Biodistribution and Toxicity of Nanomaterials *In Vivo*: Effects of Composition, Size, Surface Functionalization and Route of Exposure

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ABSTRACT

Rapid, relevant and efficient testing strategies are necessary to evaluate nanoparticle-biological interactions of emerging nanoparticles/nanomaterials due to the anticipated, unprecedented growth of the nanotechnology industry. Here we present an approach that utilizes a dynamic whole animal (embryonic zebrafish) assay to identify *in vivo* responses to nanomaterial exposure and to define physicochemical properties that result in adverse biological consequences. Our results demonstrate the utility of this model as an effective and accurate tool to assess the biological activity and toxic potential of nanomaterials in a short period of time with minimal cost.

Keywords: nanotoxicology, carbon fullerene, metal oxide, fluorescent, gold nanoparticles

1 PROACTIVE NANOTECHNOLOGY

Atomic level manipulation, a hallmark of the promising nanotechnology industry, should permit control over nanomaterial physicochemical properties as well as their interactions with biological systems. Thus, it should be feasible to minimize adverse biological interactions once the physicochemical properties that dictate those interactions are identified. Engineers and scientists must work together to provide this critical information to industry so that they can proactively design nanomaterials with enhanced performance and minimal hazard.

2 MODEL SYSTEM FOR TESTING NANOPARTICLE-BIOLOGICAL INTERACTIONS

Numerous biological models have been employed for toxicological evaluations. Like many models, much of the anatomy and physiology of fish is highly homologous to humans [1]. Zebrafish have been successfully used as an *in vivo* model organism for predictive toxicology and are now proving invaluable for the pharmaceutical and biotechnology industries for evaluating integrated system effects [2, 3]. A remarkable similarity in cellular structure, signaling processes, anatomy and physiology exists among zebrafish and other high-order vertebrates [4]. In addition, zebrafish possess all of the classical sense modalities, including vision, olfaction, taste, touch, balance and hearing; and their sensory pathways share an overall homology with humans.

2.1 Inherent Advantages

Numerous features of zebrafish biology (e.g. small size, rapid embryonic development, short life cycle), make this model system logistically attractive to rapidly evaluate nanoparticle-biological interactions [5]. Females produce hundreds of eggs weekly, so large sample sizes are easily achieved for statistically powerful dose-response studies [6]. This abundant supply of embryos also makes it possible to simultaneously assess the toxicity of a large number of nanomaterials in a short period of time. Zebrafish embryos develop externally and are optically transparent so it is possible to resolve individual cells *in vivo* across a broad range of developmental stages or throughout the duration of an experimental exposure using simple microscopic techniques. Resolution of specific cell populations can be increased by the use of transgenic zebrafish models that express fluorescent reporter genes in cell types of interest [7]. Finally, assay volumes using the zebrafish model are small; thus, only limited amounts of nanoparticles/nanomaterials are needed to evaluate biological responses.

2.2 Rationale for Exposure of Embryonic Life Stage

We investigate whole animal biological responses (i.e. organismal uptake, systemic distribution and toxicological effects) by detailing the effects of nanoparticle exposure on embryonic zebrafish. Our experimental design tests for nanomaterial toxicity during early vertebrate development for two important reasons. First, fundamental processes of development are highly conserved across species [8]. Second, vertebrates at the earliest life stages are often more responsive to perturbation [9]. Highly coordinated cell-to-cell communications and molecular signaling are required for normal development. Nanomaterials that interact with

molecular signaling pathways, intercellular interactions, or normal cellular processes can be identified by evaluating the response of actively developing organisms to nanomaterial exposure.

3 NANOPARTICLES/NANOMATERIALS

To investigate the relative importance of size and surface functionalization on the toxic potential of nanomaterials, we evaluated carbon fullerenes (C_{70}, C_{60} and hydroxylated C_{60}) in embryonic zebrafish. Fullerenes C_{60} have proposed uses in fuel cells, groundwater remediation, cosmetics and drug delivery. C_{70} is a common by-product of C_{60} synthesis, and will therefore likely be found in products containing C_{60} unless extensive purification steps are taken. C_{70} is slightly larger than C_{60}, as it contains 10 more carbon atoms than C_{60}. Differences in toxicity between these two fullerenes were used to evaluate the influence of size. Surface functionalization of C_{60} with hydroxyl groups produces a more water soluble derivative $C_{60}(OH)_{24}$. Similarly to C_{60}, hydroxylated C_{60} has proposed uses in groundwater remediation and drug delivery [10, 11]. Differences in toxicity between C_{60} and $C_{60}(OH)_{24}$ were used to evaluate the influence of surface functionalization.

Gold nanoparticles (AuNPs) were also used to test the influence of size and surface functionalization on toxic potential. Our choice of two core sizes (0.8 and 1.5nm) with one of three surface groups [neutral charge = 2-(2-mercaptoethoxy) ethanol (MEE), positive charge = N,N,N-trimethylammoniumethanethiol (TMAT), and negative charge = 2-mercaptoethanesulfonate (MES)] allowed us to also investigate the influence nanoparticle charge on toxic potential. During the last decade, methods have been developed to synthesize a library of ligand-functionalized gold nanoparticles (AuNPs) that have precise size, shape and purity[12]. These materials have potential applications in optics, electronics, *in vivo* molecular imaging and therapeutics.

To investigate the importance of chemical composition on nanoparticle-biological interactions, we evaluated 11 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]. We chose to evaluate commercially available materials for two important reasons. First, nanoparticles produced on a large-scale are not expected to be pure. Second, nanoparticles that are currently commercially available are already being used for industrial applications [13]. Nano-sized metals and metal oxides have unique properties useful for novel applications in electronics, healthcare, optics, technology and engineering industries. Some metallic nanoparticles, particularly bimetallics, are currently being tested for remediation of organic groundwater contamination, chelation of toxic metals and *in vivo* biomedical imaging. Nanoparticulate metal oxides also offer many advantages for sensors, catalysis and microelectronics applications.

In vivo biodistribution of nanomaterials was investigated using polystyrene and CdSe fluorescent nanomaterials (FluoSphere® and Qdots®, respectively). Qdots® (605ITK-carboxyl QDs, 605ITK-amino(PEG) QDs and 605ITK-organic QDs) were generously donated by, and FluoSphere® (0.02µm sulfate, carboxylate, and aldehyde-sulfate modified fluorescent spheres) were purchased from Invitrogen/Molecular Probes (Eugene, OR). Both nanomaterials have novel applications in the fields of biomedical imaging, drug delivery and electronics. These engineered materials demonstrate a wide range of physicochemical properties dependent upon inherent characteristics and environmental conditions. The intent of these studies was to identify how those properties affected nanoparticle uptake and biodistribution.

4 EXPERIMENTAL DESIGN

These studies aimed to investigate the effects of nanomaterial exposure on vertebrate systems using the unique advantages of the embryonic zebrafish model. Screening level toxicological testing was performed to determine *in vivo* responses to, and biological consequences of, nanomaterial exposure. Those results were used to identify inherent physicochemical properties that result in adverse biological consequences. Fluorescent nanomaterials were used to investigate how parameters such as surface functionalization, chemical composition and route of exposure (dermal, injection and oral) influence *in vivo* biodistribution.

4.1 Exposure Protocols for *In Vivo* Toxicological Assessments

Embryonic zebrafish were obtained from an AB strain of zebrafish (*Danio rerio*) reared in the Sinnhuber Aquatic Research Laboratory (SARL) at OSU. Adults were kept at standard laboratory conditions of 28°C on a 14h light/10h dark photoperiod. Embryos collected from group spawns were staged for experimental studies. To avoid barrier effects posed by the chorion (egg membrane), embryos staged at 6 hours post fertilization (hpf) were dechorionated using pronase enzyme degradation.

To simulate dermal exposure, 8 hpf embryos were continuously waterborne exposed at 28°C in individual wells of a 96-well plate (N = 24 per treatment) until 120 hpf. Exposures were started at 8 hpf to ensure coverage of gastrulation and organogenesis, the periods of development most well conserved among vertebrates. For injection exposures, 8 hpf embryos were arranged in agarose molds and injected with 2 nl nanomaterial solution using a picoliter injection system. As controls, samples were injected with reverse osmosis water lacking nanoparticles. After injection, embryos were transferred to fish water in

wells of a 96-well plate and incubated at 28°C until 120 hpf.

4.2 Biodistribution Studies

Since distribution patterns were expected to depend on the route of exposure, we employed three different modes of administering FluoSphere® (40 ppm) and Qdots® (2 nM) to embryos. To simulate exposure by the dermal route, embryos were continuously exposed from 8-96 hpf in individual wells of a 96-well plate. Uptake via ingestion (oral route) was evaluated in animals waterborne exposed later in development (144-168 hpf) when the mouth is open and functional, and dermal tissues are less permeable. The third route of exposure we considered was injection. Microinjections were performed at 8 hpf on embryos with intact chorion. After injection, embryos were allowed to mature to 96 hpf. For evaluations, embryos were anesthetized with tricaine and mounted on a glass cover slip in 3% methyl cellulose. Embryos were visualized using an inverted Axiocam fluorescent microscope and photos captured using Axiovert software.

5 EVALUATION OF BIOLOGICAL RESPONSES

The principal characteristics that may be predictive of nanoparticle-biological interactions have yet to be identified. Embryonic zebrafish exposed to a variety of nanomaterials were evaluated for mortality, morphological malformations, behavioral abnormalities and developmental progression.

5.1 Influence of Size and Surface Functionalization

Our evaluations of exposure to graded concentrations of fullerenes [C_{60}, C_{70}, and $C_{60}(OH)_{24}$] revealed that surface functionalization had a greater effect on toxicity than size. Exposure to C_{60} and C_{70} significantly increased mortality and the incidence of pericardial edema and fin malformations, while the response to $C_{60}(OH)_{24}$ exposure was less pronounced even at concentrations an order of magnitude higher.

Core size and surface functionalization both influenced the toxicity of AuNPs. We found a strong dependence on surface charge and a moderate influence of particle diameter. Exposure to positively charged AuNPs resulted in significantly higher toxicity than for negatively charged particles, while neutral particles exhibited no toxicity. AuNPs functionalized with TMAT caused a significant increase in mortality at 10 parts per million (ppm) for 1.5 nm particles and 400 parts per billion (ppb) for 0.8 nm particles. Exposure to MES-AuNPs did not result in increased mortality at concentrations up to 250 ppm; however, concentrations of 2 and 50 ppm did result in increased incidence of morphological malformations at 1.5 and 0.8nm particles, respectively. Embryos exposed to 1.5 nm TMAT functionalized nanoparticles also displayed increased incidences of malformations at 50ppm. Such malformations were observed at a much lower concentration (80 ppb) when the TMAT-AuNP size was 0.8nm.

5.2 Influence of Chemical Composition and Route of Exposure

Of eleven metal oxide nanoparticulates tested, approximately half were benign to embryonic zebrafish after a 5-day continuous waterborne exposure at concentrations ranging from 16 parts per billion (ppb) to 250 parts per million (ppm). Significant mortality was observed at 50 ppm for Er_2O_3 and Sm_2O_3, and at 250 ppm for Ho_2O_3 and Dy_2O_3. Significant morphological malformations were induced by waterborne exposure to Er_2O_3, Sm_2O_3 and Dy_2O_3 at concentrations of 10, 50 and 250 ppm, respectively. Exposure to Sm_2O_3 significantly increased the incidence of jaw, heart, eye and snout malformations at 50 ppm. Exposure to SiO_2/Al_2O resulted in a significant incidence of jaw malformations at 250 ppm. At 10 ppm, Er_2O_3 exposure elicited jaw malformations in 44% of embryos after 5 days. Exposure to 50 ppm Er_2O_3 significantly increased the incidence of jaw, heart, eye, snout, trunk and body axis malformations. Dy_2O_3 exposure significantly affected the jaw and eyes at 250 ppm. Embryonic exposure to Y_2O_3 significantly increased the incidence of jaw malformations at 10 ppm and the incidence of jaw and heart malformations of embryos exposed to 250 ppm.

Microinjections of metal oxide nanoparticle dispersions were administered to embryonic zebrafish to test the effects of exposure via an injection route. Morphological malformations elicited by waterborne exposure to nanoparticulate metal oxides were mimicked by injection exposures for Sm_2O_3 (Figure 1) and Y_2O_3. No significant morbidity or mortality was observed from any of the nanoparticulate metal oxides when embryos were injected with approximately 0.5 ng nanoparticles.

5.3 Biodistribution Evaluations

In vivo distributions were determined for embryonic zebrafish exposed (waterborne, injection, oral) to fluorescent FluoSphere® and Qdots® in order to evaluate the influence of exposure route and surface functionalization on uptake and biodistribution. A timeline for uptake from waterborne exposures was determined for FluoSphere® with carboxylated surface functionalization. Waterborne FluoSpheres® were observed in external epithelial tissues for the first 24 hours, in the vasculature by 72 hours and in the digestive tract by 144 hours. Distribution after uptake appeared to be greater for Qdots® than for FluoSpheres®, independent of the route of

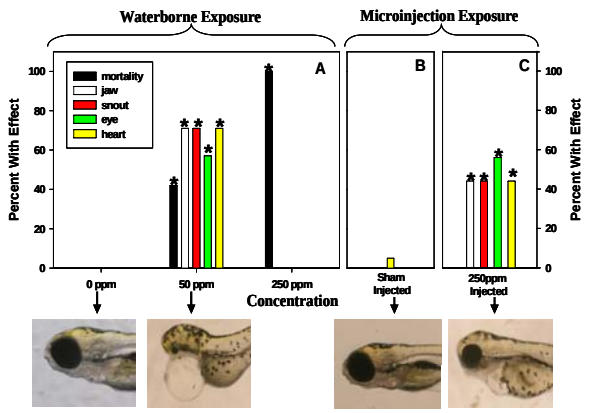

Figure 1. Morbidity and mortality of embryonic zebrafish (A) waterborne exposed to 50 or 250 ppm samarium (III) oxide, (B) sham injected with 2 nl water, and (C) microinjected with 2 nl of 250 ppm (~0.5 ng) samarium (III) oxide. * indicates significant difference from waterborne or microinjection control groups using Fisher's Exact test ($p<0.05$).

exposure (Figure 2). Uptake from a dermal route was primarily limited to the epithelial layers and the yolk sac for carboxylated FluoSpheres®, but distribution to the brain region was achieved from waterborne exposure to Qdots®. Microinjection route also shows differential uptake and distribution. FluoSpheres® administered via the oral route of exposure were retained within the gastrointestinal tract; whereas, Qdots were readily taken up across the gastrointestinal tract and distributed to the brain. A comparison of carboxylate-modified Qdots® and FluoSpheres® revealed a strong influence of chemical composition on distribution independent of the surface functional groups.

6 CONCLUSIONS

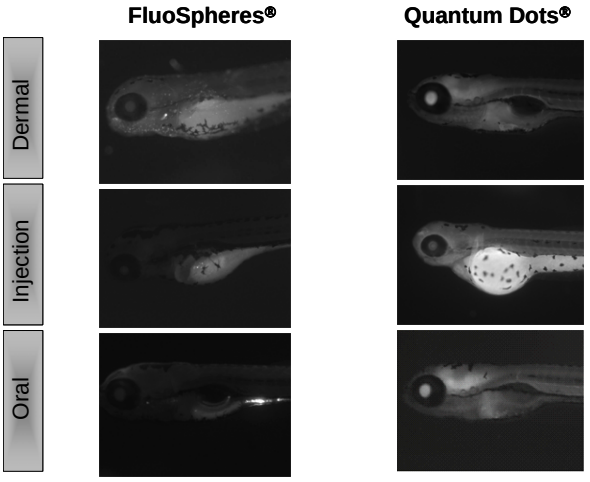

Figure 2. Biodistribution of FluoSpheres® and Quantum Dots® administered via waterborne (dermal and oral) or microinjection (injection) exposure.

Immense data gaps and conflicting reports on nanotoxicology currently prevent generalizing how nanoparticle physicochemical properties relate to biological activity and toxic potential. *In vivo* animal models, such as the zebrafish, are needed to interpret the effects of nanomaterial exposure in a whole animal context. Our results show that size was not a determining factor for the toxicity of carbon fullerenes or AuNPs. However, the size differences we evaluated were relatively small and thus limits our interpretation of the influence of size on nanomaterial toxic potential. Surface functionalization significantly affected toxicity of fullerenes and AuNPs yet did not dictate the biodistribution of fluorescent nanoparticles. Biodistribution was instead influenced by chemical composition as well as route of exposure. Chemical composition significantly influenced the toxicity of nanoparticulate metal oxides but the influence of exposure route was less pronounced, perhaps due to the amount injected. Overall, our results indicate that the zebrafish model is a powerful platform to help unravel nanomaterial structure and biological response relationships..

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