Using zebrafish to study the biological impact of metal and metal oxide nanoparticles

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Abstract: The often-surprising emergent properties include toxicity. The number of new nanoparticles is growing, requiring risk evaluation. This review highlights nanotoxicology studies that use zebrafish (*Danio rerio*) adults and embryos as model organisms. Zebrafish have been adopted because they are well-accepted vertebrate model organisms that can be obtained in large, simultaneously developing cohorts that are transparent and easy to observe. Development of organs is extremely rapid, and acute effects on internal organ formation can be observed in living animals. Zebrafish are inexpensive to maintain. Work studying metal and metal oxide nanoparticles has shown that toxicity can be caused by the nanoparticles directly, and by dissolution of the component elements. Studies have included assessments of morphological and transcriptional changes; addressed characterisation issues, uptake and possible organ/tissue targets, mechanisms of toxicity, dissolution, aggregation, and sedimentation. More recent work has explored long term effects of exposure as life cycle studies are now being reported.

Keywords: zebrafish; nanoparticles; nanotoxicity; oxidative stress.

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1 Introduction

One of the most dynamically growing areas of interdisciplinary research is the field of nanoscale engineering (Buzea et al., 2007; Caruthers et al., 2007; Farre et al., 2009; Moghimi et al., 2005). This sector of engineering focuses on the synthesis, characterisation, and application of materials that have at least one dimension in the < 100 nm range (Buzea et al., 2007; Krysanov et al., 2010; Nel et al., 2006; Oberdorster et al., 2005). Compared to larger solids, nanomaterials have more surface area in relation to their mass and novel quantum mechanical effects due to confinement of electrons to the nanosized space (Farre et al., 2009). These properties could theoretically increase reactivity and bioactivity per unit mass (Farre et al., 2009; Nel et al., 2006; Roco, 2005; Xia et al., 2006). As an extremely diverse group of compounds, nanomaterials have been produced in various shapes, sizes, compositions, surface charges, and functionalisations (Buzea et al., 2007; Grainger and Castner, 2008; Nel et al., 2006; Oberdorster et al., 2005; Shaw and Handy, 2011). The promise of novel physicochemical properties and biological characteristics has led to a scientific race to discover more efficient and reliable ways to synthesise and characterise nanomaterials. Consequently, this has resulted in an exponential increase in nanomaterial production and associated applications (Caruthers et al., 2007; Hall et al., 2007). Conversely, the development of safety regulations has been slow moving (Handy et al., 2008; Roco, 2005). This is of concern because nanomaterials are currently in use or in development, and may have the potential to cause toxicity following their release into the environment during development, manufacturing, use, and disposal (Farre et al., 2009; Handy et al., 2008; Klaine et al., 2008). The rapid development and use of nanomaterials will certainly continue, and so there is an increasing need to evaluate the materials' inherent toxicities (Klaine et al., 2008).

Additionally, this review highlights nanotoxicology studies using zebrafish adults and embryos. Studies included in this review addressed nanoparticle suspension, aggregation, sedimentation, and uptake from food and water. The studies also assessed morphological and transcriptional changes, tissue targets, and mechanisms of toxicity.

This review focuses primarily on metal and metal oxide nanoparticles, but a brief overview of the use of zebrafish in studying toxicity of carbon-based nanomaterials is

presented. We discuss steps that have been taken to mimic the natural aquatic environment in the lab to carry out nanotoxicology studies and work to evaluate oxidative stress as a mechanism of action behind some nanomaterial induced toxicities.

1.1 Zebrafish as a model organism

Whole animal toxicity studies have relied heavily on mammalian models ranging from rodents to primates. Somewhat more economical are tests with cell culture models and model invertebrates such as *Drosophila* and nematodes. While this latter approach is often less expensive and more mechanistically instructive, vertebrate models reveal complexities not found in the more reductionist systems. Zebrafish (*Danio rerio*) provide an intermediate ground: as vertebrates they maintain strong conservation with mammals, yet being small, transparent and inexpensive, they share many of the advantages of the simpler systems (Hill et al., 2005; Spitsbergen and Kent, 2003; Teraoka et al., 2003; Zon and Peterson, 2005).

The zebrafish model was first established for developmental biology and vertebrate genetics work, but has steadily gained popularity as a reliable and flexible organism for environmental and biomedical toxicology studies. Developing zebrafish are of special interest to toxicologists because of their sensitivity to chemical insults (Parng 2005; Rubinstein, 2006). Development involves an abundance of conserved molecular signalling events that coordinate cellular communication as the single cell egg grows into an embryo. Since foreign chemicals can resemble and interfere with these signalling molecules, toxic perturbations of these pathways can lead to altered development and malformations. Thus, these toxic events are more easily recognised during development, although they may also occur with more subtle results later in life. Therefore, embryonic zebrafish are highly suitable for studying biologic responses to xenobiotics, including nanomaterials. Some advantages are listed below.

- *Low cost*. Zebrafish are extremely small (adults: 3–4 cm; embryos: ~4 mm). This means that many can be kept in a relatively small space with low cost equipment (Teraoka et al., 2003; Westerfield, 2000; Zon and Peterson, 2005). In addition, for any assay, relatively low amounts of chemicals will be needed. Both of these are especially true for assays involving embryos, which live comfortably in 96 or 384 well microtiter plates.

- *Mutant lines*. Many large-scale genetic screens have yielded mutant zebrafish lines that are useful models for specific diseases. Loss or gain-of-function mutations also allow manipulation of pathways to produce disease models or test specific mechanisms. In addition, because of the small size and nutrient reserves provided in the egg, developing zebrafish can complete organogenesis with major defects that would normally terminate pregnancy in a developing mammal. An example is the silent heart mutation (*sih*) inactivating cardiac troponin (Stainier et al., 1996). This produces a developing zebrafish larva with no heartbeat or circulation. These fish continue development and can be studied for at least a week despite this catastrophic defect in the heart. In this example, the effects of defective circulation can be studied under circumstances in which other species simply would not survive to be examined.

- *Speed*. Zebrafish have an equivalent longevity to that of a lab rodent as well as a similar generation time (Pyati et al., 2007; Spitsbergen and Kent, 2003). However, fecundity is several of orders of magnitude greater. A female fish can produce 100 eggs in a single morning. Embryos complete organogenesis within only a week (Hill et al., 2005; Rubinstein, 2006; Spitsbergen and Kent, 2003; Teraoka et al., 2003). Sets of thousands of embryos developing in parallel under identical conditions can be scored manually in detail or *en masse* using robotics and computer programs. These small fish are ideal for study as individuals and also lend themselves to high-throughput screening. An early report concluded that inbreeding of zebrafish revealed recessive traits leading to malformation, making zebrafish unsuitable for toxicity testing (Piron, 1978). These problems were likely based on the use of fish obtained from pet stores. However, this demonstrates that the possible advantages of zebrafish were recognised 35 years ago.

- *Optical transparency of embryos*. Zebrafish eggs are fertilised externally in water, and from the first cell division the internal structures can be easily seen through the transparent embryo (Hill et al., 2005; Spitsbergen and Kent, 2003; Teraoka et al., 2003). This allows visualisation of early pattern formation, cell migration, and organogenesis as they normally occur and as they are altered by toxicants. Pigmentation increases with development; however, this can be prevented by chemical treatment or by using albino mutants.

- *Transgenic lines*. Transgenic reporter lines expressing cnidarian fluorescent proteins such as Green Fluorescent Protein (GFP) from promoters of interest make it simple to follow the effects of toxicants on gene expression (Kwan et al., 2007). This can be done in detail or robotically in large-scale screening. In addition, transgenic lines allow fate mapping during development as well as manipulation of gene expression in temporal or tissue-specific fashion. Such lines include heat shock or chemical induction of a protein, or the use of a promoter specific to the heart, nervous system or other tissue. Altered proteins with ether dominant negative or constitutively active effects can also be expressed. Gene knockouts have proven difficult, but techniques for producing them are being developed. In addition the injection of morpholino oligonucleotides can knock down the expression of specific proteins. Overall, this ability to tailor the zebrafish strain to the study is a major strength of the model for nanotoxicology.

1.2 Zebrafish are vertebrates

Some of the advantages listed above can be found in other model systems. Certainly nematodes and *Drosophila* are examples of powerful models, as are cultured mammalian cells. However, none of these are whole vertebrates, limiting experimental interpretation. Zebrafish development proceeds largely along mammalian lines and includes the formation of an internal skeleton and somites that have direct counterparts in humans. Tissue types inherent to mammals are also found in fish (Parng, 2005; Pyati et al., 2007). Fish possess many of the same defences against toxic chemicals as mammals, including an oxidative stress response and induction of Phase I and II metabolising enzymes (Langheinrich, 2003; Parng, 2005; Rubinstein, 2003; Zon and Peterson, 2005). In addition, the complexity of chemical distribution and metabolism as well as signalling

between organ systems is captured within the zebrafish: something that is difficult to achieve with cells in culture.

During the past decade, the zebrafish has become a favoured model for toxicology studies, and now nanotoxicologists are following this trend.

2 Zebrafish and metal-based nanoparticles

Metallic and metal oxide nanoparticles are widely used in applications as varied as biomedical imaging, fuel cells, antimicrobial coatings, and remediation of ground water and air contamination (Griffitt et al., 2008). Widespread use and large-scale production will ultimately lead to some degree of deposition of these materials in the aquatic environment (Cheng et al., 2007; Farre et al., 2009; Griffitt et al., 2008; Klaine et al., 2008). Once in the aquatic environment, the fate of these materials can be complex. The metal and metal oxide nanoparticles tend to aggregate; they may release soluble ions, and in some cases will interact with sediments and organic matter (Griffitt et al., 2009, 2008; Klaine et al., 2008).

This complexity enlarges the already enormous task of assessing the toxicity and safety of these materials. Not only are there many nanoparticles to be assessed, but also the properties of these particles can change after exposure to a normal aquatic environment. Assessment of multiple particle types under various conditions requires a system that allows for rapid simultaneous screening. The zebrafish model is the perfect fit for such screening requirements.

2.1 Elemental nanometals

Some general conclusions can be drawn from zebrafish studies of potential toxicity of nanometals. First, chemical composition is a very important factor in toxicity. Second, the release of metal ions, while often contributing to toxicity is not always sufficient to explain the toxic effects of the nanoparticles: the particle nature of the material can produce toxic effects that are distinct from metal ion toxicity. Examples of both types of toxicity identified using zebrafish are presented below.

2.1.1 Copper and nickel nanoparticles and their corresponding metal ions

Zebrafish have been used to test the idea that metal nanoparticle geometry might be a more relevant factor in producing toxicity than particle size. Ispas et al. (2009) examined the potency of different configurations of nickel nanoparticles (nNi) along with soluble nickel salts. Dechorionated embryos were exposed to lab-synthesised 30, 60, and 100 nm spherical nNi or dendritic clusters of 60 nm units at 24 hpf. The clusters were found to be more toxic than the spheres. On the other hand, the toxicity of the spheres was generally equivalent to or lower than those caused by equivalent amounts of soluble nickel salts.

Using histological methods, the authors discovered that all nNi exposures induced thinning of the intestinal epithelium as well as skeletal muscle fibre separation. However, nickel salt exposures caused skeletal muscle fiber separation only at extremely high concentrations, which led the authors to suppose that the toxicities of nNi and nickel salts were generated by different mechanisms (Ispas et al. 2009). In contrast another group using adult zebrafish found that nNi toxicity was due to dissolution (Griffitt et al., 2008).

Another study using copper nanoparticles indicated that metal dissolution was not sufficient to account for the observed nanometal toxicity. Adult female zebrafish, exposed for 48 h to commercially available 80 nm copper nanoparticles (nCu), led Griffitt et al (2007) to conclude that the nCu had toxic properties that could not be explained by dissolution of component metal ions. While dissolution of the nCu was observed, the Cu ions and nCu produced different patterns of morphological defects and gene expression changes. In experiments focused on gills of fish exposed to either soluble copper or nCu, differences in toxicity patterns indicated specific toxicity attributable to the nanoparticles themselves. The authors speculated that this included ionoregulatory toxicity, because one effect noted was a decrease in Na^+/K^+-ATPase activity.

A separate study by the same group using adult female zebrafish found that the gills responded differently to copper and silver nanoparticles (nAg) (Griffitt et al., 2009). The authors deduced that the toxic responses were due to different mechanisms. These authors claimed, however, that nAg and nCu toxicities did not occur due to dissolution (Griffitt et al., 2008). Nonetheless, the authors found that nanoparticles were less toxic than their corresponding soluble ions if the dose metrics are based on mass. The authors stressed that nanotoxicity does not appear to be a standard response to particles having nanoscale dimensions, but to the actual chemical composition of the nanometals.

One additional study in which zebrafish were used to investigate the toxicity of commercial nCu involved exposure of zebrafish embryos from 8 to 96 hpf (Bai et al., 2010a). The results were in agreement with other nCu toxicity studies, in that the particles showed aggregation and dissolution of ionic Cu. Along with mortality in the high dose exposures, observed sublethal effects included slowed hatching, decreased embryo length, formation of edema, and a bent tail. The authors concluded that the dissolved copper species contributed to, but could not account for all of the nCu toxicity.

2.1.2 Silver and gold: using zebrafish to explore the effects of size and solubility

One of the fastest growing areas in the nanotechnology industry is the use of nAg (Chen and Schluesener, 2008; Mueller and Nowack, 2008). Zebrafish have played an important role in assessing nAg toxicity. Two studies examined the toxicity of commercially available nAg (~20 nm) and found skin and blood vessel penetration (Yeo and Kang, 2008; Yeo and Yoon, 2009). One of these studies explored the distribution of Ag^+ ions and found penetration of organelles, nuclei, and the yolk; Ag^+ ion exposure also led to a lower survival rate in embryos (Yeo and Yoon, 2009). Toxic effects included a decreased hatching rate, apoptosis, a weak heartbeat, curved tail, pericardial edema, and a thin, string-like heart. Thus, distinguishing between the toxicity of the nAg themselves and the Ag^+ ions released is difficult.

To explore the relative roles of metal nanoparticles and metal ions released by dissolution, Powers et al. (2010) used citrate- or polyvinylpyrrolidone-coated 10 or 50 nm nAg and compared the resulting toxicity to that produced by dissolved Ag^+. They found that both Ag^+ and citrate-coated nAg delayed hatching, while only Ag^+ slowed swim bladder inflation and led to malformations and mortality. Behavioral assessments which used sequences of light/no light patterns and subsequent swimming responses showed that embryos became hyperresponsive to light changes after exposure to Ag^+, while citrate-coated particle exposures had no effect on the normal light response. Embryos treated with nAg coated with PVP had no observable physiological or morphological defects, but had changes in swimming patterns and responses to light. The smaller nAg-

PVP led to hypoactivity, while the larger led to hyperactivity in response to light. The authors argued that the behavioral toxic responses might not depend on Ag+ release but on size and surface coating instead.

Choi et al. (2010) exposed adult zebrafish to a commercially available preparation of 5–20 nm nAg which was treated to prevent or delay the release of Ag^+ ions. After the 24 h exposure the particles had formed agglomerates (50-300 nm in diameter) in cytoplasmic locations, most notably around the plasma and nuclear membranes. Metallothionein mRNA was found to be present in livers of exposed fish, which was indicative of possible Ag^+ leaching *in vivo* or generation of free radicals by the particles. Cellular changes such as disruption of hepatic cell cords and apoptotic changes were identified in livers of treated fish. Due to increases in malondialdehyde, a lipid peroxidation product, and total glutathione, the authors reasoned that the toxicity of nAg in zebrafish livers could be a result of oxidative stress and apoptosis.

Asharani et al. (2008) used nAg (5–20 nm) capped with either starch or BSA and found concentration-dependent hatching delays and slowed blood flow as well as increases in lethality, apoptosis, pericardial edema, and bradycardia. They reported that Ag^+ ions induced no toxic effects and transmission electron micrographs of the exposed embryos revealed nAg in the brain, heart, yolk, and blood. Another study by Asharani et al (2011) used newly fertilised zebrafish embryos and exposed them to gold (nAu) (15–35 nm), nAg (5–25 nm), and platinum nanoparticles (nPt; 3-10 nm) capped with polyvinyl alcohol (PVA). Results from the PVA-capped nAg were similar to the BSA- or starch-coated particles from the first study: nAu was found to be inert, while nPt caused hatching delays, decreased heart rate, and axial curvatures. The authors observed that the each type of particle was taken up, and that nAg was most toxic, while nAu was the least. In these studies the coatings are likely to have not only affected the surface properties of the nanoparticles but also would be expected to protect the particles from metal ion dissolution.

Uncovering mechanisms of nanotoxicity could be aided by emerging tools such as the capability to trace the path of nanoparticles in real-time. Two separate studies by the same group examined the transport of nanoparticles into the chorionic space using both nAg and nAu nanoparticles synthesised in the lab (Browning et al., 2009; Lee et al., 2007). Nanoparticles were transported through chorion pore canals, while embryonic development appeared to be affected stochastically due to the random diffusion and distribution of particles. The only difference between the two studies was that nAg was found to induce concentration-dependent toxic effects such as cardiac malformations (Lee et al., 2007), while nAu produced no clear trend of toxicity as the concentrations increased (Browning et al., 2009). Thus, the authors concluded that nAu is much more biocompatible than nAg and that nanoparticle toxicity is highly dependent on the nanometal's chemical composition.

More recently, Nel and colleagues used zebrafish embryos and cell lines to show that surface defects in nanoplate structures composed of Ag were instrumental in altering the toxic response (George et al., 2012). While coating the nanoplates with cysteine to passivate the surface resulted in reduced toxicity, the authors concluded that the reduced toxicity caused by surface defects was due to some other cause than altered release of Ag ions.

Another study exposed zebrafish embryos to nAg and nAu in four parallel sizes of 3, 10, 50, and 100 nm (Bar-Ilan et al., 2009). Embryos were exposed from 4–120 hpf to

particles synthesised in the lab with no surface functionalisation. The results consistently showed that nAg and nAu induced significantly distinct toxicity profiles, with the former being toxic and the latter being inert in all sizes. Observed toxic effects in nAg-exposed embryos included increased lethality; a nondepleted and opaque yolk; jaw, tail, and circulatory malformations; stunted growth; tissue degradation; and bent spines. Residual chemicals from the syntheses were found not to account for the observed nAg toxic effects. This study verified nanometal uptake using instrumental neutron activation analysis. The results of this study indicate that the toxic effects of the nAg can be primarily explained by the dissolved Ag+ released by the particles.

In a recent study, Harper et al. (2011) exposed fish to surface functionalised gold nanoparticles of known size and shape. They found that the toxic responses was quite different depending on these two variables. This study provides support for the idea that on a scaffold that does not readily erode to produce positive soluble products, the surface coating is of great importance.

A recent study by Cowart et al. followed the effects of nAg particles of two different sizes on developing zebrafish (Cowart et al., 2011). They found that the particles did not cause a defensive metabolic upregulation. By using different doses, they came up with a dose response relationship important in that data was expressed by mass and by particle number. By mass, the smaller 12 nm particles were more potent, but by particle number the larger 21 nm particles were more potent in impacting survival. Ag ion dissolution might explain this relationship. On a mass basis, the smaller particle, with more surface area, would be expected to dissolve more rapidly than the larger particles, and be more potent in delivering toxic Ag ions. On a particle basis, the larger 21 nm particle would deliver approximately 5 times more Ag than the smaller 12 nm particle. However, other models involving biological interactions with particles of different sizes are also possible. The use of both scales produces a clearer picture of the experimental results, even if that picture is more complex.

Despite being generally found to be inert, in one study nAu produced toxic responses in adult zebrafish (Geffroy, 2011). Exposure to nAu (12 and 50 nm) for 36 or 60 days resulted in altered transcript composition, mitochondrial dysfunction in brain and muscle tissues, and transcript changes indicative of apoptosis, oxidative stress, and DNA repair. Tissue accumulation was low, detected at only the highest dose. Although there are some disparities between the conclusions from the various nAg and nAu studies, possibly due to differences in particles, exposure paradigms, and dosing metrics, the nAg exposures generally are associated with more severe toxicity than that produced by nAu.

Taken together, these papers demonstrate the utility of zebrafish in studying the potential toxicity of nanosised metal particles.

2.2 Nano metal oxides

Metal oxide nanoparticles are a diverse group of materials that include titanium, zinc, cerium, aluminum, or iron. These are found in a wide range of applications including personal care products, nanomedicine, and environmental remediation programs (Shaw and Handy, 2011; Zhu et al., 2008). Zebrafish have been used in a variety of ways to assess the safety/toxicity of metal oxide nanoparticles. In general the results have suggested that cerium oxide and titanium dioxide nanoparticles produce minimal toxicity in developing zebrafish. However, recent work has shown that light intensity can make a radical difference in the toxicity of nanosised titanium dioxide ($nTiO_2$). Zinc oxide

nanoparticles have been shown to induce toxicity in developing and adult zebrafish, but some of the toxicity can be accounted for by zinc ions that are released from the nanosurface. Although there are differences in toxicity between the metal oxide nanoparticles, most have been found to have low bioavailability in acute exposure.

2.2.1 Zinc oxide nanoparticle toxicity in developing zebrafish: ion dissolution

Zebrafish embryos have been used to evaluate the toxicity of zinc oxide nanoparticles (nZnO). Three groups exposed blastula stage embryos to commercially available nZnO for 96 h (Bai et al., 2010b; Zhu et al., 2009, 2008). All three studies noted a decreased hatching rate, and Bai et al. found that nZnO (30 nm) led to reduced body length and tail malformations (Bai et al., 2010b).

Zhu et al. (2008) compared the toxicities of commercially available bulk and nanosised ZnO, TiO_2, and Al_2O_3. The TiO_2 and Al_2O_3 produced no toxicity in either bulk or nano forms. On the other hand, both nZnO and bulk ZnO induced similar toxic effects. Another comparison of nZnO and $nTiO_2$ with bulk materials used a 96 h exposure in adult zebrafish. In this study, the toxicity of $nTiO_2$ was greater than its bulk counterpart. However, for ZnO the LC_{50}s were similar for nZnO, bulk ZnO, and ionic Zn. (Xiong et al., 2011). This suggests that some if not all of the toxicity of nZnO could be due to released Zn ions.

Yu et al. exposed adult zebrafish for 96 h to 30 nm nZnO, bulk ZnO, and ionic zinc and found similar toxicity produced by bulk ZnO and nZnO (Yu et al., 2011). The authors concluded that zinc ions released by both forms of ZnO produced the observed toxicity.

To further explore Zn ion toxicity from nZnO, Xia et al. (2011) assessed the effects of iron doping on the surface of nZnO. This change of particle matrix was predicted to decrease Zn dissolution. Indeed, this approach reduced dissolved zinc and prevented toxicity. In contrast to the non-doped particles, the iron-doped nZnO no longer inhibited embryonic hatching from the egg chorion.

Some of these studies pointed towards oxidative stress as a mediator of nZnO toxicity. Experiments with adult zebrafish found not only higher concentrations of hydroxyl radicals in the nanoparticle suspensions compared to suspensions of the bulk materials, but also identified markers of oxidative stress in the liver, gut, and gills of the exposed fish (Xiong et al., 2011). Another study showed that even though in a highly aggregated state nZnO could still generate hydroxyl radicals (Yu et al., 2011).

2.2.2 Titanium dioxide nanoparticle toxicity in adult zebrafish

Nanosised titanium dioxide ($nTiO_2$) has been widely considered inert (Nel et al., 2006). This view is exemplified by the fact that in several zebrafish studies $nTiO_2$has been used as a nontoxic negative control (Griffitt et al., 2009, 2008; Zhu et al., 2008). One such study, conducted by Griffitt et al., used adult females exposed to various metal-based nanoparticles for 48 h (Griffitt et al., 2009). The $nTiO_2$ utilised in the study was commercially available and had a primary particle size of ~20.5 nm. Although no visible morphological toxicity was found, a microarray experiment performed on the gills of these $nTiO_2$-exposed fish demonstrated altered expression of genes involved in ribosomal function. The authors speculated that these gene expression changes might produce observable effects in a longer-term study.

Another focused on this type of chronic $nTiO_2$ exposure by conducting a 6-month study with 10 nm anatase-phase lab-synthesised particles and found a concentration- and time-dependent inhibition of growth (Chen et al., 2011). Through inductively coupled plasma-mass spectrometry (ICP-MS), this group was able to show that the particles accumulated in the liver, heart, brain, and gills. In accordance with the previously observed altered gene expression in the gills of fish exposed to $nTiO_2$ (Griffitt et al., 2009), this group found gill injuries that included thickening and edema of gill lamellae.

A shorter, chronic study using adult zebrafish exposed to commercially available anatase $nTiO_2$ for 13 weeks showed impaired reproduction, with a 29.5% reduction in the cumulative number of eggs collected (Wang et al., 2011). Furthermore, the embryos that were produced by the exposed fish had a low survival rate. The group identified a defect in folliculogenesis in the $nTiO_2$-exposed zebrafish and showed that the particles were able to enter the ovary and subsequently disrupt oocyte development.

2.2.3 Titanium dioxide and cerium oxide nanoparticle exposures in developing zebrafish: studies showing minimal toxicity

Besides using the adult zebrafish to conduct nanotoxicity studies, many groups have employed the embryo model to study the toxic effects of nanomaterials. Most $nTiO_2$ exposure studies using embryonic zebrafish have reported that the particles are biologically inert. However, some groups have found indicators of minimal toxicity. An important indicator of a toxic effect in embryos is hatching success: the embryo escaping from the acellular egg chorion envelope, normally at somewhere between 48–72 hpf. In one study, hatching success, survival, and malformations were assessed in zebrafish embryos exposed to commercially available waterborne Degussa P25 $nTiO_2$ (Chen et al., 2011). None of these were affected by the exposure. However, larval swimming velocities were significantly affected. Because $nTiO_2$ has the potential for producing reactive oxygen, the authors attempted to alleviate the toxicity by co-exposure to N-acetylcysteine, an antioxidant, and glutathione precursor. These pretreatments did not prevent the effects of $nTiO_2$ on swimming, suggesting that factors besides oxidative stress contribute to the behavioural changes. Because this group found behavioural toxicity, but no effects on morphology, they argue that perhaps those provide more sensitive means for detecting toxicity.

Although most studies use the water column for exposures in order to simulate environmental exposures, some authors microinject zebrafish with particles to bypass barriers to uptake and produce uniform dosing. Jovanovic et al. (2011) injected 48 hpf embryos with anatase $nTiO_2$. At 96 hpf, gene expression was assessed by microarray, and it was found that there were changes in genes related to circadian rhythm, kinase activity, intracellular trafficking, and immune response. Comparing the gene expression profiles induced by $nTiO_2$ to those produced by hydroxylated fullerenes ($nC_{60}(OH)_{24}$), it was discovered that although both nanoparticles downregulated similar gene sets, different clusters of genes were upregulated.

Cerium oxide nanoparticles have been found to be mostly inert. A study assessing toxicity using embryos exposed to commercially available 14, 20, and 29 nm $nCeO_2$ for 72 h found no signs of toxicity (Van Hoecke et al., 2009). Although some metal oxide nanoparticles are seemingly inert, further investigations should be conducted to identify physicochemical properties that could potentially cause toxic effects.

2.2.4 Titanium dioxide nanoparticles are toxic in developing zebrafish when illuminated

Many metal oxides act as semiconductors and can be photoactivated. This captured light energy can decay through contact with water and oxygen into the formation of reactive oxygen species (ROS) (Chatterjee and Mahata, 2002; Dunford et al., 1997; Wamer et al., 1997). ROS in turn can cause the formation of adducts on essential macromolecules such as lipids, proteins and nucleic acids. Therefore, light can potentially alter the toxic properties of substances such as $nTiO_2$. Bar-Ilan et al. (2011) exposed zebrafish embryos to Degussa P25 $nTiO_2$ for 5 d with and without illumination intended to mimic sunlight. The combination of illumination and $nTiO_2$ exposure produced stunted growth, jaw, yolk, and tail malformations, edema, and decreased survival. Thus, photoactivation transformed inert nanoparticles into lethal ones. Several lines of evidence confirmed that toxicity was due to increased ROS, and prior exposure to N-acetylcysteine, an antioxidant, prevented much of the toxicity.

2.2.5 Zebrafish, oxidative stress, and nanotoxicity

The increased surface area and reactivity of nanoparticles can in some circumstances lead to interactions with macromolecules that cause cellular damage (Nel et al., 2006; Oberdorster et al., 2005; Petersen and Nelson, 2010; Xia et al., 2009). Additionally, it may be that the ability to generate ROS *in vivo* is a property shared by many different types of nanoparticles (Choi et al., 2010; Oberdorster et al., 2005; Usenko et al., 2008). It is therefore not surprising that many zebrafish nanotoxicology studies discuss oxidative stress as a possible mechanism for the reported toxic effects. However, in many cases the mechanisms by which nanoparticles cause oxidative stress are not known (Oberdorster et al. 2005).

Zebrafish embryos are responsive to oxidative stress within the first 24 h of life (Kelly et al. 1998; Kobayashi et al., 2002; Kobayashi and Yamamoto, 2005; Li et al., 2008). Furthermore, oxidative stress can be observed in living zebrafish as it occurs, through the use of live imaging with ROS indicator dyes. More quantitative assays including the measurementof DNA adducts such as 8-OHdG, or the induction of antioxidant gene pathways using quantitative PCR are also well developed.

As in mammals, defense against oxidants in zebrafish is mediated by the transcription factor NF-E2-related factor-2 (Nrf2). Nrf2 binds to a *cis*-acting enhancer sequence known as the antioxidant response element (ARE), found upstream of genes encoding phase II detoxifying and antioxidant proteins (Kobayashi et al., 2002; Kobayashi and Yamamoto, 2005; Lee and Johnson, 2004; Li et al., 2008). As mentioned above, the activation of these genes can be directly measured. In addition, a transgenic zebrafish line, Tg(ARE:eGFP), has recently been generated in which GFP expression is controlled by ARE sequences (Bar-Ilan et al., 2011).

2.2.6 Metal oxide nanoparticles and bioavailability

For the first 48–72 hpf, embryos are surrounded by an acellular envelope, known as the chorion (Bonsignorio et al., 1996). Openings of 0.5–0.7 μm are present at the chorionic surface (Rawson et al., 2000), which in theory, could allow particles or aggregates of particles into the space between embryo and chorion and potentially access to the

organism itself. Dechorionation, or removal of the chorion enzymatically or manually, can result in greater uptake and access to the embryo earlier in development. In the phototoxicity studies described just previously, it was found that the egg chorion acted as a barrier to $nTiO_2$ uptake and toxicity (Bar-Ilan et al., 2011).

Besides using microinjection and water exposures, some research groups have started to assess oral exposure routes. In one such study by Johnston et al. (2010), zebrafish were exposed to nZnO (<100 nm) or $nCeO_2$ (<25 nm) for 7 d via the water column, and rainbow trout were exposed to $nTiO_2$ (<100 nm) via the water column (9 d) or through feeding (pellets for 21 d). In the water exposure groups, $nTiO_2$ was preferentially localised to rainbow trout gills, while significant amounts of $nCeO_2$ were found in the livers of zebrafish. Through dietary exposure, $nTiO_2$ was found in the gut and gills of rainbow trout. Overall, the authors have observed that metal oxide nanoparticles appear to have low bioavilability, but more studies are needed to confirm this as a general property.

2.3 Chalcogenides

Zebrafish have also been used to examine the toxicity of chalcogenide nanoparticles including lead sulfide nanoparticles (nPbS) and cadmium-selenide core/zinc-sulfide shell quantum dots (QDs). Truong et al. (2011) used a 120 h embryo assay to study the effects of nPbS. Dechorionated embryos were exposed to two sets of nanoparticles (3–3.5 nm): either coated with sodium 3-mercaptopropanesulfonate (MT) or coated with 2,3-dimercaptopropanesulfornate (DT). The nPbS-MT was more potent in producing toxicity with 100% mortality during the exposure, while the same concentration of nPbS-DT produced 5% mortality during the same time period. The nPbS-MT was found to decompose into ionic Pb more readily than nPbS-DT, and at concentrations as low as 20 ppm, the nPbS-MT exposures led to embryos with bent bodies and a malformed jaws and snouts. Despite the greater release of Pb ions, the nPbS-MT was more potent in causing mortality than the equivalent amount of lead ions. Therefore, the toxicity was likely the result of combined Pb toxicity and direct effects of the nPbS-MT. In contrast nPbS-DT caused less mortality than the equivalent concentration of Pb ions, leading the authors to conclude that the particles themselves were less toxic than the Pb they contained, consistent with the lower Pb release.

In using developing zebrafish to study the toxicity of cadmium-selenide core/zinc-sulfide shell quantum dots, King-Heiden et al. (2009) showed that the QDs produced some of the same effects that were produced by dissolved Cd. While this suggested that Cd plays a role in the toxicity of the cadmium-selenide core/zinc-sulfide QDs, the potency of the QDs was much greater than could be explained by CdCl dissolution. One possibility is that the QDs aid the Cd entry into target cells through a Trojan horse mechanism.

In the study described above, the toxicity was strongly influenced by the organic surface coating surrounding the QDs. Embryos were exposed to cadmium-selenide core/zinc-sulfide shell QDs functionalised with poly-L-lysine (PLL), methoxy-terminated PEG350-thiol (PEG350-OCH_3), or PEG5000-thiol terminated with carboxylate, methoxy, or amine functional groups (i.e., PEG5000-COO-, PEG5000-OCH_3, and PEG5000-NH_3). The different functional groups were selected to examine the effects of ligand chain length and terminal functional group on the toxicity. Exposure to QDs functionalised with PEG350-OCH_3 showed low potency, while the PLL-QDs were far more toxic

(King-Heiden et al., 2009). This was likely due at least in part to the toxicity of PLL itself.

2.3.1 Zebrafish matching the lab to the environment

Most studies using aquatic organisms have focused on particles in laboratory buffers and solvents. However the real aquatic environment is far more complex, with added factors including dissolved or suspended natural organic matter, light, generally low ionic strength in fresh water and high ionic strenghth in marine waters, and differing pH. In many cases the focus has been on solvent systems that retain monodisperse suspensions. However, studies examining nanomaterials under simulated environmental conditions are now emerging. Zebrafish will play an important role in these studies because they bridge an important gap: they can be maintained and studied under controlled conditions at high density, yet these conditions can model the real environment and the fish are an ideal model of an aquatic vertebrate.

One such study by Domingos et al. (2009) evaluated the effects of the natural organic matter known as Suwanneee River Fulvic Acid (SRFA) and the influence of pH and ionic strength on the stability of 5 nm anatase phase $nTiO_2$ suspensions. The group found that at a given pH, increased ionic strength led to increased particle aggregation. Furthermore, particles were less aggregated if SRFA was present. In general, the authors noted that the particles were stable under relevant environmental conditions.

Numerous studies have shown that natural organic matter can alter the behavior of nanoparticles, mostly by inhibiting aggregation. Hu et al. (2011) studied the combined effects of $nTiO_2$ and humic acid on the bioaccumulation of cadmium, a well-known environmental metal contaminant. Overall, the group found that cadmium associated with humic acid was less bioavailable, while cadmium associated with $nTiO_2$ was more bioavailable.

In some studies, TiO_2NPs have been used as inert controls. However, under normal sunlight the properties of TiO_2NPs would be expected to change. The study by Bar-Ilan et al (2011) attempted to incorporate light that resembled natural sunlight into experiments in which developing zebrafish were exposed to $nTiO_2$. As mentioned above, this greatly increased toxicity. Under illuminated conditions, the humic acid co-exposure has only a small effect on toxicity. In contrast, humic acid produced a significant increase in toxicity in the absence of illumination. The presence of dissolved organic matter decreased particle aggregation, which could account for some of the increased toxicity.

Another approach has been to carry exposure studies through whole-life experiments in which normal growth, behaviour, and survival to adulthood are recorded. In such a study, Tanguay and colleagues found that developmental exposures produced effects seen in young larvae that persisted into adulthood (Truong et al., 2012).

2.3.2 Many aquatic filter feeders survive on a diet of suspended nano-scale microbes

Daphnia are a useful environmental model because they feed on nanosized algae by filtering the surrounding medium at an average of 16.6 ml/h, hence interacting with a substantial amount of the aquatic environment (Lovern and Klaper 2006). *Daphnia* are also a good model for the types of organism eaten by small fish. Thus, the food web

study of Zhu et al. (2010), in which *Daphnia* were cultured with Degussa P25 $nTiO_2$ for 24 h and fed to adult zebrafish, is a very important model. Although the particles were transfered from *Daphnia* to zebrafish through dietary exposure, no evidence of biomagnification was found in these experiments. Because the authors discovered that there was a higher body burden of $nTiO_2$ in the dietary exposures, they reasoned that food consumption might be an important way for higher trophic level aquatic organisms to get exposed to nanomaterials.

3 Conclusions

The studies described illustrate the usefulness of the zebrafish as a model. However, interest in aquatic nanotoxicology predates the use of zebrafish, and other fish species have proven useful as well. For example the work of Federici and Ramsden with trout presaged much of what was done later using zebrafish to study TiO_2NPs (Federici et al. 2007; Ramsden et al. 2009). Numerous articles have been published using the trout as well as the *Medaka* to study nanoparticle toxicity. The size of trout can be an advantage in yielding larger tissue samples for assay. In addition trout physiology and pathology has long been studied. However, a single developing trout requires at least as much space as a plate carrying hundreds of developing zebrafish, with each well potentially representing a different exposure, transgenic zebrafish line, or mutant. Furthermore, zebrafish organogenesis is almost an order of magnitude faster than that of trout in cold water, allowing a string of zebrafish experiments to take place during the time it takes to follow one cohort of developing trout. *Medaka* is another fish species proven useful. The major advantage that zebrafish have over *Medaka* is the wide acceptance of the zebrafish as a biomedical model. This brings with it the resources of rapidly expanding technology and thousands of research reports with zebrafish as the focus.

The short and easily studied maturation of the organs is useful in identifying the toxicity produced by exposures lasting only a few hours or days. As long-term exposures are studied, the ability to establish simultaneously developing cohorts with large sample sizes, low requirements for space and low costs will prove very useful. The large variety of different nanomaterials being made or contemplated requires an approach capable of examining many different variables such as composition, surface preparation, shape, size, etc. This complexity ultimately produces a need for high throughput toxicology. Zebrafish are well suited to this requirement.

In addition to being a facile laboratory model, zebrafish also represent organisms that live in the aquatic environment; an environment subject to contamination. Because of the role that zebrafish play in biomedical research, it is sometimes forgotten that zebrafish are an excellent model for studying the effects of nanomaterials on aquatic organisms. In this regard, it is important to note that the aquatic environment exists as it is found in nature. Efforts to alter solvent systems and particle surface properties solely to maintain a monodisperse suspension of particles may prove useful in physical modeling, but are poor representations of the environment. Therefore, studies that focus on nanomaterials, as they will be found, after weathering, aggregation and interaction with natural materials are important to consider. In this regard, the food web approach mentioned above, while still restricted by a laboratory setting is noteworthy.

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