Differential Distribution and Toxicity of Nanomaterials *In Vivo*

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Opportunity for Nanotechnology: The innovative field of nanotechnology is most likely to gain societal acceptance if environmental and human health considerations are thoroughly investigated and those results are used to optimize safety and performance together to produce effective and non-toxic profitable technologies. Industrial and scientific communities must work together to integrate toxicological and safety evaluations into nanomaterial research and development schemes so that actual risks of nanomaterials are defined and adverse environmental consequences are minimized. Thus far, major research efforts and resources have focused on discovering applications of novel nanoparticles; yet little research has aimed at the health and safety consequences of nanoparticle exposure (Colvin 2003). Our current knowledge about the toxicological effects of nano-scale materials is insufficient to direct the rational development of safer, non-toxic products. Since the biological activity of nanomaterials will likely depend on inherent physicochemical properties not routinely considered in toxicity studies (e.g. particle size and size distribution, agglomeration status, interactions with environmental and biological moieties), it is important that chemical engineers work together with toxicologists to provide critical information on the potential biological and environmental impacts of the newly emerging nanotechnology industry.

It is anticipated that nano-scale materials will interact with biological systems in a different way than their bulk counterpart since their properties and attributes (i.e. magnetic, optical, tensile strength) appear to be unique to their size. Materials reduced to the nano-scale (at least one

dimension in the 1-100 nm range) exhibit unique physical and chemical properties that may be desirable for medical, industrial and scientific applications but may be deleterious for human health and the environment. Areas of primary concern in terms of toxicity of nanoparticles include but are not limited to their: 1) high redox activity (Hoet et al. 2004), 2) ability to partition into cell membranes especially mitochondria both *in vitro* (Huang et al. 2004; Jia et al. 2005; Oberdorster et al. 2005) and *in vivo* (Hoet et al. 2004; Lam et al. 2004; Oberdorster 2004; Warheit et al. 2004), 3) capacity to translocate from the olfactory nerve into the olfactory bulb via a neuronal translocation pathway (Oberdorster et al. 2005), 4) activity as ion channel blockers (Park et al. 2003), 5) diversity in structure, and 6) observed cytotoxicity and bioactivity (Jia et al. 2005; Tsuchiya et al. 1996). However, other nanomaterials have beneficial effects and exhibit decreased toxicity when reduced to the nano-scale. Nanoparticles composed of cerium or yttrium oxide were shown to protect nerve cells from oxidative stress via direct antioxidant properties they exhibit (Schubert et al. 2006). *In vitro* studies using osteoblasts (bone-forming cells) revealed a reduction in toxicity of nano-sized alumina and titania compared to conventional micron-sized particles (Gutwein and Webster 2004). It is unknown if any generalizations can be made about the toxicity and/or pharmacological efficacy of nano-scale materials on the basis of structural characteristics, physicochemical properties and/or features of the bulk materials.

An *In Vivo* Approach to Evaluate Biodistribution and Potential Toxicity: Given the anticipated growth of the nanotechnology industry and the deficiency of toxicological information, there was an obvious need for the development of rapid, relevant and efficient testing strategies to evaluate the biological activity and toxic potential of novel nanomaterials.

Here we present an approach that utilizes a dynamic whole animal (*in vivo*) assay to reveal whether a nanomaterial is potentially toxic at multiple levels of biological organization (i.e. molecular, cellular, systems, organismal). Embryonic zebrafish were chosen as a model organism for evaluating integrated system-level effects because: 1) zebrafish are vertebrates that share many cellular, anatomical and physiological characteristics with higher vertebrates, 2) numerous effects can be assessed visually (non-invasive) over the course of development due to the transparent nature of the embryos; 3) embryos develop rapidly with most body organs formed by 48 hours post-fertilization (hpf) so developmental endpoints can be evaluated promptly; 4) females produce hundreds of eggs weekly so large sample sizes are easily achieved for statistically powerful dose-response studies; and 5) many routes of exposure (i.e. ingestion, injection and dermal) can be assessed individually or in combination. Early developmental life stages are often uniquely sensitive to environmental insult, due in part to the enormous changes in cellular differentiation, proliferation and migration necessary to form required cell types, tissues and organs. Since molecular signaling underlies all of these processes and most toxic responses result from disruption of proper molecular signaling; early developmental life stages are perhaps the ideal life stage to determine if nanomaterials are potentially toxic.

Our working hypothesis is that the inherent properties of some engineered nanomaterials make them potentially toxic. Embryonic zebrafish toxicity assays were performed to define *in vivo* responses to nanomaterials and identify physicochemical properties that lead to adverse biological consequences. We evaluated the potential toxicity of commercially available carbon fullerenes (C_{70}, C_{60} and hydroxylated C_{60}) and dispersions of nanoparticulate metal oxides (aluminum oxide, titanium (IV) oxide, zirconium (IV) oxide, cerium (IV) oxide, gadolinium (III)

oxide, dysprosium (III) oxide, yttrium (III) oxide, homium (III) oxide, samarium (III) oxide, silicon dioxide, alumina doped, and erbium (III) oxide), and studied the *in vivo* biodistribution of polystyrene and CdSe fluorescent nanomaterials (FluoSphere® and Qdots®, respectively).

Toxic Potential of Carbon Fullerenes [C_{70}, C_{60} and $C_{60}(OH)_{24}$]: In order to evaluate the toxic potential of carbon fullerenes, embryonic zebrafish were evaluated for mortality, morphological malformations and developmental progression following waterborne exposure to graded concentrations of C_{60}, C_{70}, and hydroxylated-C_{60} [$C_{60}(OH)_{24}$]. For C_{60} and C_{70} exposures, concentrations above 200 parts per billion (ppb) resulted in 100% mortality during the first 48 hours of exposure. $C_{60}(OH)_{24}$ exposure did not result in significant mortality until the exposure concentration was above 800 ppb. LC_{50} calculated for C_{60}/C_{70}- and $C_{60}(OH)_{24}$-exposures were approximately 200 ppb and 800 ppb, respectively. Embryonic exposure to 200 ppb of C_{60} and C_{70} resulted in delayed development (approximately 12-20 hours), fin malformation, pericardial edema and yolk sac edema. Neither C_{60} nor C_{70} induced sublethal effects at concentrations higher than 200 ppb due to the high mortality observed at these concentrations within the first 24 hours of exposure. Low concentrations of $C_{60}(OH)_{24}$ (<500 ppb) did not elicit a significant response; whereas, concentrations over 500 ppb induced pericardial edema, yolk sac edema and fin malformations. The specificity of the malformations observed in developing fin regions is indicative of signaling perturbation during early development. Exposure to 1000 ppb $C_{60}(OH)_{24}$ resulted in an overall swelling of embryos and delayed development (approximately 15-20 hours). These results are consistent with published cell culture evaluations and support the predominant belief that hydroxylated-C_{60} is less toxic than underivatized C_{60}. This means that the toxicity of C_{60} can be diminished through appropriate functionalization.

Toxic Potential of Metal Oxide Nanoparticles: Embryonic zebrafish were waterborne exposed to nanoparticle dispersions of metal oxides to determine their relative toxic potential. Approximately half of the nanoparticulate metal oxides (aluminum oxide, titanium (IV) oxide, zirconium (IV) oxide, cerium (IV) oxide, gadolinium (III) oxide) tested were benign to embryonic zebrafish after a 5-day continuous exposure at concentrations ranging from 16 ppb to 250 parts per million (ppm). Significant mortality occurred when embryos were exposed to 250 ppm of holmium (III) oxide or dysprosium (III) oxide, and 50 ppm of erbium (III) oxide or samarium (III) oxide. In addition to the increase in mortality, erbium (III) oxide, samarium (III) oxide and dysprosium (III) oxide elicited significant morphological malformations at 10, 50 and 250 ppm, respectively. Yttrium (III) oxide and alumina-doped silicon dioxide caused significant morphological malformations at 10 and 250 ppm, respectively, but did not cause an increase in mortality.

Differential Distribution of Nanomaterials: *In vivo* distribution of fluorescent nanomaterials was dependent on core structure, surface functionalization (amino-polyethylene glycol, carboxyl, organic, sulfate, aldehyde-sulfate) and route of administration (oral, injection, dermal). In order to investigate the influence that core structure and surface functionalization parameters have on uptake and biodistribution, zebrafish were waterborne exposed to carboxylate, sulfate or aldehyde-sulfate functionalized polystyrene nanoparticles (FluoSpheres®), or carboxylate, organic or polyethylene glycol functionalized CdSe quantum dots (Qdots®) from 144 to 168 hpf. The timing of this exposure targeted both dermal and oral routes of administration. Given the short duration of these exposures, dermal uptake was negligible. FluoSpheres®

accumulated and were retained within the gastrointestinal (GI) tract; whereas, all Qdots® were absorbed from the gut and distributed to other body regions. Qdots® functionalized with poly-ethylene glycol showed a strong distribution pattern to the brain. Distribution patterns correlated more with the composition of the core structure than surface functionalization.

To gain information on the influence that the route of exposure has on nanoparticle uptake and biodistribution, embryos were waterborne exposed or microinjected with (FluoSpheres®) functionalized with carboxyl, sulfate or aldehyde-sulfate groups. Data showed that the FluoSpheres® were readily taken up into developing zebrafish independent of surface functionalization. After 2 days of waterborne exposure, fluorescence was observed primarily in external epithelial (epidermal) tissues. After 3 days, fluorescence was observed in the heart and vasculature throughout the animal. By day 6, fluorescence was observed in the GI tract and excrement of the embryos, reflective of their newly acquired ability to eat. While waterborne exposure to FluoSpheres® resulted in the above-mentioned pattern of biodistribution, microinjections of the same FluoSpheres® showed a different distribution pattern. Once injected, the polystyrene nanoparticles were not distributed throughout the animal, even after 6 days; but instead, were retained within the yolk and GI tract of embryonic zebrafish. Surface functionalization played a minor role in uptake and biodistribution of these particular nanomaterials. Route of exposure was more predictive of biodistribution than surface functional groups. There were notable differences in the distribution pattern of FluoSpheres® with sulfate functional groups, with a delay in the time it took to enter the general circulation. Since our methods of evaluation are not currently quantifiable, the significance of this difference has yet to be determined.

Conclusions: Our research demonstrates the utility of the embryonic zebrafish model as an effective and accurate tool for rapidly assessing nanomaterial toxicity at minimal cost. This model is also well-suited to identify areas of relative distribution of nanomaterials that are fluorescently labeled. Information gained from this dynamic whole animal assay can be immediately applied to predict effects in other systems since genetic, cellular and physiological processes are highly conserved between zebrafish and humans, especially early in development. Furthermore, that information is useful as feedback for engineers designing novel nanomaterials, such that they can take into consideration potential toxicity and ensure the development of materials that have the least amount of toxic potential.

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