Nanomaterials in the Environment: From Materials to High-Throughput Screening to Organisms

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ABSTRACT One of the challenges in the field of nanotechnology is environmental health and safety (EHS), including consideration of the properties of engineered nanomaterials (ENMs) that could pose dangers to the environment. Progress in the field of nanomaterial development and nanotoxicology was presented at the International Conference on the Environmental Implications of Nanotechnology at the California NanoSystems Institute (CNSI) on the UCLA campus on May 11–14, 2010. This event was cohosted by the University of California Center for the Environmental Implications of Nanotechnology (UC CEIN) and the Center for the Environmental Implications of NanoTechnology (CEINT) based at Duke University. Participants included scientists and scholars from various backgrounds, including chemistry, biology, engineering, nanomaterial science, toxicology, ecology, mathematics, sociology, and policy makers. The topics of discussion included safety evaluation of ENMs from an environmental perspective, nanotoxicology, ecotoxicology, safe design of ENMs, environmental risk assessment, public perception of nanotechnology, application of ENMs in consumer products, and many more. The UC CEIN presented data on their predictive toxicological approach to the assessment of ENM libraries, which were designed and synthesized to develop an understanding of the material properties that could lead to hazard generation at the cellular and organismal levels in the environment. This article will focus on the first metal oxide ENM library that was introduced to harmonize research activities in the UC CEIN, with particular emphasis on the safety assessment of ZnO on cells and organisms. Methods of decreasing the observed toxic effects will also be discussed as an integral component of the UC CEIN’s activity in developing safer nanomaterials to lessen their environmental impacts.

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maintains balance between the rate of knowledge generation at the biomolecular and cellular levels versus more relevant but costly experiments in more complex organisms (e.g., microorganism relevant to the test). Thus, whereas traditional toxicological approaches study one material at a time, UC CEIN has implemented as one of its goals the development and introduction of high-throughput technology to expedite the toxicological assessment of ENMs.

In this focus article, we outline the progress we have made in the UC CEIN in developing ENM libraries and high-throughput screening (HTS) approaches that attempt to link hazardous material properties to possible adverse biological outcomes in more complicated life forms (e.g., mice or other animal models relevant to the test). This brief overview reports the progress that was made and presented at the annual international meeting in association with the Center for the Environmental Implications of NanoTechnology (CEINT). From the different classes of nanomaterials, we will focus on the hazard assessment of one metal oxide, ZnO. This material has received considerable attention from physical and biological scientists because of its high production volume and frequent use in consumer products. We will examine the characteristics of the nanoparticles and their toxic effects on cells and organisms as determined by HTS and individual organism screening and discuss the chemical methods of designing safer nanomaterials based on the associated mechanism of toxicity.

Figure 1. Strategy for determining the toxicity of various nanomaterials. Libraries of materials are first characterized to establish the properties of the materials. After HTS toxicity screening, the data can be analyzed and displayed as heat and self-organizing maps, with fluorescence signals seen in yellow and red corresponding to increased toxicity. At the same time, the mechanism of toxicity will be determined and linked to the physicochemical properties of nanomaterials. Nanomaterials are then prioritized with regard to further toxicity screening. Model organisms in various trophic levels will be used to examine the toxicity of nanomaterials. This information will be used to build the structure—activity relationships established using cell studies and confirmed in vivo; new materials can be synthesized based on safe design principles. These new materials are added to the combinatorial libraries, and tested to verify the hypothesized reduced toxicity.

Development of Compositional and Combinatorial Libraries To Explore Potentially Hazardous Nanomaterial Properties. With a view toward developing a predictive toxicological model, CEIN’s initial aim was to establish a small metal oxide library that would be introduced in the Center to harmonize protocols for ENM physicochemical characterization, implementation of HTS, fate and transport studies, and studies of the toxicological effects in different organisms and across aquatic versus terrestrial environments (Figure 1). Three metal oxides—zinc oxide, titanium dioxide, and cerium dioxide—were chosen for in-house synthesis as well as purchased from commercial sources because of their high production volume and frequent use in consumer products. These materials were distributed to the different working groups in UC CEIN for a wide range of research activities as was outlined at the conference by the Center Director, Prof. Andre Nel.

In order to develop structure—activity relationships, comprehensive characterization is needed to delineate the primary materials properties that should be considered in nanomaterial hazard assessments. Center members outlined the synthesis and characterization of the first compositional library. This included a description of the flame spray pyrolysis process that was used in-house by Dr. Suman Pokhrel in Prof. Lutz Mädler’s group at Bremen University, Germany, as well as commercial acquisition of large stocks of nanomaterials that could be used for the environmental studies. Dr. Zhaoxia Ji described the primary characterization of the metal oxides in their as-prepared form as well as under the exposure conditions for cellular, organism (e.g., zebrafish embryos), and more complex...
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If these unstabilized suspensions were used for nanotoxicity studies, they would lead to decreased signal-to-noise ratios and to incorrect dose estimations that could complicate interpretation of test results. Special attention was paid to select biologically and environmentally relevant dispersing agents to improve nanoparticle dispersion and stabilization in various media. Nontoxic, biologically relevant dispersants such as bovine serum albumin (BSA) and fetal bovine serum (FBS) emerged as the most promising candidates for biological studies in mammalian tissue culture media, while natural organic material (NOM) proved to be useful for the stabilization of nanomaterials in environmental model systems. Dr. Zhaoxia Ji described the development of a nanoparticle dispersion protocol comprising sonication and the use of stabilizing agents (Figure 2) in various biological media. The best approach in tissue culture media is to stabilize the nanoparticle by sonication in BSA and FBS, which coats the nanoparticle surface and decreases the hydrodynamic radius in comparison to that of nanoparticles not treated with a dispersant. Dr. Ji also demonstrated that the binding interactions of BSA with the metal oxide nanoparticle surface likely involves a Ca$^{2+}$ bond that is sensitive to interference by phosphate in the culture medium, leading one of the bacterial culture media with high phosphate content to interfere with the dispersal effect of BSA. The importance of particle stabilization during the performance of HTS was illustrated by Dr. Saji George, who showed the improvement of signal-to-noise ratios and enhanced dose—response assessment of proper particle dispersal.

(e.g., rodents) animal studies. The standard techniques for characterizing the as-prepared materials in their powder form included assessment of chemical composition, crystallinity, size, shape, surface area, etc., through the use of techniques such as X-ray diffraction, Fourier transform infrared spectroscopy, electron energy loss spectroscopy, transmission electron microscopy, high-resolution transmission electron microscopy, and BET methods. While these methods are key to interpreting structure—activity relationships, it was also necessary to characterize the nanoparticles under different exposure conditions, which included the use of mammalian tissue culture media, bacterial broth, yeast culture media, Holfreter’s medium for the study of zebrafish embryos, and environmental media representative of freshwater, seawater, and groundwater conditions. Particle suspension and stabilization in these media must include consideration of the effects of ionic strength, mono- and divalent cations, pH and the presence of buffers, proteins, natural organic matter (NOM), etc. All of these factors could contribute to the state of particle dispersion or agglomeration, which can exert important effects on the various assays to be developed, whether the encounter at the nano/bio interface is with nanoscale or larger material aggregates, as well as the types of organisms that will be exposed in mesocosm studies (e.g., benthic versus pelagic life forms). It has been shown that dispersions of nanomaterials agglomerate when introduced into several of these systems, and, in many cases, these large agglomerates also settle rapidly. Thus, additional ENM characterization methods were required for assessment of ENM size distribution, state of dispersion, suspension stability, change in the zeta potential, and assessment of dissolution characteristics and metal ion shedding. Dr. Zhaoxia Ji, Prof. Arturo Keller, Dr. Tian Xia, and Dr. Saji George delivered talks discussing these physicochemical characterizations and the use of standardized approaches for stabilizing ENM suspensions under different biological and environmental use conditions.

Figure 2. Dispersion protocol for ENMs in biological media. To ensure that nanomaterial samples are well-dispersed for accurate HTS toxicity screening, a high-throughput dynamic light scattering technique was used to examine the effects of various types of dispersants at various concentrations. The hydrodynamic radius of the particles is measured over time to verify the optimal conditions for well-dispersed ENM working solutions.
Unlike the use of a protein-based strategy for stabilization in tissue culture medium, NOM such as alginate was shown by Prof. Arturo Keller to decrease agglomeration and was capable of acting as a potential stabilizing agent for nanoparticle environmental fate and transport studies. Dr. Tian Xia further elaborated on the practical application of this observation in the performance of nanoparticle exposure studies in zebrafish embryos that are cultured in Holtfreter’s medium. Dr. Xia demonstrated that the metal oxides could be stabilized in suspension by the introduction of alginate as an environmentally relevant NOM. In performance of these environmental and tissue culture medium suspension studies, the use of high-throughput dynamic light scattering was shown to be a useful technique for rapid assessment of the suspension stability in comparison to the more conventional dynamic light scattering approach for assessing particle agglomeration size distribution.

In freshwater systems (e.g., rivers, stormwater, lakes), the presence of NOM and the relatively low ionic strength results in rather stable dispersions of these metal oxide nanoparticles (Figure 3a). Thus, organisms in the water column are more likely to be exposed to the nanoparticles. As the ionic strength increases, even the presence of natural NOM is not sufficient to disperse the nanoparticles; in marine and estuarine environments, we expect rapid aggregation and deposition, increasing the risk of exposure for benthic organisms (Figure 3b). The metal oxide nanoparticles can be stabilized with higher levels (~10 mg/L) of alginate, a naturally present organic in these environments. Such NOMs may be generated by cultured organisms themselves; however, bacteria can directly cause dispersion of ENMs through the affinity of ENMs to cell surfaces. This phenomenon, presented by Prof. Patricia Holden and her group and shown in both laboratory media and with natural estuarine waters, has far-reaching implications regarding nanoparticle hazard assessments. For nanoparticles that are stabilized in environmental media, potential risk increases due to the prolonged exposure.

Implementation of Toxicity Screening at the Cellular and Organismal Levels. Because of the need to develop simultaneous assessment of batches of ENMs rather than a single material testing, UC CEIN is developing HTS platforms premised on the use of biological injury paradigms at the cellular and organismal levels in order to generate knowledge rapidly about the hazard that ENMs might pose at the level of the nano/bio interface. This choice reflects our hypothesis that an understanding of ENM properties that engage injury pathways that also play a role in generating pathology or disease in vivo could constitute a robust platform for knowledge generation on which to base ENM hazard rankings and the subsequent prioritization of in vivo research. Dr. Saji George presented the approach of IRG5 toward establishing an HTS platform to assess the effects of metal oxides in mammalian tissue culture cells. The approach that was taken utilized a series of sublethal and lethal cellular responses that are triggered by a number of potentially hazardous ENMs, including nanoparticles that participate in the generation of reactive oxygen species (ROS) and oxidative stress, cationic injury, and shedding of toxic metal ions. Dr. George described the development of a multiparametric assay that utilizes a series of compatible fluorescent dyes that contemporaneously measure ROS production, intracellular calcium flux, mitochondrial membrane depolarization, and cell death leading to membrane disruption in 384-well plates. We chose to study the effect of ENMs initially in transformed macrophages and alveolar epithelial cell lines to develop the assay, which has subsequently also been implemented in primary cell types. Cellular seeding of the well plates, preparation of ENM working solutions, ENM additions to the assay plate containing target cells, and preparation and addition of analytes were carried out with automated liquid-handling devices in the CNSI HTS facility. Cells exhibiting differential
fluorescence signals depending on the extent of toxicity induced by each of the metal oxide nanoparticles were imaged using an epifluorescence microscope. Once the images were captured, the percent of affected cells for each toxicity end point was assessed automatically using software that analyzes each cell in the image. After analysis of the HTS data, the differential toxicity of ZnO nanoparticles was clearly indicated by multiparametric response generation that was reflected in the heat maps showing dose- and time-dependent response generation in a cell-specific manner. Moreover, Prof. Lutz Madler’s laboratory demonstrated the importance of ZnO dissolution in triggering sublethal and cytotoxic effects. In contrast, no significant toxicity was observed for TiO$_2$ and CeO$_2$ nanoparticles under dark tissue culture conditions. However, TiO$_2$ has the capacity to generate ROS under exposure to UV or near-UV light, and the band gap energy has been hypothesized to determine the phototoxicity of semiconductor ENMs. This property has made TiO$_2$ the subject of intensive applied research in water treatment and other infectious processes. Moreover, this property is also relevant to environmental UV exposure conditions as will be illustrated in the discussion of introducing bright sunlight conditions when assessing the potential harm for effects of TiO$_2$ nanoparticles in phytoplankton.

Following HTS of the metal oxides in mammalian cells, the toxicity of these materials was investigated in aquatic organisms and plants. In the case of ZnO nanoparticles, Prof. Arturo Keller and the investigators in the fate and transport group presented data that the dissolution of these nanoparticles in natural waters occurs on the order of hours, such that the most likely exposure for ecological receptors is to increased levels of Zn$^{2+}$ rather than to the intact particles. This effect was demonstrated by Prof. Gary Cherr and his laboratory for sea urchins, and by Prof. Hunter Lenihan and his laboratory for four common species of coastal marine phytoplankton: *Thalassiosira pseudonana*, *Skeletonema marinoi*, *Dunaliella tertiolecta*, and *Isochrysis galbana*. Toxicity to phytoplankton resulted in a significant reduction in population growth rate at concentrations above 223–428 µg/L. The same marine phytoplankton species were also used to assess the toxicity of TiO$_2$. In marine environments, the photoactivity of TiO$_2$ has been shown to decrease significantly relative to laboratory or freshwater studies. This is due to two effects: the aggregation of TiO$_2$ due to the high ionic strength of seawater, which reduces the available surface area for photoactivity, and the coating of the available surface with NOM, which competes for photons. ROS generation from photoactivation of TiO$_2$ can damage organisms through a variety of interrelated effects, including lipid peroxidation and DNA damage. Lack of TiO$_2$ toxicity has been demonstrated by others for bacteria and freshwater crustaceans. CEIN researchers have shown similar trends for marine phytoplankton and sea urchins. TiO$_2$ nanoparticles did not affect population growth of the four phytoplankton species, likely due to relatively low UV exposures in these experiments. Further tests with the same species of marine phytoplankton, however, have shown that exposure to natural levels of UV light induces TiO$_2$ toxicity, significantly depressing growth rates at concentrations as low as 1 mg/L (unpublished data). Clearly, UV exposure in experiments needs to be carefully considered when evaluating TiO$_2$ toxicity.

To assess the potential hazard of the metal oxides in the terrestrial environment, the toxicity in several types of plants, specifically the effects on seed germination and root growth, was studied by the laboratory of Prof. Jorge Gardea-Torresdey. At a concentration of 2000 mg/L ZnO, inhibition of germination was seen for corn (*Zea mays*) but not for the other various plant species studied. In examining root growth, a variety of adverse outcomes were observed at different concentrations of ZnO in soybean (*Glycine max*), mesquite (*Prosopis juliflora-velutina*), palo verde (*Parkinsonia florida*), tumbleweed (*Salsola tragus*), and corn (*Zea mays*).

These differential toxic effects of ZnO nanoparticles, which extend across mammalian cells, environmental organisms, and plant life, were also examined using zebrafish embryos, an emerging model for aquatic toxicity with high-throughput capabilities. Dr. Tian Xia demonstrated inhibition of embryo hatching by ZnO without any morphological changes, suggesting that the dissolution of ZnO nanoparticles could have an effect on one of the hatching enzymes being produced by a chorionic gland. In contrast, TiO$_2$ and CeO$_2$ did not show any adverse effect on zebrafish embryos.

**Implementation of a Safe Design Strategy That Lessens the Toxicity of ZnO Nanoparticles.** Following toxicity assessment, it is important to use the knowledge to develop safer nanomaterials, including re-engineering in such a way as to reduce or to eliminate the observed toxic effects of the native particles. In order to design these safe ENMs, we first looked at the toxic mechanisms of the nanoparticles of interest and began synthesizing nanomaterials that would alter how the ENMs behave in the environment, as shown in Figure 4.

On the basis of thorough physicochemical characterization of the nanoparticles, we identified the importance of particle dissolution and shedding of toxic Zn ions in ZnO-induced toxicity in mammalian cells. To study the dissolution property of ZnO, we performed detailed analyses of ZnO nanoparticles in water, PBS, and cell culture media using inductively coupled plasma mass spectrometry. The results showed that ZnO nanoparticles were highly soluble in these media. To study ZnO dissolution in cellular studies, we used fluorescently labeled ZnO to treat the cells and performed confocal microscopy. We found that no labeled ZnO particles could be seen in the acidic lysosomal compartments of RAW 264.7 cells, whereas fluorescent particles could be observed in a less acidic caveolar compartment in BEAS-2B cells. These data showed that dissolution of ZnO nanoparticles inside cells could play an important role in cellular toxicity. On the basis of the importance of dissolution to ZnO nanoparticle toxicity, we hypothesized that lowering the rate of dissolution could decrease its toxicity. Because mixed zinc—iron oxides are significantly more...
resistant to proton-assisted dissolution than is pure zincite, we investigated the possibility of deliberate doping of ZnO with iron to reduce dissolution. Using flame spray pyrolysis, Prof. Madler’s laboratory generated a panel of ZnO nanomaterials with an increasing atomic percentage of iron in the crystal matrix and characterized the materials to ensure crystallinity and uniform distribution of iron. As postulated, Dr. Pokhrel demonstrated that iron doping enhanced the aqueous stability of ZnO through strengthening of iron versus zinc binding to oxygen. Iron is typically found to be present as high spin Fe$^{2+}$/H$^+$ ions substituted for Zn$^{2+}$/H$^+$ at lattice sites, and based on the crystal field splitting of the Fe 3d states, we infer Fe$^{2+}$ to be more strongly bound than Zn$^{2+}$. Consistent with this mechanism, we observed reduced dissolution in buffered solution for iron-doped nanomaterials.

The panel of iron-doped ZnO was assessed using the HTS platform described in Figure 1. Consistent with the low dissolving nature of iron-doped ZnO, Dr. George found that reduction in the cytotoxicity of ZnO followed with an increasing atomic percentage of iron. We then tested the toxicity of these nanoparticles in vitro using a multiparametric rapid throughput screening assay and found that the cytotoxicity decreased as the percentage of Fe doping increased, proving that dissolution indeed plays an important role in cytotoxicity. Furthermore, several investigators performed in vivo tests using the Fe-doped ZnO library, including in zebrafish. The zebrafish studies looked at embryo hatching and mortality rates as well as the generation of morphological defects. Dr. Xia found that ZnO could inhibit hatching of zebrafish embryos without causing changes in viability or morphology. Dr. Xia also found that iron doping, similar to the effect of a metal chelator, DTPA, interfered in the inhibitory effects of ZnO on zebrafish hatching. These data showed that dissolution plays an important role under both in vitro and in vivo conditions.

**SUMMARY AND FUTURE ENM STUDIES**

At the UC CEIN, we have developed strategies for examining the toxicity and potential environmental impact of ENMs. This methodical study, as outlined in Figure 1, allowed the Center to assess potential hazards by first examining toxic effects using a HTS assay with mammalian cells. Once the potential risks were determined by this method, ENMs were distributed to various groups within UC CEIN for further testing. Studies using aquatic organisms, plant life, and higher organisms broaden our knowledge of the risks of ENMs in the environment. Using this information, we can then develop combinatorial libraries by synthetic strategies that allow us to tailor ENMs toward examination of specific structural properties. These combinatorial libraries with precisely controlled properties allow us to gain mechanistic understanding of the nano/bio interface.

Starting with three metal oxides, we are moving to expand the list to include other classes of nanomaterials, Figure 4. Safe design of ENMs. Once the mechanism of toxicity is determined for a particular nanomaterial, methods for reducing this toxicity can be proposed. In the case of ZnO toxicity, where dissolution of Zn$^{2+}$ is the cause of toxicity, it was proposed to dope the nanoparticles with iron to slow the dissolution process. Using the doped materials, the reduced toxicity is confirmed by comparing the results of the undoped particles with the doped materials by monitoring toxic end points in RAW 264.7 cells as a function of an increasing percentage of atomic iron in the re-engineered nanoparticles.
including carbon-based nanomaterials, silica-based nanomaterials, and other metal and metal oxide nanoparticles. Premised on the current knowledge about the unique material characteristics of each class, a compositional library will be created. For example, to understand the effects of dissolved metal ions (as compared to direct nanoparticle surface interactions with biological systems), metal and metal oxide nanoparticles are encapsulated in mesoporous silica \(^3\) and the toxicity is compared to that of bare particles. Further examples of specific physicochemical properties that can be modified to expand these combinatorial libraries are summarized in Table 1.\(^3,6\)

By creating combinatorial libraries that expand the scope of discovery for specific material properties, we can develop the structure—activity relationships that are required to understand the environmental toxicity of ENMs. Panels of ENMs can be created in order to emphasize a particular characteristic and these toxic profiles can be examined to further refine our knowledge. Systematic examination of toxicity as it relates to these defined structure—activity relationships allows us to develop synthetic strategies for the safer design of nanomaterials.

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**REFERENCES AND NOTES**


- Protein aggregation and fibrillation
- Modifying structure to reduce stiffness; reduce aspect ratio
- Coating or functionalization surface
- Modifying charge, size, hydrophilicity; inducing ionic hindrance
- Polymeric or inorganic shells
- Induce particle aggregation; modify size and/or surface charge
- Modify material to exert antioxidant effects through altered electronic states

\(^*\) Adding to combinatorial libraries by systematically varying single physicochemical properties and examining toxicity for these materials, toxic mechanisms can be determined for particular ENM properties. Reprinted with permission from ref 36. Copyright 2009 Nature Publishing Group.

<table>
<thead>
<tr>
<th>material examined</th>
<th>mechanism of toxicity</th>
<th>compositional library with systematic variation of predominant toxic characteristics</th>
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<tbody>
<tr>
<td>carbon nanotubes (MWCNT)</td>
<td>frustrated phagocytosis leads to inflammation and oxidative DNA damage</td>
<td>modify structure to reduce stiffness; reduce aspect ratio</td>
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<tr>
<td>Al(_2)O(_3)</td>
<td>ROS generation</td>
<td>coat or functionalize surface</td>
</tr>
<tr>
<td>Au (nanoparticles and nanorods)</td>
<td>disruption of protein formation</td>
<td>modify charge, size, hydrophilicity; induce ionic hindrance</td>
</tr>
<tr>
<td>CdSe</td>
<td>dissolution of toxic Cd and Se ions</td>
<td>polymeric or inorganic shells</td>
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<tr>
<td>Si(_3)</td>
<td>ROS generation, protein unfolding; Membrane disruption</td>
<td>induce particle aggregation; modify size and/or surface charge</td>
</tr>
<tr>
<td>CeO(_2)</td>
<td>Protein aggregation and fibrillation</td>
<td>Modify material to exert antioxidant effects through altered electronic states</td>
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Summary of Toxic Mechanisms Associated with Various Nanomaterials and Proposed Techniques Available To Vary Physicochemical Properties of ENMs

**TABLE 1.**


31. Eilersma, F.; Witkamp, G. J.; Vanrosmalen, G. M. Kinetics and Mechanism of Reductive Dissolution of Zinc Ferrite in H$_2$O and D$_2$O.