and systems that have novel properties and functions because of their small and/or intermediate size; and (3) the ability to control or manipulate on the atomic scale.

There is very little information available regarding the safety of manufactured ''nanomaterials,'' which for the purposes of this article will include nanoparticles, nanotubes, nanowires, fullerene derivatives, and other nanoscale materials. Environmental and other safety concerns about nanotechnology have previously been raised (Dagani, 2003; Masciangioli and Zhang, 2003; Service, 2003). Effects resulting from the use of nanotechnology might arise as a result of the chemical composition of the nanoparticles, the characteristics of the products made from them, or aspects of the manufacturing processes that are used to generate them. The large surface area and the associated increased reactivity of some nanoparticles may facilitate broad transport in the environment as a result of greater persistence, or they may impact biological systems from interactions with cellular material. In the case of nanomaterials, size matters and could facilitate and exacerbate effects caused by the composition of the materials themselves.

Some research and testing has been done on inhalation and dermal exposure to nanoparticles and other ultrafine particles. However, the current research on ultrafine particles may not be applicable to the evaluation of the safety or risk from manufactured nanoparticles, because the ultrafine materials that have been studied are neither a consistent size nor pure in chemical or structural composition.

In order to develop the data that will be required to generate risk assessments and safety evaluations for nanomaterials, more research is needed to identify validated methods and

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[1] To whom correspondence should be addressed. Fax: (202) 659-3859. E-mail: kthomas@ilsi.org.

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techniques for characterizing and testing them. In addition, information that helps to elucidate the mechanisms of action for these materials will provide more insight into the hazards associated with them. Exposure data characterizing realistic exposure scenarios for nanomaterials are essential for the development of risk assessments that will adequately inform public health decision making.

Inhalation Exposure Route for Evaluating Nanomaterials

An improved fundamental understanding of the behavior of airborne nanomaterials is critical for the development of accurate exposure assessments. There are considerable data available on aerosol generation, distribution, and deposition based on the aerodynamic diameter of small particles. Much less is known about the disposition and fate of particles in the nanoscale range (<100 nm) in the body. Aerosolization of primary nanoparticles is likely limited to combustion and spray dispersion processes. Mechanical grinding processes have low yields of particles in the nanoscale range, though aerosolization of micron-sized aggregates or agglomerates is possible. Cohesive forces (between nanoparticles) and adhesive forces (with surrounding media) strongly influence the state and fate of the primary particles in gas, liquid, or solids. Airborne discrete nanoparticles predominantly move by convection and diffusion. Particles in this size range deposit in the respiratory tract predominantly by diffusion (James *et al.*, 1991).

Once deposited, nanoparticles may cross biological membranes and access tissues that would not normally be exposed to larger particles. Ferin *et al.* (1992) reported preferential translocation of nanoscale titanium dioxide (TiO_2) particles into lung interstitium, and Semmler *et al.* (2004) reported on the kinetics of iridium, including translocation to secondary organs. Aggregates of nanoparticles would likely be subject to normal macrophage clearance mechanisms. Indeed, Oberdorster *et al.* (1992) demonstrated that following intratracheal instillation, ultrafine TiO_2 particles were phagocytized by alveolar macrophages, which prevented both the pulmonary inflammatory reaction and the interstitial access of the ultrafine particles. However, inflammation has been observed with some nanoparticles in the respiratory tract, likely reflecting the relatively large surface area. Bermudez *et al.* (2004) demonstrated accentuated inflammation with ultrafine TiO_2. In that study, inhalation of 10 mg/m^3 of TiO_2 for 13 weeks resulted in pulmonary overload in rats and mice with inflammation similar to that seen with higher mass doses of fine TiO_2.

Effects beyond the respiratory tract may also occur from exposure to nanomaterials. Advances in this area have been made with studies on ultrafine particles associated with air pollution. Dockery *et al.* (1993) studied air pollution effects and reported that increased mortality was most strongly associated with fine particulates. This observation led to a number of studies on respiratory and cardiovascular effects. Oberdorster *et al.* (2005) provided an overview of this area in relation to nanoparticles, noting work describing their fate in the lungs and potential pro-inflammatory and oxidative stress-related cellular responses.

An understanding of the exposure and effects of discrete nanoparticles is important information that toxicologists need to fully understand nanoscale materials. However, with the broad applications of nanotechnology, a simple focus on discrete nanoparticles is not adequate. Cohesive forces maintain nanoparticles as aggregates and agglomerates, markedly affecting their propensity to become airborne, as well as their aerodynamic diameter. Uses of nanomaterials in liquids and composites may severely limit or preclude airborne exposure. Disaggregation, deagglomeration, and dissolution in biological fluids are important factors potentially contributing to a complete understanding of nanoparticle fate. Although inhalation is a critical route of exposure in some cases, a full understanding of exposure and fate will require an assessment of the technology applications and physical state of the nanomaterials. Exposures to respirable aggregates of primary particles may be relevant in some cases, while in others it may not be relevant. Key questions remain in determining what conditions will lead to potential exposure, as well as determining the relevant metrics (concentration, and also perhaps particle number, surface area, and surface characteristics). Routine histopathology of major tissues and organs may prove to be a good basis for assessment of experimentally exposed animals, though other endpoints may also be useful to consider.

Dermal Exposure Route for Evaluating Nanomaterials

Skin is a complex dynamic organ that has several functions, the primary one being to act as a barrier to the external environment. The skin is the largest organ of the body and serves as a primary route of environmental and/or occupational exposure; it is one of the principal portals of entry by which environmental toxicants or nanomaterials can enter into the body. At present, there is no information on whether nanoparticles can be absorbed across the stratum corneum barrier or whether systemically administered particles can accumulate in dermal tissue. Skin is unique because it provides an environment within the avascular epidermis where particles could potentially lodge and not be susceptible to removal by phagocytosis. The ability for nanomaterials to traverse the skin is a primary determinant of their dermatotoxic potential. That is, nanomaterials or nanoparticles must penetrate the stratum corneum in order to exert toxicity in lower cell layers. The quantitative prediction of the rate and extent of percutaneous penetration (into skin) and absorption (through skin) of topically applied nanomaterials is complicated because the processes driving nanoparticles into skin may be different from

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those governing chemicals. Anatomically, nanomaterial absorption may occur through several routes, as the majority of lipid-soluble particles may move through the intercellular lipid pathway between the stratum corneum cells (intercellular), through the cells (transcellular), or through the hair follicle or sweat ducts (transappendageal).

One of the major decisions to be faced in assessing the skin absorption and toxicity of nanomaterials is how to conduct the experiments. Should *in vitro* cell cultures, flow-through diffusion cells, or perfused skin model systems be used? *In vivo* studies conducted in rat or preferably pig skin (since it is anatomically, physiologically, and biochemically similar to man) would be ideal. However, there are limitations in obtaining the quantity and quality of some nanomaterials to conduct *in vivo* studies. Therefore, in some cases it may be best to study their interactions *in vitro* in order to estimate the *in vivo* starting dose for toxicity. *In vitro* studies have shown that multi-walled carbon nanotubes (not derivatized or optimized for biological applications) are capable of localizing within and initiating an irritation response in human epidermal keratinocytes, which are a primary route of occupational exposure (Monteiro-Riviere *et al.*, 2005).

Ultimately, the experimental design of these studies must consider the fact that the absorption of nanomaterials may not be similar to chemical absorption, because nanoparticles are made of different materials, such as carbon, cadmium, and heavy metals, and are of different sizes, such as quantum dots and clusters. Furthermore, nanoparticles occur in many shapes, including single- or double-walled tubes, crystals, and spheres, in a range of surface modifications, and are prepared in different vehicles. All of these properties will affect how they can traverse through the stratum corneum. Some of the major questions that need to be answered include the following:

- Can manufactured nanoparticles gain access to the epidermis? Would such particles preferentially locate in the lipids of the stratum corneum after topical exposure? As noted above, there is evidence that keratinocytes exposed to nanomaterials can elicit an early inflammatory response. Can nanoparticles gain access to tissue spaces, a prerequisite for systemic toxicity? Does the concept of partitioning, central to predicting chemical absorption, apply to nanoparticles?
- What are the toxicological consequences of ''dirty'' nanoparticles (catalyst residue) becoming lodged in the epidermis? It is the relative biological isolation in the lipid domains of the epidermis that has allowed for the delivery of drugs to the skin using lipid nanoparticles and liposomes. Larger particles of zinc and titanium oxide used in topical skin-care products are able to penetrate the stratum corneum barrier of rabbit skin, with the highest absorption occurring from water and oily vehicles (Lansdown and Taylor, 1997), and this should also apply to manufactured nanomaterials.
- What are the potential considerations for exposure to metallic nanoparticles? The physical properties of these materials would allow them to catalyze a number of biomolecular interactions, which potentially could produce adverse toxicological effects. The difference between nanoparticles and ''traditional'' hazardous chemical exposure is that decontamination of nanoparticles would be significantly more difficult than that of chemicals, because solubilization or dilution, the two hallmarks of post-exposure decontamination, might be less efficacious for these solid structures.
- Ultimately, what data are needed for defining the two essential components of any risk assessment: systemic exposure after topical administration and cutaneous hazard after topical or systemic exposure? If carbon nanoparticles are accidentally modified, or if exposure occurs before cleansing, this could have untoward consequences if they gain entry to tissues.

A single study will not definitively answer all of the pertinent questions relative to dermal risk assessment of nanomaterials, but studies addressing the above questions should be able to provide an insight into the nature of the potential hazards of nanoparticles, and an initial estimate of dermal exposure parameters that can be used to design more definitive studies.

Nanomaterial Hazard Identification

Although engineered nanoparticles have not been systematically tested, a few inhalation and epidemiology studies using ambient ultrafine particles have yielded some results from which preliminary conclusions can be drawn (Oberdorster and Utell, 2002). Additionally, there are a few limited toxicology studies that have addressed the effects of nanomaterials in a variety of organisms and environments (Oberdorster, 2004; Yamakoshi *et al.*, 1999). Pharmaceutical applications have also been a source of information regarding the potential translocation of nanoparticles from the site of exposure to distal areas of the body (Cui and Gao, 2003; Weber, 1999). Therefore, several studies using nanoparticles have shed some light on the kinetics and distribution of nanomaterials once inhaled or ingested.

Commonly employed human and environmental toxicity testing approaches have been applied to assess ultrafine materials. Numerous studies evaluating potential inhalation, oral, and dermal toxicity have been conducted on nanomaterials such as carbon particles, silica, fullerenes, magnesium oxide, zinc oxide, and titanium dioxide (Baggs *et al.*, 1997, Beckett *et al.*, 2005, Bermudez *et al.*, 2004, Conner *et al.*, 1988, Dick *et al.*, 2003, Driscoll *et al.*, 1996, Ferin *et al.*, 1992, Jani *et al.*, 1994, Johnston *et al.*, 2000, Kuschner *et al.*, 1997, Nelson *et al.*, 1993, Oberdorster *et al.*, 2000, Pflucker *et al.*, 2001, Schulz *et al.*, 2002, Shvedova *et al.*, 2003). As our understanding of the toxicology of nanoparticles continues to grow, these standard toxicology tests will allow for better comparisons and conclusions in determining their effects.

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In the testing of nanomaterials, there needs to be an emphasis on the characterization of the materials themselves. Essential parameters to consider for material characterization should include such physicochemical properties as size distribution, agglomeration state, crystalline structure, chemical composition, and shape. Appropriate controls such as including micron-sized materials of like chemistry and benchmark materials will need to be included in tests, at least until a sufficient database has been generated to address the question of whether (and if so, how) nanoparticle toxicity differs from that of larger particles. Toxicity studies on engineered nanomaterials such as fullerenes, single-walled carbon nanotubes, multi-walled carbon nanotubes, and nanoscale metal oxides such as TiO_2 and nanometer-diameter low-solubility particles support the need to carefully consider how nanomaterials are characterized when evaluating their potential biological activity (Brown *et al.*, 2000, 2001; Monteiro-Riviere *et al.*, 2005; Shvedova *et al.*, 2003; Warheit *et al.*, 2004; Yamago *et al.*, 1995).

The appropriate route of exposure and the appropriate endpoints need to be considered in the design of toxicity studies for nanomaterials. One of the most important portals of entry for nanoparticle exposure could be the gastrointestinal tract. Uptake of nanoparticles via the gastrointestinal tract has been documented in oral feeding and gavage studies using particles ranging from 10 to 500 nm (Hillyer and Albrect, 2001; Jani *et al.*, 1994). As discussed above, inhalation is another important route of exposure for many nanomaterials. Effects from inhalation of micron-sized particles are generally restricted to the lung, or portal of entry, and have no systemic distribution. Therefore, traditional inhalation toxicology studies have been typically restricted to studying effects directly related to the lung when studying the effects of particles. However, the lung is a major route of exposure for gases, vapors, and liquid aerosols that can produce systemic exposures, so it is not uncommon for inhalation studies to include systemic evaluations. Because more is known today regarding the kinetics and distribution of nanoparticles, functional endpoints can be expanded beyond the traditional route of entry effect to include systemic effects.

Determining the appropriate dose is crucial in evaluating the true risk of these materials. Overload conditions or exceeding the maximum tolerated dose should be avoided. In determining appropriate dose, the use of exposure levels relevant to human and environmental exposures should be utilized.

A tiered approach can be implemented for evaluating nanomaterials. *In vitro* testing, followed by escalation to more complex testing models, may render useful information in the evaluation of these materials in lieu of chronic bioassays. Short-term mechanistic studies, *in vitro* studies, and ultrafine-particle epidemiological studies can provide important enhancements to traditional inhalation toxicity assays. Integrating this information increases our confidence in the hazard identification of nanoparticles, and when coupled with exposure considerations, the risk assessment of nanomaterials.

Mode of Action Considerations for Nanoscale Materials

The characterization of the potential health impact of engineered materials produced through nanotechnology is an emerging issue of considerable discussion and debate. Determination of the mode of action (MOA) for effects observed with nanomaterials will be a key issue in understanding data obtained from toxicological evaluations and its extrapolation for the determination of potential human health risk.

Some of the key MOA considerations for nanoscale materials include: (1) do their unique physicochemical properties translate into unique MOAs?; (2) what are the best experimental strategies to obtain data that can identify key events with which to evaluate these MOAs?; (3) can evaluation of a core set of parameters and/or model materials be used to determine MOAs that can be applied to emerging nanomaterials?

Nanomaterials cannot simply be considered as a single homogeneous class. In addition, our current understanding of the MOA of nanomaterials is very limited, since research to date has only been conducted on a few example materials. There are a number of parameters that will be key to understanding the MOA for a given material, including the size/shape/aspect ratio, hardness/deformability, composition, surface area and surface chemistry, types of coatings/modifications, and stability. Given that nanomaterials vary greatly based on these parameters, extrapolation of a MOA from one material to another will need to be made with extreme caution until more knowledge is gained on a broader class of materials.

Much of the current concern for potential toxicological effects of materials produced through nanotechnology is based on a paradigm founded in the toxicology of inhaled particles. While MOAs associated with pulmonary toxicity may be applicable to some nanomaterials, it is likely that there will be others, and that multiple modes may occur for the same material. Some modes will be defined by perturbations at the cellular and molecular level, and will be driven by the composition of the nanomaterial/coating/modification itself, with other parameters regulating access of the molecular species to a potentially sensitive target site. Conversely, some modes will be driven by the morphology of the material and the physical interaction of the material with its biological environment. It is clear we cannot separate issues of pharmacokinetics from potential MOAs, since one of the unique aspects of nanomaterials compared with their bulk counterparts is a difference in absorption and distribution and clearance as a result of size and coating variations.

The field is currently too much in its infancy to be able to predict modes based on physiochemical parameters, and therefore identification of MOAs will come through research and evaluation of the action of these species in suitable experimental models. Given that nanomaterials fall between molecular species and physical entities, the diversity of parameters

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that could contribute to a MOA need to be fully evaluated so that the associations of effects to a given parameter are valid. Adequate characterization of materials in an experimental system through collaborations and partnerships with analytical chemists is therefore a key issue in this field.

In this context, there is a real concern that inadequate material characterization may lead to the identification of toxicities and potential MOAs that have little to do with the nanomaterial under investigation. Because of this concern, some have called for the development of standard/reference nanomaterials for use by the toxicology research community. While this approach would be beneficial for the evaluation of those specific materials, the diversity of these standards will need to be sufficiently broad so that the extrapolation of these findings to a larger class of materials can be fully evaluated. An immediate benefit of implementing this approach would be the development of minimum standards for reporting the characterization of nanomaterials in publications, in much the same way that minimal standards were recently adopted for the reporting of microarray and toxicogenomics data.

Summary and Conclusions

The science of toxicology has always provided the foundation for understanding the interactions between chemistry and biology. While the use of nanomaterials is relatively new in commercial products, the philosophical basis for performing a toxicological evaluation of these materials is not expected to be different from other materials. A basic tenet of any study designed to develop an understanding of the toxic effects of a material on biological systems is to understand the physicochemical properties of that material. Consequently, the unique physical-chemical characteristics of engineered nanomaterials that lead to their distinctive properties will likely also contribute to the hazards associated with these materials. Therefore, the approach to addressing the safety of these materials will best be conducted via multidisciplinary teams. While this suggestion is certainly not unique to toxicology, addressing the safety of nanomaterials will require significant contributions from the chemists who developed them because of the critical role that full material characterization will play in the interpretation of the studies to address hazard and characterize exposure.

Scientists must also accept that it is still very early in the toxicological evaluation and characterization of the safety of nanomaterials, and there are few data on the safety of nanomaterials at the present time. There is no question that this situation is rapidly changing. As noted by Thomas and Sayre (2005), the Federal agencies participating in the NNI funded an estimated $106 million in research on the health and environmental aspects of nanomaterials in 2004. There is also little debate over the fact that even though it is recognized that nanomaterials exhibit unique properties that clearly distinguish them from their bulk counterparts, many of the methods, tests, assays, and principles that have been the cornerstones of traditional approaches to safety assessment can also be applied to the design of the studies characterizing nanomaterial safety.

There is a good foundation on how to proceed with characterizing the safety of nanomaterials that has been developed through several years of studying materials such as fine and ultrafine particles, increasing understanding of the lungs and the skin as portals of entry and as potential target organs, and improving approaches to characterize the important roles played by absorption, distribution, metabolism, and excretion. It is generally thought that it is unlikely that nanomaterials will manifest new toxic manifestations in spite of their unique physical-chemical properties. Therefore, many traditional methods and approaches will likely be applicable to studies of nanomaterials, especially if it is acknowledged that not all nanoscale materials are the same and that potential impacts due to the unique properties of these materials are to be expected.

It is clear that toxicologists must apply the very best science in characterizing the safety of nanomaterials. The development of a comprehensive strategy to address nanomaterial safety must integrate the state-of-the-science for *in vitro* methods, and must include a consideration of MOAs at the onset. Understanding of the MOA for nanomaterials must be developed at the same time that information is compiled about the toxicity profile(s). This is in contrast to the retrospective approach that has been traditionally taken by toxicologists and/or risk assessors. It also appears clear that a diverse toxicological strategy that covers a continuum of effects from short-term *in vitro* tests to subchronic and chronic studies needs to be considered when evaluating nanomaterials. Whether this strategy can take on the characteristics of a tiered approach is being debated. However, there is no debate over the conclusion that no one study should be interpreted as definitive, and that the ultimate approach to the consideration of nanomaterial safety will depend on a consideration of the weight of evidence.

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