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# Comparative metal oxide nanoparticle toxicity using embryonic zebrafish

Leah C. Wehmas[a], Catherine Anders[b], Jordan Chess[b], Alex Punnoose[b], Cliff B. Pereira[c], Juliet A. Greenwood[d], Robert L. Tanguay[a,*]

[a] Department of Environmental and Molecular Toxicology, Environmental Health Sciences Center, Oregon State University, 1007 Agriculture & Life Sciences Building, Corvallis, OR 97331, USA
[b] Department of Physics and the Interdisciplinary Graduate Program in Biomolecular Sciences, Boise State University, 1910 University Drive, Boise, ID 83725, USA
[c] Department of Statistics, Oregon State University, Corvallis, OR 97331, USA
[d] Department of Biochemistry and Biophysics, Environmental Health Sciences Center, Oregon State University, 2011 Agricultural & Life Sciences Building, Corvallis, OR 97331, USA

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ABSTRACT

Engineered metal oxide nanoparticles (MO NPs) are finding increasing utility in the medical field as anticancer agents. Before validation of *in vivo* anticancer efficacy can occur, a better understanding of whole-animal toxicity is required. We compared the toxicity of seven widely used semiconductor MO NPs made from zinc oxide (ZnO), titanium dioxide, cerium dioxide and tin dioxide prepared in pure water and in synthetic seawater using a five-day embryonic zebrafish assay. We hypothesized that the toxicity of these engineered MO NPs would depend on physicochemical properties. Significant agglomeration of MO NPs in aqueous solutions is common making it challenging to associate NP characteristics such as size and charge with toxicity. However, data from our agglomerated MO NPs suggests that the elemental composition and dissolution potential are major drivers of toxicity. Only ZnO caused significant adverse effects of all MO particles tested, and only when prepared in pure water (point estimate median lethal concentration = 3.5–9.1 mg/L). This toxicity was life stage dependent. The 24 h toxicity increased greatly (~22.7 fold) when zebrafish exposures started at the larval life stage compared to the 24 h toxicity following embryonic exposure. Investigation into whether dissolution could account for ZnO toxicity revealed high levels of zinc ion (40–89% of total sample) were generated. Exposure to zinc ion equivalents revealed dissolved $Zn^{2+}$ may be a major contributor to ZnO toxicity.


## 1. Introduction

Engineering materials at the nanoscale results in unique characteristics valuable for applications in electronics, personal care products, environmental remediation and medicine [1,2]. The semiconducting properties of metal oxide nanoparticles (MO NPs) such as zinc oxide (ZnO) and titanium dioxide ($TiO_2$) make them particularly popular for use in commercially available sunscreens and cosmetics to block ultraviolet radiation when they are <50 nm in size [3]. Engineered MO NPs are finding

* Corresponding author. Tel.: +1 541 737 6514; fax: +1 541 737 6074.
*E-mail addresses:* wehmasl@onid.orst.edu (L.C. Wehmas), catherineanders@u.boisestate.edu (C. Anders), jchess@uoregon.edu (J. Chess), apunnoose@boisestate.edu (A. Punnoose), pereira@science.oregonstate.edu (C.B. Pereira), julie.greenwood@oregonstate.edu (J.A. Greenwood), robert.tanguay@oregonstate.edu (R.L. Tanguay).

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increasing utility in the medical field ranging from use as antimicrobial agents [3–6] to diagnostic imaging [7–13] and potential cancer treatment [5,9,14–16]. While scaling down the size of materials to the nanometer realm imparts useful traits, they are then within a size range to interact with biomolecules, such as proteins and nucleic acids, or organelles such as mitochondria, causing damage that could interfere with biological functions [1,2].

To date, most anti-cancer applications with engineered MO NPs have been demonstrated using cell lines [9,10,15,16]. Specifically, *in vitro* studies indicate ZnO nanomaterials generate reactive oxygen species, perturb calcium homeostasis within the mitochondria, disrupt cellular membranes, induce apoptosis and generate an inflammatory response [14,15,17,18]. $TiO_2$ nanomaterials, in the absence of photoactivation, require high parts per million exposure concentrations to affect gene transcription, cause DNA and chromosomal damage, and stimulate inflammation [19–21]. Upon photoactivation, $TiO_2$ nanomaterials generate more reactive oxygen species resulting in greater cytotoxicity to mammalian cells and bacteria [3,4]. Conversely, some cerium dioxide ($CeO_2$) nanomaterials scavenge reactive oxygen species enhancing cell survival in the presence of an oxidant [18,22], but these results are controversial as others have found the opposite effect [23,24]. Unfortunately, differences in experimental design and exposure concentrations can make cross study comparisons difficult, and evaluating the toxicity of these materials under culture conditions with a single or even a few cell types cannot adequately simulate a living dynamic organism, which can metabolize, sequester and excrete compounds. Before *in vivo* efficacy of these MO NPs as medical agents can occur, a better understanding of whole-animal nanomaterial toxicity is required. This could enable the engineering of safer nanomaterials for therapeutic applications. Despite a multitude of data on NP toxicity, data gaps still exist and the limited sample availability, common with nanomaterials under development, make *in vivo* nanotoxicology assessment particularly challenging using traditional mammalian models.

The embryonic zebrafish model has emerged as an inexpensive and efficient alternative for *in vivo* nanotoxicity screening [25,26]. This is, in part, due to the high degree of genetic conservation, anatomical and physiological similarity between zebrafish and humans particularly throughout development. Additionally, the small size, rapid growth and transparency of zebrafish embryos makes them conducive for moderate to high-throughput screening methods. Toxicity assays can be conducted in 96-well plates in which morbidity and mortality are visually assessed over a short duration. Multiple routes of nanoparticle exposure including epithelial absorption (primary), ingestion, and respiration (gill uptake) can be assessed along with identification of potential windows of developmental susceptibility to NPs. The small quantity of test material required to investigate *in vivo* toxicity in zebrafish is particularly advantageous.

Our laboratory assesses nanotoxicity using a well-defined five-day embryonic zebrafish assay [26–29]. Research by others assessing MO NP toxicity with the zebrafish model has primarily focused on characterizing ecotoxicological health risks [30–39]. These assays typically employ zebrafish embryos with intact chorions, an acellular envelope surrounding the embryo, which can obstruct NP uptake in a size dependent manner and potentially confound interpretation of concentration response results [30,39]. Furthermore, NPs are frequently coated with various natural organic matter to mimic aqueous environmental conditions, which will alter bioavailability [40–43]. Because our laboratory is interested in these NPs for medical applications, we enzymatically removed the chorion to mitigate barriers of NP absorption, and we do not coat the MO NPs with natural organic matter. Zebrafish exhibit a high degree of tolerance to varying water chemistry parameters such as salinity and pH allowing us to carefully adjust exposure conditions, such as reducing medium salt content to diminish agglomeration and enhance particle absorption [44,45].

The objective of these studies was to assess and compare the *in vivo* toxicity of seven semiconductor MO NPs made from zinc oxide (ZnO), titanium dioxide ($TiO_2$), cerium dioxide ($CeO_2$) and tin dioxide ($SnO_2$). In this article, we report the first *in vivo* toxicity assessment of these novel MO NPs compared to bulk controls using the embryonic zebrafish assay under two medium conditions of differing ionic strengths. While these MO NPs possess similar primary mean diameters and spherical shapes, they differ in physicochemical properties such as band gap, hydrodynamic size, charge, chemical composition and ionic state of metal ions, and reactive oxygen species generation. We hypothesized that differences in MO NP toxicity will depend on these physicochemical properties. In this study we investigated how hydrodynamic size and charge of uncoated, non-functionalized MO NPs in a waterborne suspension affected zebrafish toxicity. Most MO NPs caused little to no toxicity in our assay under either medium condition except ZnO. Similar to other aqueous systems, MO NP agglomeration complicates toxicological studies making it challenging to study primary particle characteristics, as these particles are not well dispersed, and ions present in the suspension medium can further enhance agglomeration and impede dispersal. However, it is important to note that agglomeration is an important parameter in particle hazard assessment. Despite agglomeration of all our MO NPs, particularly in the high ionic strength embryo medium, we successfully compared how size and charge were associated with the toxicity of three MO NPs: $TiO_2$ (TC009), $CeO_2$ (QK055) and ZnO, as they created stable suspension in low ionic strength water. While all three MO NPs possessed similar hydrodynamic sizes and similar, high positive charges under our assay conditions, only ZnO was significantly toxic to embryonic zebrafish. This data suggests that for MO NPs suspended in water, elemental composition or dissolution are principally important for producing toxicity in zebrafish.

## 2. Materials and methods

### 2.1. Nanoparticle synthesis

All MO NPs were produced through in-house synthesis. Bulk samples were purchased from commercially available