CHAPTER 5

TOXICITY TESTING OF NANOMATERIALS

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Abstract: The large-scale production and consumer exposure to a variety of nanotechnology innovations has stirred interest concerning the health consequences of human exposure to nanomaterials. In order to investigate these questions, in vitro systems are used to rapidly and inexpensively predict the effects of nanomaterials at the cellular level. Recent advances in the toxicity testing of nanomaterials are beginning to shed light on the characteristics, uptake and mechanisms of their toxicity in a variety of cell types. Once the nanomaterials have been satisfactorily characterized, the evaluation of their interactions with cells can be studied with microscopy and biochemical assays. The combination of viability testing, observation of morphology and the generation of oxidative stress provide clues to the mechanisms of nanomaterial toxicity. The results of these studies are used to better understand how the size, chemical composition, shape and functionalization may contribute to their toxicity. This chapter will introduce the reader to the impact of nanomaterials in the workplace and marketplace with an emphasis on carbon-based and metal-based nanomaterials, which are most commonly encountered. While most purified carbon nanomaterials were nontoxic to many cell lines, many metal nanoparticles (e.g., silver or manganese) were more toxic. Other side- effects of nanoparticle interactions with cells can also occur, such as increased branching and dopamine depletion. Further investigation into the characteristics, uptake and mechanisms of nanomaterial toxicity will continue to elucidate this fascinating and rapidly growing area of science.

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INTRODUCTION

The emergence of the new field of nanotoxicity has spurred great interest in a wide variety of materials and their possible effects on living systems. In particular, carbon-based and metal-based nanomaterials are being pursued for novel applications in industry and healthcare and for military purposes. Due to the increasing risk of exposure, in vitro systems have been widely used to estimate nanomaterial toxicity or biocompatibility. This chapter will focus on the recent advances in the in vitro toxicity testing of nanomaterials through characterization, the monitoring of internalization and the elucidation of the cellular mechanisms at work.

Nanotechnology and the Health Risk

Nanotechnology involves the creation and manipulation of materials at the nanoscale, to create products that exhibit novel electrical, catalytic, magnetic, mechanical, thermal, or optical features. Engineered nanomaterials are defined as materials with features in the range of 1-100 nm (10^{-7} to 10^{-9}m) in length or diameter. A variety of nanomaterial innovations have infiltrated the market place, in products such as titanium oxide (TiO_2) and zinc oxide (ZnO) in sunscreens for enhanced product transparency, silver (Ag) in bandaids and cosmetics for antibacterial activity and carbon (C) nanomaterials in nanocomposites (Table 1).[1] Concomitantly, highly specialized industrial applications of nanomaterials are being developed, such as aluminum (Al) as a fuel additive, manganese (Mn) as a catalyst or in battery technology, carbon nanotubes (CNT) as drug, gene, or protein carriers,[2] quantum dots (QDs) and iron (Fe) nanoparticles as imaging probes, Ag as an antiviral agent[3] and polymers as novel scaffolds.[4]

There is a great concern about the health consequences of nanomaterials, due to the increased probability of contact with engineered nanomaterials in both commercial products and industrial settings. Therefore, determining the toxicity of nanomaterials is a fundamental question relating to their extremely small size, high surface area and increased surface reactivity (i.e., redox ability) as compared to larger materials.[5,6] The small size of nanomaterials permits their translocation across cell membranes into critical organelles

Table 1. Applications of commonly used nanoparticles

Nanoparticle Type	Abbreviation	Applications
Carbon nanotube (single or multiwalled)	CNT (SWNT, MWNT)	Cell delivery, biosensors
Silver	Ag	Antimicrobial
Quantum dot	QD	Fluorescent imaging
Aluminum	Al	Fuel additive
Iron	Fe	Magnetic imaging
Titanium dioxide	TiO_2	Paint, water treatment, food, cosmetics
Zinc oxide	ZnO	Transparent sunscreens
Manganese oxide	MnO	Catalysis, batteries

such as mitochondria.[7] This same small size may also allow the nanomaterials to evade the cells responsible for their clearance, leading to biopersistence. Studies have shown that ultra-fine particles (<100 nm) are more toxic than larger particles made of the same material, partly due to a decrease in their clearance functions and their persistence in tissues.[8]

In support of these studies, there is a great amount of evidence that some nontoxic, micron-sized particles become toxic once they are reduced in size to the nano-scale.[9-16] The physical and chemical properties which contribute to this can include changes in solubility and surface area, as well as novel surface chemistry. The small size of nanomaterials changes their physical behavior from classical physics to quantum physics with decreasing particle size, which affects solubility, transparency, color, absorption or emission wavelength, conductivity, melting point and catalytic behavior.[17] Studies have shown that combustion particles with very low metal content, such as carbon black, cause inflammation, purely due to their surface characteristics and not their solubility.[18,19] The small size also leads to greater interactions with individual cells and their bio-molecules, which are on a similar size scale to that of the nanomaterials. However, some studies show that not all nanomaterials are more toxic than fine-sized particles of similar chemical composition. For example, pulmonary exposure in rats to uncoated TiO_2 nanorods (200 nm × 30 nm) compared to TiO_2 nanodots (<30 nm) did not produce greater inflammation than fine-sized TiO_2 particles (270 nm), whereas naive TiO_2 appears less inflammatory than hydrophobic TiO_2.[20,21] A suggested set of nanomaterial characteristics considered valuable prior to toxicity testing include size distribution of primary particles, shape, surface area, composition, surface chemistry, surface contamination, surface charge, crystal structure, particle physicochemical structure, agglomeration state, porosity, method of production, heterogeneity, storage conditions, and concentration.[13]

Occupational Exposure to Nanomaterials

Occupational exposure is likely to occur through dermal contact, ingestion or inhalation. Therefore, nanomaterials could potentially have an impact on the deep regions of the lungs, such as the alveolar region, where the barrier between the alveolar wall and capillaries is only 500 nm thick.[22] For example, inhaled nanomaterials may become lodged in the lung and remain there, unable to be cleared, as with asbestos. Thus, some of the same properties that make nanomaterials useful, are also properties that may contribute to toxicity and make them a health hazard under certain conditions. Recently, low concentrations of CNTs and other nanomaterial particulates were found in the air during industrial processes, but a full assessment of exposure levels has yet to be made.[24] Aerosol release during the handling of unrefined single-walled nanotubes (SWNTs) suggests that concentrations released in laboratories are lower than 53 g/m^3 and glove deposit concentrations are between 0.2-6 mg/hand.[24] Current US Occupational Safety and Health Administration (OSHA) and National Institute for Occupational Safety and Health (NIOSH) standards limit the exposure of silver compounds (including solubles, metal dusts and fumes) to $0.01 mg/m^3$. This limit is intended to reduce the occurrence of argyria, a permanent discoloration of the skin, in the occupational setting.[25] Another common health effect of workers exposed to silver dust and particulates is upper and lower respiratory infection. Overall, the toxicity of silver heavily depends upon its form. For example, liquid silver nitrates and chlorides are found to be extremely toxic, but silver oxides are not. Drake and Hazelwoood also reported that exposure to large amounts of silver iodide does not lead to argyria.[25] Other studies involving humans have shown that elevated levels of manganese may increase

the risk of developing Parkinson's disease.[26] In environments where large amounts of manganese powder are being produced, such as steel, nonsteel alloy, battery, welding and fuel additive factories, the exposure risk is greatly increased.[27]

The Characterization of Nanomaterials

In order to understand the characteristics of nanomaterials that can contribute to toxicity, they are first assessed in the as-synthesized form, prior to use in in vitro systems and after dispersion in the appropriate aqueous media for cell dosing (Fig. 1). Some of the most common techniques for initially determining composition and concentration include energy dispersive X-ray analysis (EDS), atomic absorption spectroscopy (AAS), or inductively coupled plasma spectroscopy (ICPS). The Brunauer, Emmett and Teller (BET) technique and inverse gas chromatography (IGC) can be used to estimate size and surface area, while scanning electron microscopy (SEM), transmission electron microscopy (TEM) and atomic force microscopy (AFM) are routinely used for evaluating size and morphology (http://kristall.uni-mki.gwdg.de/english/docs/BET.htm).[28-29] Other studies with environmental SEM or AFM can permit the examination of wet materials, while techniques such as dynamic light scattering (DLS), zeta potential and UV-visible spectroscopy are routinely performed in solution to provide size, charge and composition characteristics (http://www.malvern.com).[30]

After sufficient information is obtained on the characteristics of the nanomaterial, in vitro measurements commence, such as concentration-dependent effects on viability. In our studies, oxidative stress and apoptosis have been assessed in skin, lung and neuronal cell lines. The results of such preliminary studies can then be examined in animal systems

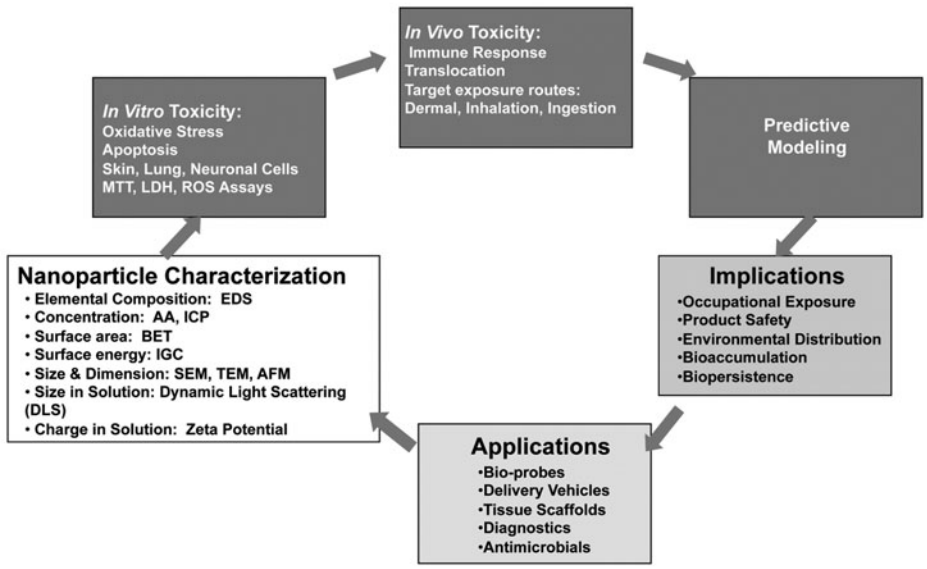


Figure 1. The integration of the areas studied in nanomaterials research.

(in vivo) for their effects on immune responses or on translocation to other areas after dermal, inhalation, or oral uptake. Once an adequate amount of data are collected, predictive modeling through computer-based approaches can be used to extrapolate the in vitro results to in vivo situations.[31] Toxicokinetic modeling describes the absorption, distribution, metabolism and elimination of xenobiotics (foreign materials) within an organism, as a function of dose and time. Toxicokinetic models can be divided into two main categories, namely, data-based compartmental models and physiologically-based compartmental models. Other quantitative structure–activity relationship (QSAR) models have been explored for structurally-related materials. However, many challenges still remain, including predicting the chronic effects that lead to conditions such as cancer, hematotoxicity, hepatotoxicity, lung fibrosis, nephrotoxicity and neurotoxicity, on the basis of in vitro studies. Additionally, most cellular responses are dependent upon dose and exposure time, where a low dose over a long period of time may result in an adaptive or even beneficial/protective effect.[31]

After careful consideration, the implications of the research outcome can be used to set safe limits for exposure in the work environment, in consumer products and in environmental waste. How the nanomaterials are distributed, accumulate and persist in the environment, are also matters of great concern. At the same time, these negative factors must be weighed against the benefits of using the nanoparticles, e.g., as antimicrobials, bio-probes, delivery vehicles, diagnostics and tissue scaffolds.

IN VITRO TOXICITY ASSESSMENT

Both in vitro cell culture and animal studies are being used to evaluate nanomaterials for their toxicity or potential to induce cell death.[31,32] In general, in vitro assays consist of subcellular systems (i.e., macromolecules, organelles), cellular systems (i.e., individual cells, coculture, barrier systems) and whole tissues (i.e., organs, slices, explants). Although in vitro data are not a substitute for whole animal studies, the use of relatively simple in vitro models with endpoints that reveal a general mechanism of toxicity can be a basis for further assessment of the potential risk of exposure to nanomaterials. For example, data reported on the toxicity testing of a series of high energy chemicals in an in vitro model,[27,33] were used to derive a baseline for extrapolation to a human health risk assessment.[34] The toxicity data obtained from such in vitro systems has been used to screen, rank and predict the acute hazards and mechanisms of compound interactions with animals or humans. This "basal toxicity" is defined as the ability of a compound to cause cell death as a consequence of damage to basic cellular functions. It can be used to define the concentration ranges of chemicals or nanomaterials which produce a toxic effect. The data obtained from basal toxicity studies have been found to be in good correlation with acute toxicity in animals and humans after studies involving diverse arrays of chemicals and assay systems.[35] However, kinetic factors and target organ specificity were parameters that weakened the correlation. Therefore, in vitro studies are conducted as a starting point and are very useful, because of their ability to rapidly and inexpensively produce results which may uncover the underlying toxic mechanisms of the selected chemicals, without the use of animals. The limitations of in vitro methods include: the transformation or immortalization of the cell lines, which may alter the properties and sensitivities of the cells; selective toxicity, in which some cell types are more sensitive than others; the isolation of the cells from their natural environment; and the difficulty encountered

in studying integrated groups of cells or organ systems. In our laboratory, rat alveolar macrophages were one of several cell lines selected for assessing the level of toxicity of nanomaterials and exploring possible mechanisms of toxicity after internalization that would be encountered after inhalation. Human lungs contain approximately 10^9 alveolar macrophages, which are found in the alveolar sacs, deep within the lungs.[36] Other cell lines which were tested for nanomaterial toxicity included murine neuroblastoma cells, PC-12 rat pheochromocytoma cells, which were derived from adrenal gland tumor cells and murine keratinocytes.

The Toxicity of Carbon-Based Nanomaterials

The interaction of carbon nanomaterials in a variety of cell types has been recently examined and reviewed, with uncertain conclusions. Many of these studies involve the use of established in vitro toxicity assays, based on the breakdown of the cellular permeability barrier, reduced mitochondrial function, changes in cell morphology, or changes in cell proliferation. The tests used in our laboratory involve microscopic examination and early biochemical endpoints, such as the MTT viability assay, lactase dehydrogenase (LDH) leakage and the production of reactive oxygen species (ROS). Because working with nanomaterials is different from that with other test materials, we have made minor modifications to the MTT assay, such as the incorporation of an additional centrifugation step to remove the nanomaterials from the solution, before the microplate reading step.[33,37,58]

Our recent viability results with nanodiamonds (NDs) ranging in size from 2-10 nm, showed that they are not cytotoxic to a variety of cell types, such as neuroblastoma, macrophage, keratinocyte and PC-12 cells.[37] Figure 2 shows the MTT viability results in neuroblastoma cells after a 24h exposure to acid or base functionalized NDs compared to those for 20 nm fine carbon black nanoparticles or the positive toxicity control, micron-sized cadmium oxide (CdO). Additional results showed that the cells did not produce significant ROS and they were able to grow on ND substrates with similar substrate growth observed by neurites grown on patterned carbon nanotubes.[23] These findings have stimulated the possible use of biocompatible carbon nanoparticles in advanced medical systems such as nanorobots or as fluorescent biolabels.[36,38]

While these NDs appear to be biocompatible, the parameters that are thought to influence the toxicity of carbon nanomaterials in general include their length, mass, functionalization and functionalization density.[42-44,46-48] Jia et al compared the relative cytotoxicities to macrophages of SWNTs, multi-walled carbon nanotubes (MWNTs) and fullerenes.[43] They found that the SWNTs significantly impaired the phagocytosis of macrophages at doses as low as 0.38 $\mu g/cm^2$, whereas the MWNTs and the fullerene, C60, induced injury only at the high dose of 3.06 $\mu g/cm^2$. The cytotoxicities appeared to follow a sequence on a mass basis: SWNTs > MWNTs > quartz > C60.

Investigations into the impact of surface chemical functionalization on toxicity have been performed in various cell types.[42,46,48] For example, human dermal fibroblasts incubated with water dispersible, functionalized SWNTs, showed that SWNT-phenyl-SO_3H, SWNT-phenyl-SO_3Na and SWNT-phenyl-$(COOH)_2$ were more biocompatible and had greater functionalization density compared to an unfunctionalized, surfactant-stabilized SWNT.[42] However, SWNT-phenyl-$(COOH)_2$ reduced cell viability to a greater extent than did SWNT-phenyl-SO_3H. In particular, the toxicity observed in cells incubated with SWNT-phenyl-$(COOH)_2$ was significantly higher than that in the controls, at concentrations ranging from 10-200 $\mu g/ml$ while SWNT-phenyl-SO_3H, with the highest

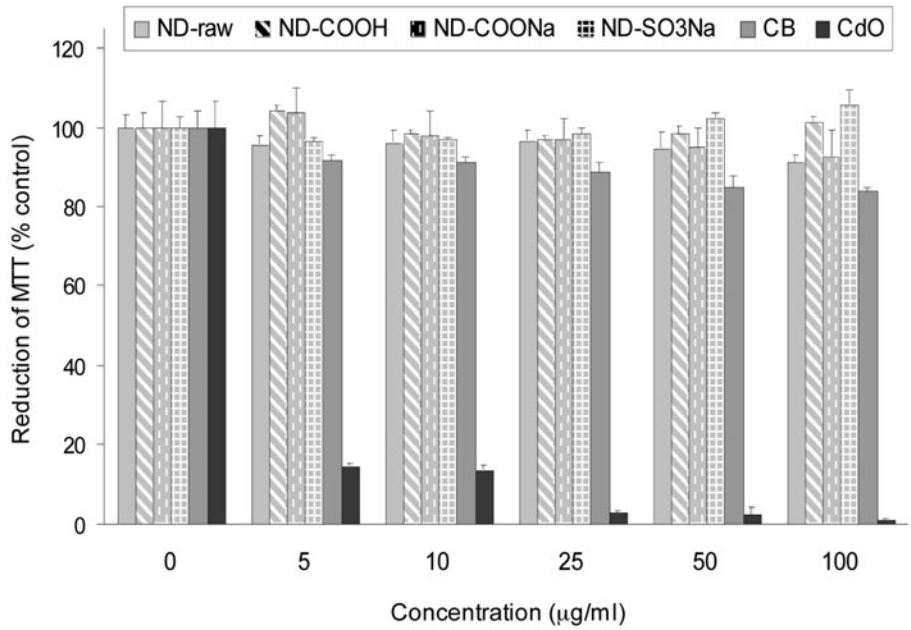


Figure 2. A cytotoxicity evaluation with neuroblastoma cells incubated with various nanoparticles for 24 h, as assessed with the MTT assay.[37] Reproduced with permission from Schrand et al. J Phys Chem Lett B 2007; 111(1):2-7.[37]

functionalization density, was not toxic up to the highest concentration tested, 2 mg/ml. Two other notable studies found that surface acid functionalization (the addition of carbonyl C = O, carboxyl (COOH) and/or hydroxyl [OH]) of carbon nanomaterials played a role in the cytotoxic effects in lung tumor cells and human acute monocytic leukemia cells, respectively.[46] After exposure to 0.02 μg/ml for 2 days, the viability of the lung tumor cells was reduced in the following order: carbon black (CB) > CNFs > MWNTs with MWNTs being the least toxic to the cells. The authors suggested that the MWNTs, having the highest aspect ratio out of the three materials, may have fewer dangling bonds, which preferentially occur at lattice defects or endcaps, compared to CB, where they are at high density. Cytopathological analysis showed that, after 1 day of incubation with 0.02 μg/ml of MWNTs, the cells lost their mutual attachments and retracted their cytoplasm, while their nuclei were more condensed, which was indicative of toxicity. Therefore, the conclusions to this study were that carbon nanomaterials are generally cytotoxic, with increasing toxicity after acid functionalization. Another research group found that the acid functionalization of hat-stacked carbon nanofibers, through the addition of carboxyl groups, led to only weak changes in toxicity to human acute monocytic leukemia cells and human embryonic kidney cells, while there was no difference between the toxicities to macrophages of MWNTs between 500 nm and 5 microns in length.[47-48]

While some studies have demonstrated the biocompatibility of unmodified carbon nanotubes with fibroblasts, osteoblasts and human umbilical vein endothelial cells, as well as murine and human macropages,[49,53-57] other studies with murine alveolar macrophages

have shown that carbon nanotubes are just as toxic as asbestos,[50,52] can inhibit cell growth or induce apoptosis in human embryonic kidney cells[39] and can generate an irritant and oxidative stress response with keratinocytes.[40,41,45] Therefore, based on the variety of carbon nanomaterials and cell types tested, these studies suggest that, to be least toxic to cells, an ideal material would be of small size, low mass and appropriately functionalized.

The Toxicity of Metal-Based Nanomaterials

The toxicity of silver nanoparticles in liver cells, germ-line stem cells and alveolar macrophages is greater than that of most carbon nanomaterials and many other metal nanomaterials. The toxicity of silver nanomaterials generally increases with decreasing size and with increasing concentration because of oxidative stress.[27,59-60] Research with C18-4 germ line stem cells showed that they were more sensitive to 15nm silver nanomaterials than were either BRL-3A liver cells or CRL-2192 alveolar macrophages. After 24 h of exposure to 15 nm Ag nanoparticles, the effective concentrations that decreased viability and increased LDH leakage respectively, by 50% were 8.75 μg/ml and 2.5 μg/ml for stem cells, 24 μg/ml and 50 μg/ml for BRL-3A liver cells and 28 μg/ml and 15 μg/ml for alveolar macrophages.[27,59-60] Other signs of toxicity included reductions in mitochondrial membrane potentials and in glutathione (GSH) levels and the activation of pro-inflammatory cytokines after exposure to 15nm silver nanoparticles.[27,60]

A variety of other metal nanomaterials have been screened for their toxicities through assays that measure LDH leakage through the plasma membrane. Because LDH is not typically found extracellularly in normal, healthy cell cultures, extracellular measures of LDH can be used as an indicator of membrane damage and subsequent cell viability. The LDH release of BRL 3A rat liver cells was measured after a 24h exposure to various nanoparticles (Fig. 3). The results demonstrated that exposure to micron-sized CdO dramatically increased membrane leakage (Fig. 3A) compared to silver nanoparticles (Fig. 3B) and also caused a dose-dependent increase in membrane leakage with concentration. Compared to other metal/metal oxide nanoparticles (Fig 3C), silver nanoparticles significantly increased LDH leakage at concentrations of 250 μg/ml.

The Uptake of Nanomaterials

The evaluation of nanomaterial uptake into cells answers many fundamental questions regarding toxicity by verifying the internalization of the nanomaterials, their location inside the cell and the amount that can be internalized over a certain period of time. The evaluation of nanomaterial uptake with in vitro cell culture has been monitored with fluorescent microscopy, flow cytometry, or fluorescent-activated cell sorting (FACS).[60-71] Other methods for quantitatively determining the uptake of nanomaterials include confocal microscopy,[49,73-74] ICPS,[52] radio-active labeling,[74-77] and AFM.[73] While newly-developed nanomaterials may be used purely as bio-labels for applications such as photodynamic therapy,[69] factors influencing their uptake can include size, concentration, temperature and surface properties, such as functionalization or charge. Additionally, it may be possible to distinguish active versus passive uptake by differences in kinetics. In our laboratory, we use advanced techniques in light and electron microscopy, such as ultrahigh resolution light microscopy and wet imaging under high vacuum conditions, in addition to observations on thin sections in TEM that can rapidly and accurately describe the uptake of nanomaterials into cells.

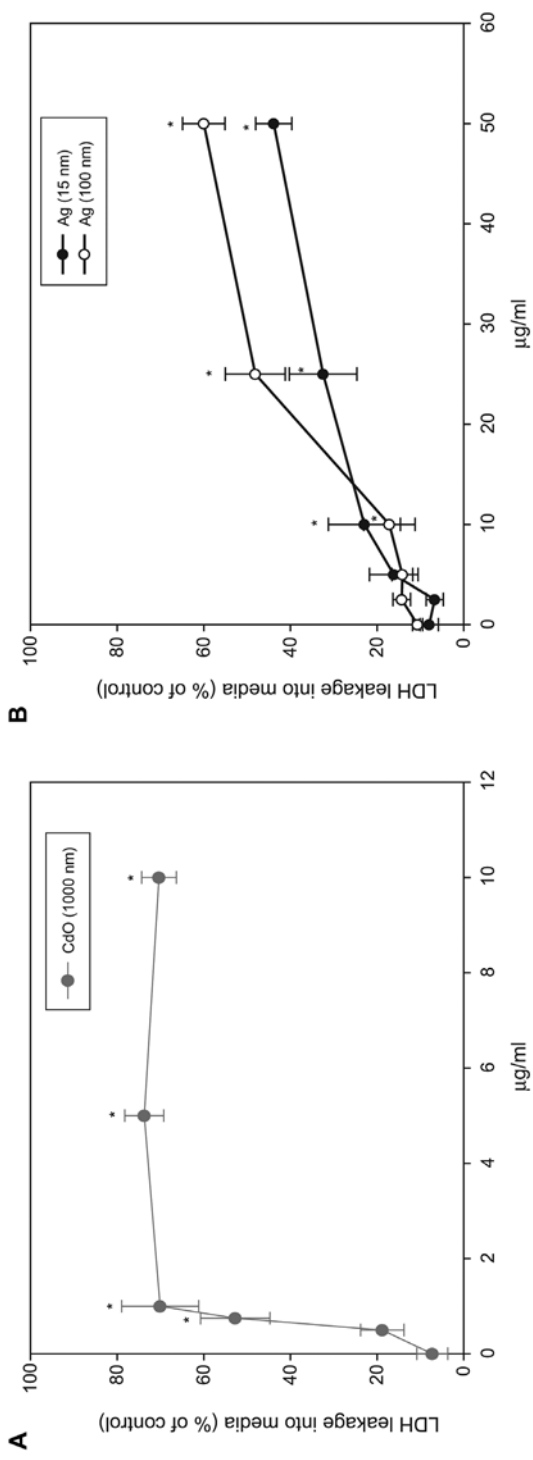
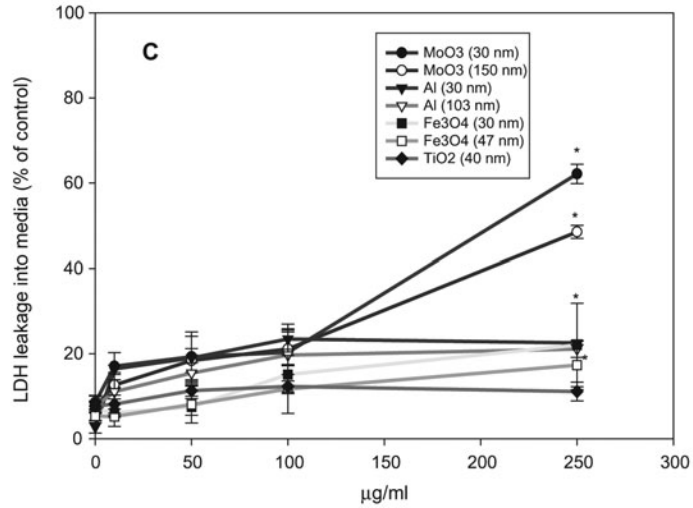


Figure 3. (A and B seen here, C on next page). The effects of nanoparticles on LDH leakage by BRL 3A rat liver cells after a 24 h exposure. The data are expressed as the mean ± SD of three independent experiments.[27] (*) indicates a statistically significant difference compared to the control ($p < 0.05$). Reproduced with permission from Hussain et al. Toxicol In Vitro 2005; 19:975-983.[27]



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Morphological Observations

Because some nanomaterials exhibit pronounced toxicity while others appear biocompatible, light microscopy can be used to visualize overall cell morphology and nanomaterial interaction. In our studies, we used the CytoViva™ 150 Ultra Resolution Imaging (URI) system to observe changes in living cell morphology and uptake after exposure to nanomaterials.[78-79] This new technology surpasses the accepted 250 nm limit for light microscopy, by minimizing the spot size and stray light, which increases the efficiency of the metal halide lamp, resulting in resolution less than 150 nm.[80-81] Additionally, the unique light scattering capability brightly illuminates internal cell structures or agglomerates of metal nanoparticles inside living cells.[78-79,81] For example, PC-12 cells incubated for 24 hours with 50 µg/ml of MnO show distinct bright spots, which represent nanoparticle agglomerates (Fig. 4). This information complements the toxicological studies that showed that 40 nm MnO nanoparticles produced much greater levels of ROS than either Mn^{2+} or 15 nm silver nanoparticles.[78]

Another recent advance for imaging cells in a more natural state with minimal sample preparation, is the Quantomix capsule for wet SEM imaging under high vacuum conditions (http://www.quantomix.com/). This technology completely isolates the cell, tissue and other samples from the vacuum in the microscope chamber, making it possible to image and obtain X-ray composition data for fully-hydrated samples at a resolution as low as 10 nm.[82-85] The thin, yet electron and vacuum stable, transparent polyimide membrane permits the direct growth of cells and visualization into the interior of whole cells, whereas traditional secondary electron imaging in SEM only allows the visualization of surface topography. In order to gain greater contrast under the backscattered electron imaging mode, heavy metal staining can be performed directly in the capsule—without the additional steps that would be required for typical electron microscopy sample preparation, such as dehydration or critical point drying. Therefore, cell samples can be prepared quickly with preserved integrity in a hydrated form and labeled with nano-sized metal-based probes

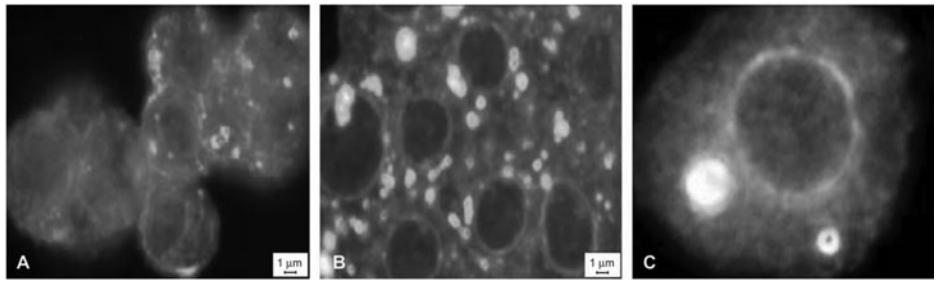

Figure 4. The uptake and distribution of manganese oxide nanoparticles associated with PC-12 cells after a 24 h incubation, visualized by high-illuminating inverted microscopy.[78] A) Control cells, B) Exposed cells and C) and enlargement of a single cell, showing bright areas that are agglomerated MnO nanoparticless. Reproduced with permission from J Toxicol Sci 2006; 92(2):456-463.[78]

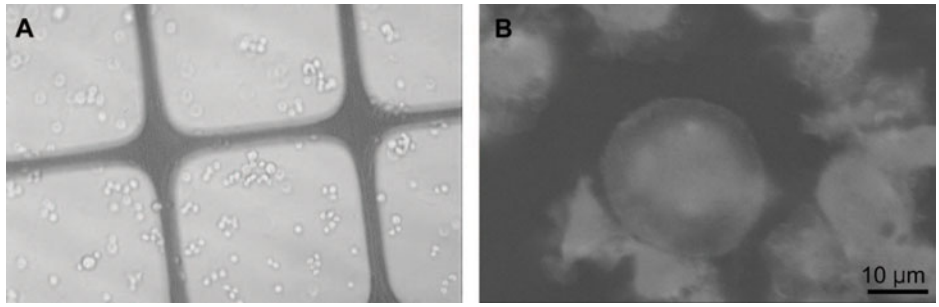

Figure 5. Demonstrations of (A) macrophage seeding and (B) SEM imaging after uranyl acetate and lead citrate staining in a Quantomix capsule for wet imaging under high vacuum conditions.

for the localization of antigen—antibody complexes or the uptake of nanoparticles. In our studies, we grew cells directly on a thin polymer membrane, which could be visualized with both light microscopy and SEM after uranyl acetate staining (Fig. 5).

By comparison, TEM preparation of cells consists of fixation with glutaraldehyde/paraformaldehyde, postfixation with osmium tetroxide, dehydration through a graded series of ethanols, embedding in resin, curing and thin sectioning. We have examined the presence of a variety of nanoparticles inside alveolar macrophages, neuroblastoma cells and keratinocytes. Although the cells may have different mechanisms of nanoparticle uptake, the nanoparticles still accumulate inside the cells, thereby reducing cell viability. Additionally, many of the nanoparticles are found inside cytoplasmic vesicles or freely in the cytoplasm, suggesting that mechanisms of uptake other than phagocytosis, such as endoycytosis or diffusion, are involved.

Fluorescence microscopy is another technique that can be used to examine the morphology of cells after incubation with nanoparticles. We recently observed increased branching of the actin cytoskeleton in ND-exposed cells (Fig. 6B) dual stained for actin and nuclei compared to the untreated controls (Fig. 6A), but there was no obvious uptake into the nuclei. It is not known whether the cells are merely responding to the presence of the nano-sized particles with increased neurite extension or whether the internalization of

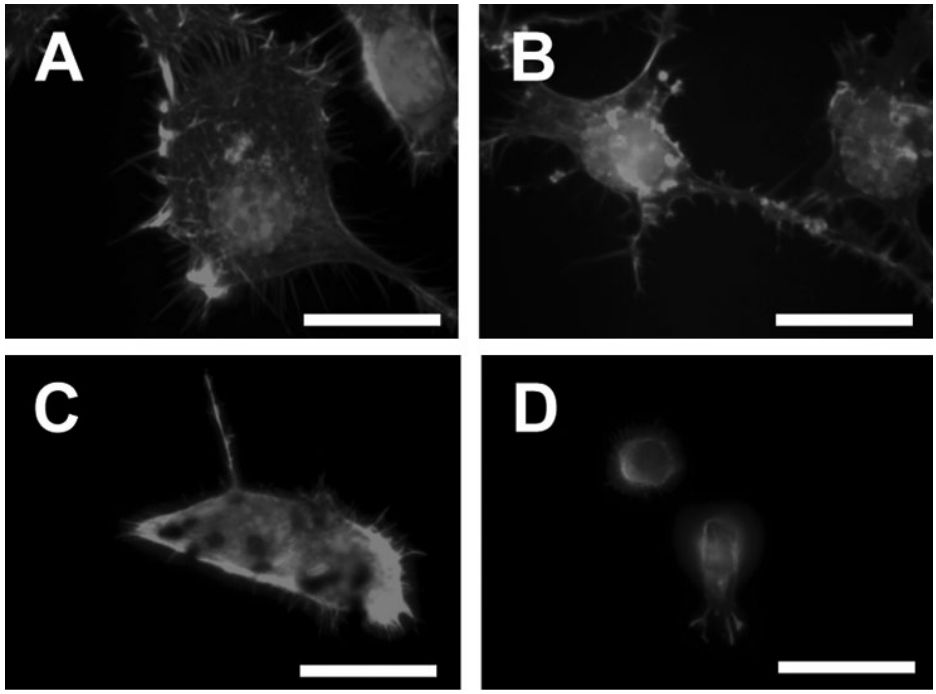

Figure 6. Fluorescence microscopy of neuroblastoma cells incubated with nanoparticles. Shown are cells incubated with nanoparticles for 24 h, then dual- stained for actin (red) and nuclei (blue) to reveal changes in cytoskeletal architecture. (A) Control, (B) 100 μg/ml ND-raw, (C) 100 μg/ml CB and (D) 2.5 μg/ml CdO. Scale bars are 10 um.[37] Reproduced with permission from Schrand et al. J Phys Chem Lett B 2007; 111(1):2-7.[37] A color version of this image is available at www.landesbioscience.com/madamecurie.

the NDs induces differentiation or other signaling pathways by an inside-out mechanism. Increased branching was not observed in cells incubated with fine CB nanoparticles (Fig. 6C), which suggests that there is a unique mechanism at work. By contrast, cells incubated with the positive control, CdO, showed reduced staining and cell shrinkage, which was indicative of toxicity (Fig. 6D).

MECHANISMS OF NANOMATERIAL TOXICITY

Collectively, the results of in vitro studies have identified oxidative stress-related changes in gene expression and cell signaling pathways as the underlying mechanisms of ultrafine particle effects, as well as roles for transition metals and certain organic compounds on combustion-generated ultrafine particles.[86] The interpretation of these studies is often difficult, due to differences in particle chemical composition, cell type, duration of exposure, endpoint and dosage. For example, 100 μg/ml, the highest concentration that we used in our studies, is not likely to be encountered in vivo, but provides information on the possible effects of high nanoparticles doses on mechanistic processes and modes of action.

The generation of intracellular ROS was determined by using dichlorofluorescin diacetate (DCFH-DA),[87] with minor modifications, as previously described by Hussain and Frazier.[33] In neuroblastoma cells incubated with carbon nanomaterials, there was no noticeable generation of ROS, except with CB nanoparticles. This suggests that, although carbon has generally been considered inert, it may react to produce some stress to the cells.[37] In BRL-3A cells and macrophages, there were significant increases in ROS generation after exposure to 10-50 μg/ml of Ag-15 nm for 6 h or 24 h, respectively, which suggests that the mechanism of toxicity is via an oxidative stress pathway. The dose-dependent increase in ROS generation following the exposure of BRL 3A cells for 6 h to 15 nm or 100 nm Ag at concentrations of 0, 5, 10, 25 and 50 μg/ml, is shown in Fig. 7A. Both sizes of silver nanoparticles significantly increased ROS generation compared to the untreated controls. The qualitative assessment of ROS generation was conducted by using fluorescence microscopy and showed increases in brightness after exposure to 25 μg/ml of Ag nanoparticles (Figs. 7B and 7C).

More-recent studies have shown that the chemical reactivity of the nanoparticles alone may be capable of generating ROS in the absence of a cellular environment. Therefore, the inherent reactivity of the nanomaterial must be taken into consideration as another characteristic which may be responsible for nanomaterial toxicity. Another factor to consider is the cell-specificity of effects, which may lead to particular diseases. The likely primary targets for nanomaterial exposure in the laboratory are the lung and skin, but cancerous conditions have not been linked to either organ after exposure and may be more related to the individual's health status.[17] The pathway of oxidative stress may be responsible for many conditions, such as Parkinson's disease, Alzheimer's disease and liver, heart and intestinal disease, but there have been no systematic studies which have directly linked exposure to nanomaterials to diseases in humans. Additionally, the dosing procedures and tumor-related effects following exposure of the lung may be limited to rats, as they have not been shown in other rodent species, which makes extrapolation to humans problematic.

CONCLUSION

Recent advances in the tools and techniques used for the toxicity testing of nanomaterials have permitted a more rapid and more thorough analysis of a variety of particles and cell types. Interdisciplinary technology, such as ultrahigh resolution light microscopy and capsules for wet imaging by SEM, combined with modifications to standard microscopic and biochemical techniques, has provided opportunities for collaboration on nanoparticle toxicology involving biologists, toxicologists and materials scientists. The characteristics of nanomaterials that can be used to predict their toxicity are still being elucidated, but our own work and that of many other groups, has shown that chemical composition, size, shape and functionalization are contributing factors. Further investigations into the characteristics, uptake and mechanisms of nanomaterials toxicity are expected to influence many fields of science for generations to come. However, at this point, no generalities have been identified, so nanomaterial toxicity should be evaluated on a case-by-case basis.

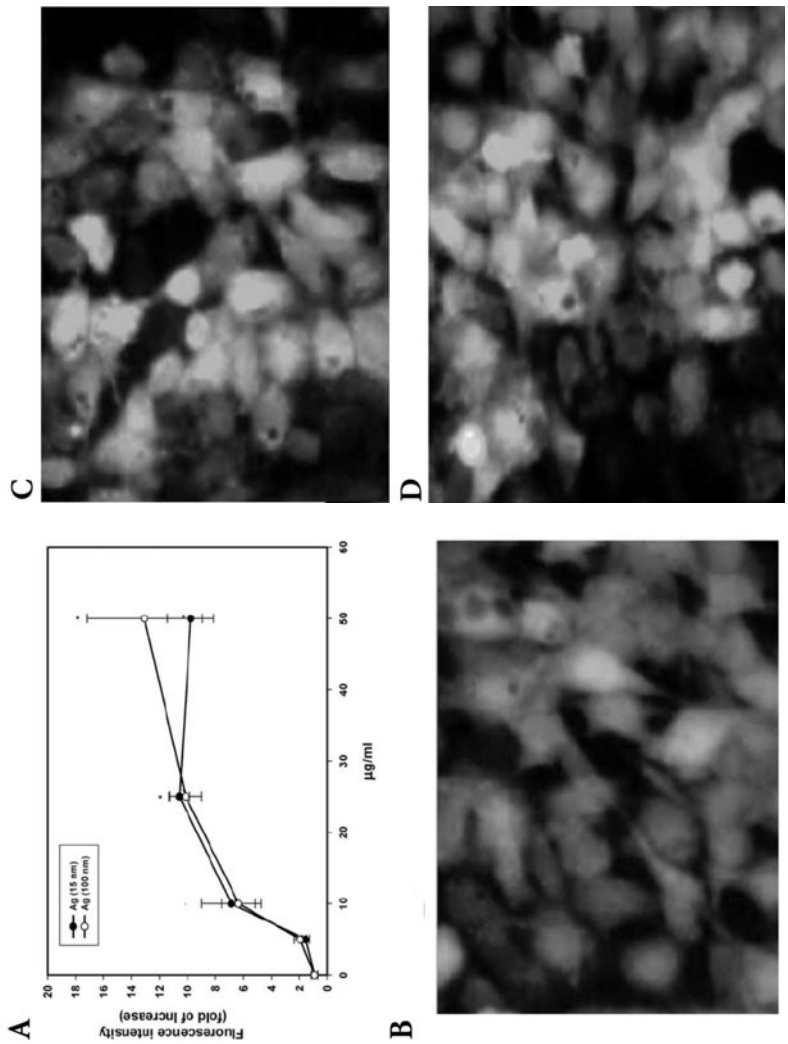

Figure 7. Effects of incubation for 6 h with 15 nm or 100 nm Ag nanoparticles on ROS generation in BRL- 3A rat liver cells. (A) The generation of ROS, assessed with DCFH-DA and expressed as fold increase relative to the untreated controls. The data are expressed as the mean ± SD of three independent experiments with (*) indicating a statistically significant difference compared to controls ($p < 0.05$). B-D) The qualitative characterization of ROS generation with DCFH-DA staining, by using fluorescence microscopy after a 6h-incubation with (B) Control, (C) 25 µg/ml Ag-15nm and (D) 25 µg/ml 100nm nanoparticles.[27] Reproduced with permission from Hussain et al. Toxicol In Vitro 2005; 19:975-983.[27]

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