

# Toxicity Assessment of Iron Oxide Nanoparticles in Zebrafish (*Danio rerio*) Early Life Stages

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## Abstract

Iron oxide nanoparticles have been explored recently for their beneficial applications in many biomedical areas, in environmental remediation, and in various industrial applications. However, potential risks have also been identified with the release of nanoparticles into the environment. To study the ecological effects of iron oxide nanoparticles on aquatic organisms, we used early life stages of the zebrafish (*Danio rerio*) to examine such effects on embryonic development in this species. The results showed that  $\geq 10$  mg/L of iron oxide nanoparticles instigated developmental toxicity in these embryos, causing mortality, hatching delay, and malformation. Moreover, an early life stage test using zebrafish embryos/larvae is also discussed and recommended in this study as an effective protocol for assessing the potential toxicity of nanoparticles. This study is one of the first on developmental toxicity in fish caused by iron oxide nanoparticles in aquatic environments. The results will contribute to the current understanding of the potential ecotoxicological effects of nanoparticles and support the sustainable development of nanotechnology.

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## Introduction

Manufactured nanomaterials, defined as materials with at least one dimension between 1 nm and 100 nm [1], possess enhanced or even unique physicochemical properties, such as nanoscale size effects, quantum effects, increased surface area, and higher surface curvature as well as unique electric, thermal, mechanical, and imaging properties [2,3]. These excellent characteristics promise them a wide variety of applications and an increasing production. As of the end of 2011, more than 1300 nanotechnology products had appeared on the market [http://www.nanotechproject.org/inventories/consumer/analysis\_draft/. Accessed 02/25/2012]. And, it is estimated that nanotechnology will generate a market worth US\$1 trillion by 2015 [4–6].

Iron oxide nanoparticles (NPs), with the main forms being of magnetite ( $\text{Fe}_3\text{O}_4$ ) and hematite ( $\alpha\text{-Fe}_2\text{O}_3$  and  $\gamma\text{-Fe}_2\text{O}_3$ ), have attracted extensive interest for application because of their superparamagnetic properties and high catalytic abilities [7,8]. The related fields for the application of iron oxide NPs include terabit magnetic storage devices, pigments, catalysis, sensors, high-sensitivity biomolecular magnetic resonance imaging, tumor therapy, drug and gene transfer to cells, and labeling of macromolecules and cells [9–12]. Moreover, iron oxide NPs can be used as an adsorbent in the removal of metals from aqueous solutions [13–15]. A catalytic property of iron oxide NPs, which is widely used in laboratory tests and in the treatment of wastewater, has also been reported recently [16]. The increasing production and use of iron oxide NPs will inevitably result in a greater

exposure risk for both people and the environment. Thus, it has become essential to assess the potential health and environmental effects of iron oxide NPs on humans, non-human biota, and ecosystems.

Until recently, most studies on the potential effect or toxicity of iron oxide NPs have focused on mammals (such as mice and rats) and/or on different types of cell lines [17–21]. However, to date, very few studies have investigated the ecotoxicity of iron NPs, particularly in aquatic systems [22–24]. Zhu et al. (2008) were the first to report that pumpkin plants (*Cucurbita maxima*) grown in an aqueous medium containing iron oxide ( $\text{Fe}_3\text{O}_4$ ) NPs could absorb, translocate, and accumulate NPs in the plant tissues [25]. More recently, Nations et al. (2011) have reported that iron oxide ( $\text{Fe}_2\text{O}_3$ ) NPs decreased the snout-vent length (SVL) of *Xenopus laevis* tadpoles at a concentration as low as 0.001 mg/L. The SVL increased at 1 mg/L  $\text{Fe}_2\text{O}_3$  NPs and then steadily decreased at higher concentrations (10, 100, and 1000 mg/L). The total body length of *X. laevis* tadpoles exposed to 1000 mg/L  $\text{Fe}_2\text{O}_3$  NPs was also significantly reduced ( $p \leq 0.033$ ) compared with controls [23]. In addition, Garcia et al. (2011) conducted a series of acute ecotoxicity tests, including phytotoxicity using several plant seeds, aquatic toxicity using *Daphnia magna*, and a bioluminescent test (Microtox<sup>®</sup>) using the bioluminescent marine bacterium *Vibrio fischeri* as a model organism. Their results showed that iron oxide ( $\text{Fe}_3\text{O}_4$ ) NPs ( $\leq 0.67$  mg/mL) exhibited low or no toxicity on plant seeds. However, *D. magna* ( $\text{LC}_{50} = 23 \times 10^{-4}$  mg/mL) and *V. fischeri* ( $\text{EC}_{50} = 0.24$  mg/mL) demonstrated extreme sensitivity to iron oxide NPs, which indicates the high toxicity of iron oxide NPs in

aquatic environments [24]. Another form of iron oxide NPs, Fe<sub>2</sub>O<sub>3</sub> NPs, even at a low concentration of 1 mg/L, was also able to affect the hematological, biochemical, ionoregulatory, and enzymological parameters in an Indian major carp, *Labeo rohita*, upon a 96-h static exposure [26]. This finding suggests a high potential toxicity of iron oxide NPs in aquatic environments [26]. The above pioneering studies indicate that the release of iron oxide NPs into the environment may be harmful to various eco-relevant organisms, which highlights the need for further research into the environmental impact and biological effects of iron oxide NPs.

To further assess the ecological effect, especially the aquatic toxicity, of iron oxide NPs, we conducted an early life stage (ELS) test using zebrafish (*Danio rerio*) embryos and larvae as model organisms. The ELS test is currently one of the most widely used tools in environmental science research, especially for investigating the toxicity and teratogenicity of chemicals that could significantly affect environmental and human health [27,28]. The present study also aimed to assess whether conventional standardized tests, such as the ELS test, are useful in determining the ecotoxicity of NPs when no or insufficient data are available.

## Materials and Methods

### Iron oxide NPs and characterization

Uncoated alpha-Fe<sub>2</sub>O<sub>3</sub> ( $\alpha$ -Fe<sub>2</sub>O<sub>3</sub>) nanoparticles (nFe<sub>2</sub>O<sub>3</sub>) with a published particle size of 30 nm were purchased from Nanjing High Technology NANO CO., Ltd. (Nanjing, China). Supplied as a red powder, the nanoparticles had a purity of  $\geq 99.5\%$  and a specific surface area of 38.57 m<sup>2</sup>/g. Stock solutions (1000 mg/L) of nFe<sub>2</sub>O<sub>3</sub> were prepared by stirring nFe<sub>2</sub>O<sub>3</sub> vigorously in zebrafish culture medium (consisting of 64.75 mg/L NaHCO<sub>3</sub>, 5.75 mg/L KCl, 123.25 mg/L MgSO<sub>4</sub>·7H<sub>2</sub>O, and 294 mg/L CaCl<sub>2</sub>·2H<sub>2</sub>O) prepared according to International Organization for Standardization (Geneva, Switzerland) standard 7346-3:1996 [29], using a magnetic agitator at room temperature for 2 h. The morphology of nFe<sub>2</sub>O<sub>3</sub> was observed under a transmission electron microscopy (TEM, Hitachi H-7650, Japan).

The actual size distributions of nFe<sub>2</sub>O<sub>3</sub> in the culture medium were determined using a dynamic light scattering device (DLS; Brookhaven Instrument Corporation, Holtsville, NY, USA). Before carrying out the DLS measurements, no sound or ultrasound was applied to agitate the particles in the culture medium. Suspensions (10 mg/L) were introduced into a polystyrene disposable cuvette; and the size measurements were conducted immediately according to the manufacturer's guidelines. All DLS measurements were performed at 26°C, which was set in accordance with the water temperature in the exposure experiments. Thereafter, the average size of the nFe<sub>2</sub>O<sub>3</sub> aggregates was documented.

### Zebrafish culture and embryo selection

The detailed procedure for zebrafish culture and embryo selection is provided in a previous paper [28]. In brief, zebrafish adults with a roughly 2:1 male/female sex ratio were kept in a 250-L full glass aquarium under the following conditions: 26°C  $\pm$  1°C, 14-h/10-h light/dark cycle. Spawning was triggered once the light was turned on in the morning and completed within 30 min. At 4–5 h post-fertilization (hpf), embryos were collected and rinsed several times with the culture medium to remove residues on the egg surface. Healthy embryos at the blastula stage were then selected for subsequent experiments.

### Exposure process

Test solutions were prepared immediately prior to use by diluting the stocks of nFe<sub>2</sub>O<sub>3</sub> with the culture medium. During the preparation of the diluted solution, the stock solution/mixture was continuously stirred with a magnetic stirrer to maintain the suspension at as stable a concentration as possible. The embryo toxicity test design was followed according to a standard guideline [30] and the methods of Schulte and Nagel [31]. The embryo toxicity test was initiated as soon as the intact fertilized eggs were selected. Twenty-four eggs (blastula stage) were transferred to the test wells of a 24-well multiplate (Costar® 24Well Cell Culture Cluster, Corning Incorporated, NY, USA). Twenty wells were prepared with 2 mL nFe<sub>2</sub>O<sub>3</sub> test solution (treatment) each. The remaining four wells (control) were prepared similarly, with the culture medium replacing the test solution. The concentration gradients of nFe<sub>2</sub>O<sub>3</sub> tested in this study were 100, 50, 10, 5, 1, 0.5, 0.1 mg/L, and the water control. The experiment was performed in triplicate (i.e., 12 embryos were used in the water control and 60 embryos in the exposure group) for each treatment. The wells were covered with transparent plastic films to ensure a constant concentration. All the plates containing experimental embryos were placed in a fish room with controlled light and temperature conditions (i.e., 26°C  $\pm$  1°C with a 14-h/10-h light/dark cycle). At the end of the experiment, water samples were collected and immediately tested for quality assessment. All the experimental protocols were approved by the Animal Welfare and Ethics Committee of Tsinghua University (Shenzhen), China (No. 2012-XSZ-F58).

### Embryo-larval toxicity test

Throughout the whole exposure period after fertilization, the development status of the zebrafish embryos and larvae was observed under an inverse microscope ( $\times 10$ –40, DMLL, Leica Corp., Germany) and documented at specified time points ( $t = 6, 12, 24, 36, 48, 60, 72, 84, 96, 120, 144,$  and 168 h). The endpoints used to assess developmental toxicity included embryo/larva survival and embryo hatching rate. Malformations were described and documented among the embryos and larvae from both the control and treated groups. Inhibitory tendencies were also noted and described among the embryos and larvae from both the control and treated groups using a stereomicroscope ( $\times 0.67$ –6.7, Olympus, Japan).

### Statistical analysis

All experiments were repeated three times independently. Data were recorded as the mean with the standard deviation (SD). For the embryo/larval bioassays, a one-way analysis of variance (ANOVA) with Tukey's multiple comparisons was used to detect significant differences between the control and treated groups. A  $p < 0.05$  was considered statistically significant. The EC<sub>50</sub> (hatching delay) and LC<sub>50</sub> (mortality) values as well as their associated 95% confidence intervals (95% CI) were calculated using a tsk method (US EPA Tsk Analysis Program, Ver.1.5 <http://www.epa.gov/nerleerd/stat2/tsk.zip>). The no observed effect concentration (NOEC) value was designated as the highest tested concentration that had no statistically significant effect within the exposure period when compared with the control.

## Results and Discussion

### Characterization of nFe<sub>2</sub>O<sub>3</sub> NPs

In this study, the addition of nFe<sub>2</sub>O<sub>3</sub> to the culture medium induced the formation of aggregates. Figure 1 shows a confirmatory TEM image of the large aggregations of nFe<sub>2</sub>O<sub>3</sub> at

concentration of 10 mg/L. Further determined by DLS, the average size of the  $n\text{Fe}_2\text{O}_3$  aggregates was calculated to be 1.025  $\mu\text{m}$ , which is far larger than the  $n\text{Fe}_2\text{O}_3$  primary particle size of 30 nm. Moreover, these  $n\text{Fe}_2\text{O}_3$  aggregates were found to settle out of the water column rapidly. We did not monitor the sedimentation rate, but after 24 h a layer of  $n\text{Fe}_2\text{O}_3$  aggregates was observed at the bottom of the exposure well in the 24-well culture multiplate. The solution above was clearer, and it appeared as though all  $n\text{Fe}_2\text{O}_3$  aggregates had settled out.

### Survival

The survival of zebrafish embryos and larvae exposed to different concentrations of  $n\text{Fe}_2\text{O}_3$  was determined at specific time points. As shown in Figure 2A, 0.1 mg/L to 10 mg/L of  $n\text{Fe}_2\text{O}_3$  exhibited no toxicity to zebrafish embryos or larvae. Both 50 and 100 mg/L of  $n\text{Fe}_2\text{O}_3$  demonstrated toxicity, killing 75% and 45%, respectively, of the zebrafish embryos/larvae at the end of the exposure (168 hpf). As a result, the 168-h NOEC of  $n\text{Fe}_2\text{O}_3$  was less than 50 mg/L, and the 168 h  $\text{LC}_{50}$  was calculated to be 53.35 mg/L (95% CI: 28.21–100.89). Moreover, survival of the embryos was more than 90% at 48 hpf, but it declined sharply to 25% at 168 hpf. This result suggests that the zebrafish development toxicity of  $n\text{Fe}_2\text{O}_3$  is time and dose dependent.

### Hatching rate

The hatching rates of zebrafish embryos exposed to different concentrations of  $n\text{Fe}_2\text{O}_3$  at different development stages are shown in Figure 2B. The  $n\text{Fe}_2\text{O}_3$ -treated embryos showed a dose-dependent effect on the hatching rates under laboratory conditions. Compared with the control group, 0.1–5 mg/L of  $n\text{Fe}_2\text{O}_3$  did not significantly affect the hatching rate during the 168-h exposure time. However,  $\geq 10$  mg/L of  $n\text{Fe}_2\text{O}_3$  displayed significant ( $p < 0.05$ ) embryo-hatching delay and toxicity. Based on this result, the 168-h NOEC and  $\text{EC}_{50}$  of  $n\text{Fe}_2\text{O}_3$  on the

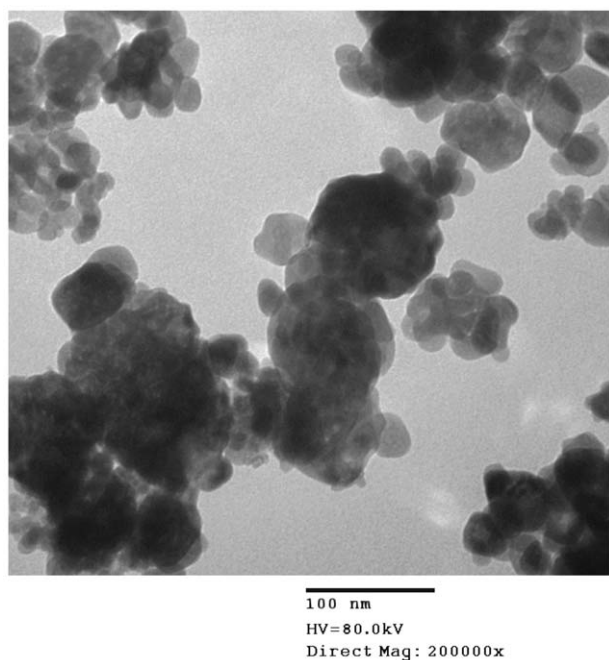
hatching rate were calculated to be 10 mg/L and 36.06 mg/L (95% CI: 20.63–63.02), respectively.

### Malformations

In this study, malformation mediated by  $n\text{Fe}_2\text{O}_3$  in the embryos and larvae from both the control and treatment groups was recorded. Malformation was not found in zebrafish embryos or larvae exposed to  $n\text{Fe}_2\text{O}_3$  at a concentration of  $\leq 10$  mg/L during the 168-h exposure time (Figure 3A). However, at a concentration of  $> 50$  mg/L, the embryos and larvae exhibited severe malformations, characterized by tissue ulceration, pericardial edema, and body arcuation (Figure 3). Some affected embryos were unable to hatch and eventually died (Figure 3). In the 50-mg/L treatment group, 12.5%, 25%, and 32.5% of the surviving embryos and larvae showed significant ( $p < 0.05$ ) tissue ulceration, pericardial edema, and body arcuation, respectively. Malformation was more serious in the embryos and larvae exposed to 100 mg/L  $n\text{Fe}_2\text{O}_3$ , although no significant difference was found from those in the 50-mg/L treatment group.

### Developmental toxicity of iron oxide NPs

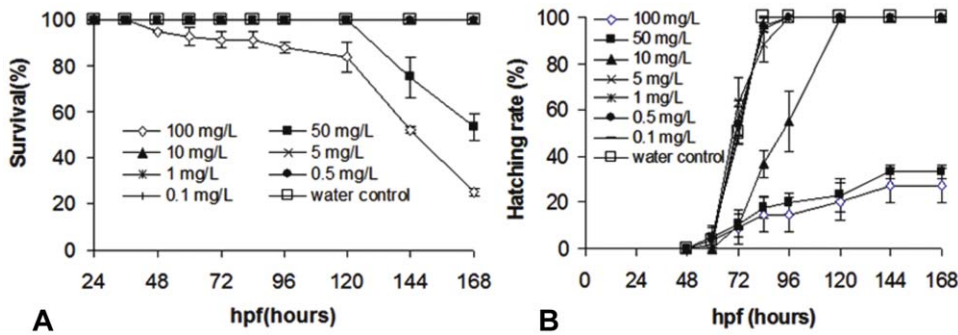
In this study, the addition of  $n\text{Fe}_2\text{O}_3$  to the culture medium resulted in the formation of aggregates that settled out of the water column very quickly. This aggregation and sedimentation phenomenon of  $n\text{Fe}_2\text{O}_3$  is similar to that of other NPs, including Cu, Ag,  $\text{TiO}_2$ ,  $n\text{ZnO}$ ,  $n\text{Al}_2\text{O}_3$ , fullerene NPs, and single-walled carbon nanotubes (SWCNTs) [32–39]. These findings revealed that aggregates or agglomerates of NPs are likely to settle out of solution and sink into sediments rather than remain in suspension. Thus, benthic organisms living in sediments or at the bottom of aquatic environments could be potential targets of NPs released into the environment. Zebrafish embryos are demersal and can settle to the bottom of the water column, which allows a mimicking of the direct contact between benthic biota and NPs in the sediment. Therefore, zebrafish embryos may serve as an effective model to explore the potential ecological effects underlying the toxicity of NP aggregates that settle out of the water column. In our experiment,  $n\text{Fe}_2\text{O}_3$  aggregates ( $\geq 10$  mg/L) were found to be toxic to zebrafish embryos and larvae, causing a dose-dependent mortality and hatching inhibition (Figure 2). Developmental abnormalities, such as pericardial edema, malformation, and tissue ulceration, were also found in the group exposed to 50 and 100 mg/L of  $n\text{Fe}_2\text{O}_3$  (Figure 3); these affected more than 12.5% of the surviving fish by 168 hpf. To our knowledge, the present study is one of the first to evaluate developmental toxicity in vertebrate fish caused by exposure to  $n\text{Fe}_2\text{O}_3$  in aquatic environments. Previous studies have shown that doses higher than 50 mg/kg of dimercaptosuccinic acid-coated iron oxide ( $\text{Fe}_3\text{O}_4$ ) NPs can disrupt mouse embryo development, causing a significant decrease in the growth of infant animals [11]. Moreover, Nations et al. (2011) demonstrated that although exposure to  $n\text{Fe}_2\text{O}_3$  (0.001 mg/L to 1000 mg/L) caused no mortality or significant malformation in frog (*Xenopus laevis*) embryos and larvae,  $n\text{Fe}_2\text{O}_3$  exposure did decrease tadpole SVL even under a low concentration of 0.001 mg/L [23]. These studies demonstrate that organisms in the early stages of embryonic development are usually more sensitive to toxicological effects. Thus, examining organisms at these stages can help evaluate the sublethal effect of NPs and distinguish the nature of the toxicological effect (e.g., neural toxicity and genotoxicity) [40,41].



**Figure 1. The transmission electron microscope (TEM) image of  $n\text{Fe}_2\text{O}_3$  aggregates (10 mg/L) in zebrafish culture medium.**  
doi:10.1371/journal.pone.0046286.g001

### Toxicity assessment of NPs using the ELS test

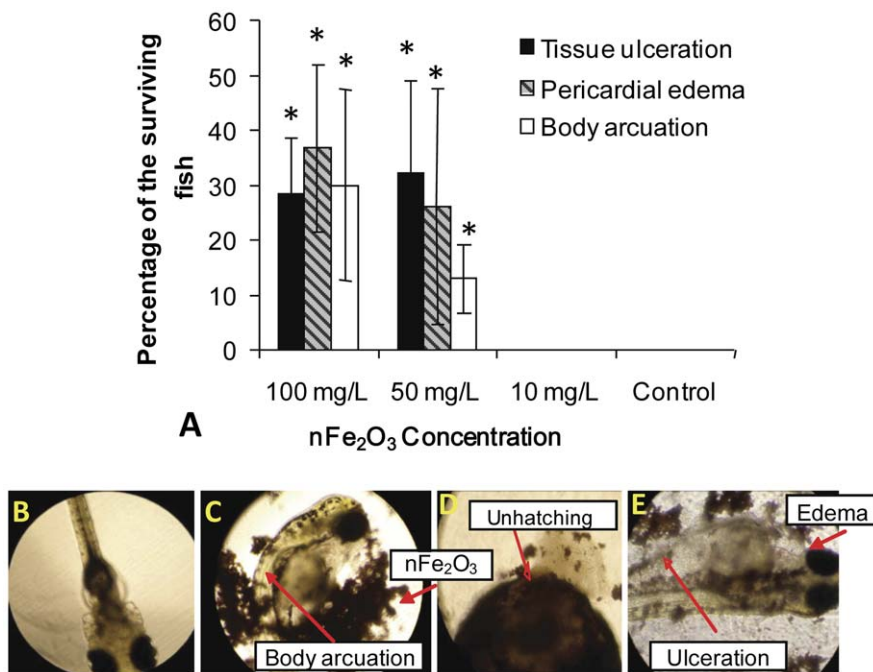
The reported results relating to the toxicity assessment of iron oxide NPs are disputed. In general, iron oxide NPs have been



**Figure 2. Survival (A) and Hatching rate (B) of zebrafish embryos exposed to different concentration of nFe<sub>2</sub>O<sub>3</sub> over 168 h.** Error bars represent ± one standard deviation from the mean of three replicates. doi:10.1371/journal.pone.0046286.g002

widely used in several fields, and they are considered non-toxic materials [24,42–44]. Kim et al. (2006) suggested that iron oxide NPs do not cause apparent toxicity to mice *in vivo* [44]. However, other researchers have reported severe toxicity in the cell system or *in vivo* model of rodents [11,17,18,45–47]. For example, nFe<sub>2</sub>O<sub>3</sub> was able to induce lung injury in rats, increase microvascular permeability and cell lysis in lung epithelia, and significantly disturb blood coagulation parameters [17]. Further investigation showed that nFe<sub>2</sub>O<sub>3</sub> entered the central nervous system, induced severe oxidative stress, and damaged nerve cells in mice [18]. The toxic effects of NPs in the environment depend on initial physicochemical properties (such as composition, size, additives, specific surface area, surface charge, and synthesis method employed), environmental factors, test organisms, and experimental methods [48–50]. Thus, a basic set of tests is warranted to determine the toxicity of certain NPs to extract reliable

conclusions about their toxicological effects. Therefore, the ELS test (used in this study) using zebrafish embryos/larvae as an animal model may serve as a good protocol to explore the potential mode of action underlying the toxicity of NP aggregates. This model offers several advantages [31,38,40,51]. First, zebrafish embryos are demersal: they settle to the bottom of the water column and make direct contact with sediments, mimicking the direct contact between the biota present in the sediments and the NPs that settle out of the water column. Second, transparency and extra-uterine development can be examined, allowing direct observation of phenotypic changes during embryonic development. Third, zebrafish share many cellular and physiological characteristics with higher vertebrates. Toxicological results can thus be compared with those from studies on developmental toxicity in mammals. Fourth, the zebrafish embryo model has been employed to study the ecotoxicology of other biohazards.



**Figure 3. Malformations (e.g., pericardial edema, tissue ulceration, and body arcuation) induced by nFe<sub>2</sub>O<sub>3</sub> at 168 hpf.** (A) Malformation percentage in the surviving fish; (B) control fish; (C) hatching fish with body arcuation, treated with 50 mg/L of nFe<sub>2</sub>O<sub>3</sub> aggregates; (D) unhatching embryos, treated with 50 mg/L of nFe<sub>2</sub>O<sub>3</sub> aggregates, dead at 168 hpf; (E) hatching fish with pericardial edema, treated with 100 mg/L of nFe<sub>2</sub>O<sub>3</sub> aggregates. Error bars represent ± one standard deviation from the mean of three replicates. Significance indicated by: \**p*<0.05. doi:10.1371/journal.pone.0046286.g003

Therefore, although no international consensus exists about which toxicity tests should be used for NP toxicity assessment [24], the results from the ELS test may provide useful data for assessing the potential environmental effects and health risks of NPs.

### Mechanism of toxic effects of iron oxide NPs

Little information exists on the toxic effects of iron oxide NPs [8], and to our knowledge there is no published study on the developmental toxicity of  $n\text{Fe}_2\text{O}_3$  in aquatic organisms. The impact of  $n\text{Fe}_2\text{O}_3$  on zebrafish development observed in this study was found to be associated with the aggregation and sedimentation of  $n\text{Fe}_2\text{O}_3$  and with the characteristics of nanoparticles.

Accompany the aggregation and sedimentation of  $n\text{Fe}_2\text{O}_3$ , direct adherence/adsorption of  $n\text{Fe}_2\text{O}_3$  aggregates could be observed on the surface of embryos (Fig. 3). In a previous study, we found that this direct adherence/adsorption of NPs may exert a physical effect on experimental embryos, causing toxicity [52]. For example, the direct adherence/adsorption of  $n\text{Fe}_2\text{O}_3$  aggregates on the surface may cause hatching delay of embryos through a change in the surface mechanical properties or by interfering with the digestive function of the chorionic hatching enzyme [33]. He et al. showed that iron oxide nanoparticles located on the surface of *Escherichia coli* damaged the cell wall and outer membrane [53]. Moreover, direct adherence/adsorption may also interfere with nutrient exchange between the embryos and the environment. For example, the direct adherence/adsorption of  $n\text{Fe}_2\text{O}_3$  aggregates on the surface may cause depletion of oxygen exchange, resulting in hypoxia of embryos on exposure; this has been reported to cause delayed hatching and development of embryos [33]. In addition, the adherence and/or adsorption of  $n\text{Fe}_2\text{O}_3$  aggregates may also cause excessive production of reactive oxygen species (ROS) (i.e., NO and  $\text{O}_2^{\cdot-}$ ) *in vivo*, resulting in oxidative stress for the embryos, which may be critical in inducing the observed developmental toxicity [38,54].

Another crucial factor that may have affected the zebrafish embryos is the release of metal ions from the nanoparticles. The aggregation and sedimentation of  $n\text{Fe}_2\text{O}_3$  may lead to a high localized concentration. Given the direct adherence/adsorption of  $n\text{Fe}_2\text{O}_3$  aggregates on the embryo surface, there could be high levels of free iron ions in the exposed tissue. This iron overload could thus have toxic implications as an excessive accumulation of  $n\text{Fe}_2\text{O}_3$ . In particular, it could lead to an imbalance in homeostasis and aberrant cellular responses, including cytotoxicity, DNA damage, oxidative stress, epigenetic events, and inflammatory processes, which would eventually lead to the observed toxicity [55]. He et al. confirmed the dissolution of iron oxide nanoparticles and demonstrated that the iron ion and uptake of nanoparticles facilitated iron binding with proteins and DNA strands, resulting in greater mutation frequency in *E. coli* [53]. We previously demonstrated that zinc oxide particle ( $n\text{ZnO}$ ) toxicity may be attributed to both the  $n\text{ZnO}$  and the released  $\text{Zn}^{2+}$  [38]. More research is needed to evaluate the role of the direct physical effect and/or release of metal ions from nanoparticles in the case of  $n\text{Fe}_2\text{O}_3$ -mediated toxicity.

### Iron oxide NPs in real aquatic environments

The zebrafish culture medium used in the present study is not the same as a real aquatic environment (such as lakes and rivers), and in a real environment  $n\text{Fe}_2\text{O}_3$  may behave differently. Keller et al. reported that nanoscale titanium dioxide particles ( $n\text{TiO}_2$ ),  $n\text{ZnO}$ , and cerium oxide particles ( $n\text{CeO}_2$ ) were relatively stable in natural freshwater: over 90% of these nanomaterials remained

in the water body even after standing for 6 h at a concentration of 200 mg/L [56]. However, in seawater, less than 30% of these nanomaterials remained after 6 h [56]. Kádár et al. examined the aggregation and sedimentation of  $n\text{Fe}_2\text{O}_3$  in natural seawater:  $\leq 30\%$  of  $n\text{Fe}_2\text{O}_3$  remained in the seawater after 12 h [8]. Thus, the conditions in the present study were those of an “ideal” experimental situation, using standard zebrafish culture medium as a simulated aquatic environment. This was done to determine whether traditionally standardized tests, such as the ELS test, are useful in determining the ecotoxicity of NPs when no or insufficient data are available. In addition, such ideal experimental conditions allow comparisons of studies among different research groups.

Since there have been no major releases of  $n\text{Fe}_2\text{O}_3$ , little is known about the distribution level of the metal oxide NPs in real aquatic environments—except for  $n\text{TiO}_2$ , which has been detected at over 0.1 mg/L (calculated as Ti) in the runoff from an urban area of Switzerland [57–59]. Furthermore, Klaine et al. pointed out that for some kinds of NPs, such as iron oxide NPs, a large background of naturally occurring iron in the dissolved phase exists; this makes it difficult to differentiate natural from manufactured material [58]. However, nanotoxicology is a new field, and more data are needed with respect to risk assessment and management; examining whether NPs may be toxic to aquatic organisms is an important first step. The present study, which covered a wide range of  $n\text{Fe}_2\text{O}_3$  concentrations from 0.1 to 100 mg/L, was intended to address this issue and hopefully provide some guidance regarding risk assessment and acceptable safe exposure levels. Though much work remains ahead, this paper is the first report on the developmental toxicity of  $n\text{Fe}_2\text{O}_3$  in an aquatic organism. Therefore, this study should serve as the basis for future research into chemical and physical factors that control the toxicity of such nanomaterials as well as a basis for further studies to determine the necessary data to set water-quality standards to protect aquatic life. Such research will benefit risk assessment and management efforts and may contribute indirectly to the development of nanotechnology.

### Conclusions

With the increasing use of metal oxide nanomaterials in catalysis, sensors, environmental remediation, and such commercial products as ones for personal care, there is a strong possibility that these nanomaterials will ultimately enter aquatic ecosystems through wastewater discharge and washing off during recreational activities, such as swimming and water skiing, thereby impacting on the environment and human health. Our results in this study demonstrate that  $n\text{Fe}_2\text{O}_3$  aggregates caused a serious delay in embryo hatching, malformation in some zebrafish embryos and larvae, and eventually mortality. The results of this work will contribute to our understanding of the potential ecotoxicological impact of NPs released to aquatic environments. Moreover, our results also suggest that the ELS test using zebrafish embryos/larvae could be a standard method for assessing the potential toxicity of NPs, especially in consideration of the fact that NPs could accumulate in sediments.

### Author Contributions

Conceived and designed the experiments: XZ ZC. Performed the experiments: XZ ST. Analyzed the data: XZ ST. Contributed reagents/materials/analysis tools: XZ ZC ST. Wrote the paper: XZ.

## References

- American Society for Testing and Materials (2006) Standard terminology relating to nanotechnology. E 2456-06. West Conshohocken, PA.
- Colvin VL (2003) The potential environmental impact of engineered nanomaterials. *Nature Biotechnol* 21: 1166–1170.
- Scientific Committee On Emerging And Newly Identified Health Risks (SCENIHR) (2005) The appropriateness of existing methodologies to assess the potential risks associated with engineered and adventitious products of nanotechnology. Available: [http://ec.europa.eu/comm/health/ph\\_risk/committees/04scenihhr/04scenihren.htm](http://ec.europa.eu/comm/health/ph_risk/committees/04scenihhr/04scenihren.htm). Accessed 28 January 2011.
- Nel A, Xia T, Madler L, Li N (2006) Toxic potential of materials at the nanolevel. *Science* 311: 622–627.
- Shosaku K (2006) Distribution of Nanoparticles in the See-through Medaka (*Oryzias latipes*). *Environ Health Perspect* 114: 1697–1702.
- Zhang WX, Karn B (2005) Nanoscale Environmental Science and Technology: Challenges and Opportunities. *Environ Sci Technol* 39: 94A–95A.
- Huber DL (2005) Synthesis, properties, and applications of iron nanoparticles. *Small* 1: 482–501.
- Kádár E, Lowe DM, Solé M, Fisher AS, Jha AN, et al. (2010) Hutchinson, Uptake and biological responses to nano-Fe versus soluble FeCl<sub>3</sub> in excised mussel gills. *Anal Bioanal Chem* 396: 657–666.
- Pankhurst QA, Connolly J, Jones SK, Dobson J (2003) Applications of magnetic nanoparticles in biomedicine. *J Phys D Appl Phys* 36: 167–181.
- Salata OV (2004) Applications of nanoparticles in biology and medicine. *J Nanobiotech* 2: 3.
- Noori A, Parivar K, Modaresi M, Messripour M, Yousefi MH, et al. (2011) Effect of magnetic iron oxide nanoparticles on pregnancy and testicular development of mice. *Afr J Biotechnol* 10: 1221–1227.
- Zhang C, Liu T, Gao J, Su Y, Shi C (2010) Recent development and application of magnetic nanoparticles for cell labeling and imaging. *Mini Rev Med Chem* 10: 193–202.
- Grover VA, Hu J, Engates KE, Shipley HJ (2012) Adsorption and desorption of bivalent metals to hematite nanoparticles. *Environ Toxicol Chem* 31: 86–92.
- Ge F, Li MM, Ye H, Zhao BX (2012) Effective removal of heavy metal ions Cd<sup>2+</sup>, Zn<sup>2+</sup>, Pb<sup>2+</sup>, Cu<sup>2+</sup> from aqueous solution by polymer-modified magnetic nanoparticles. *J Hazard Mater* 211: 366–372.
- Gonzalez CM, Hernandez J, Peralta-Videa JR, Botez CE, Parsons JG et al. (2012) Sorption kinetic study of selenite and selenate onto a high and low pressure aged iron oxide nanomaterial. *J Hazard Mater* 211: 138–145.
- Perez JM (2007) Iron oxide nanoparticles: Hidden talent. *Nat Nanotechnol* 2: 535–536.
- Zhu MT, Feng WY, Wang B, Wang TC, Gu YQ, et al. (2008) Comparative study of pulmonary responses to nano- and submicron-sized ferric oxide in rats. *Toxicology* 247: 102–111.
- Wang B, Feng WY, Zhu MT, Wang Y, Wang M, et al. (2009) Neurotoxicity of low-dose repeatedly intranasal instillation of nano- and submicron-sized ferric oxide particles in mice. *J Nanopart Res* 11: 41–53.
- Soenen SJ, De Cuyper M (2009) Assessing cytotoxicity of (iron oxide-based) nanoparticles: an overview of different methods exemplified with cationic magnetoliposomes. *Contrast Media Mol I* 4: 207–219.
- Soenen SJ, De Cuyper M (2010) Assessing iron oxide nanoparticle toxicity in vitro: current status and future prospects. *Nanomedicine* 5: 1261–1275.
- Mahmoudi M, Laurent S, Shokrgozar MA, Hosseinkhani M (2011) Toxicity evaluations of superparamagnetic iron oxide nanoparticles: cell “vision” versus physicochemical properties of nanoparticles. *ACS Nano* 5: 7263–7276.
- Li H, Zhou Q, Wu Y, Fu J, Wang T, et al. (2009) Effects of waterborne nano-iron on medaka (*Oryzias latipes*): antioxidant enzymatic activity, lipid peroxidation and histopathology. *Ecotox Environ Safe* 72: 684–692.
- Nations S, Wages M, Cañas JE, Maul J, Theodorakis C, et al. (2011) Acute effects of Fe<sub>2</sub>O<sub>3</sub>, TiO<sub>2</sub>, ZnO and CuO nanomaterials on *Xenopus laevis*. *Chemosphere* 83: 1053–1061.
- García A, Espinosa R, Delgado L, Casals E, González E, et al. (2011) Acute toxicity of cerium oxide, titanium oxide and iron oxide nanoparticles using standardized tests. *Desalination* 269: 136–141.
- Zhu H, Han J, Xiao JQ, Jin Y (2008). Uptake, translocation, and accumulation of manufactured iron oxide nanoparticles by pumpkin plants. *J Environ Monit* 10:713–717.
- Saravanan M, Suganya R, Ramesh M (2011) Toxicity of iron oxide nanoparticles to Indian major carp, *Labeo rohita* on haematological, biochemical, ionoregulatory and enzymological alterations. 8th International Symposium on Recent Advances in Environmental Health Research. Jackson, MS, USA. Sep. 18–21.
- Frayse B, Mons R, Garric J (2006) Development of a zebrafish 4-day embryonal bioassay to assess toxicity of chemicals. *Ecotox Environ Safe* 63: 253–267.
- Zhu X, Zhu L, Duan Z, Qi R, Li Y, et al. (2008) Comparative toxicity of several metal oxide nanoparticle aqueous suspensions to Zebrafish (*Danio rerio*) early developmental stage. *J Environ Sci Heal Part A43*: 278–284.
- International Standard Organization (ISO) (1996) Water quality—Determination of the acute lethal toxicity of substances to a freshwater fish [*Brachydanio rerio* Hamilton-Buchanan (Teleostei, Cyprinidae)]—Part 3: Flow-through method. ISO 7346-3:1996. Geneva, Switzerland.
- Organisation for Economic Co-operation and Development (OECD) (1998) OECD Guideline for Testing of Chemicals 212: Fish, short term toxicity test on embryo and sac-fry stages. OECD. Paris, France.
- Schulte C, Nagel R (1994) Test acute toxicity in the embryo of zebrafish, *Brachydanio rerio*, as an alternative to the acute fish test: preliminary results. *Altern Lab Anim* 22: 12–19.
- Zhang Y, Chen Y, Westerhoff P, Hristovski K, Crittenden JC (2008) Stability of commercial metal oxide nanoparticles in water. *Water Res* 42: 2204–2212.
- Cheng J, Flahaut E, Cheng SH (2007) Effect of carbon nanotubes on developing zebrafish (*Danio rerio*) embryos. *Environ Toxicol Chem* 26: 708–716.
- Zhu X, Zhu L, Chen Y, Tian S (2009) Acute toxicities of six manufactured nanomaterial suspensions to *Daphnia magna*. *J Nanopart Res* 11: 67–75.
- Griffitt R J, Weil R, Hyndman K A, Denslow N D, Powers K et al. (2007) Exposure to copper nanoparticles causes gill injury and acute lethality in zebrafish (*Danio rerio*). *Environ Sci Technol* 41: 8178–8186.
- Zhu X, Zhu L, Li Y, Duan Z, Chen Y, et al. (2007) Developmental toxicity in zebrafish embryos after exposure to manufactured nanomaterials: buckminsterfullerene aggregates (nC60) and fullerol. *Environ Toxicol Chem* 26: 976–979.
- Zhu X, Wang J, Zhang X, Chang Y, Chen Y (2009) The impact of ZnO nanoparticle aggregates on the embryonic development of zebrafish (*Danio rerio*). *Nanotechnology* 20: 195103 (9pp).
- Asharani PV, Lian Wu Y, Gong Z, Valiyaveetil S (2008) Toxicity of silver nanoparticles in zebrafish models. *Nanotechnology* 19: 255102.
- Hallare AV, Köhler HR, Triebkorn R (2004) Developmental toxicity and stress protein responses in zebrafish embryos after exposure to diclofenac and its solvent, DMSO. *Chemosphere* 56: 659–666.
- Takeda K, Suzuki K, Ishihara A, Kubo-Irie M, Fujimoto R, et al. (2009) Nanoparticles transferred from pregnant mice to their offspring can damage the genital and cranial nerve systems. *J Health Sci* 55: 95–102.
- Ju-Nam Y, Lead JR (2008) Manufactured nanoparticles: an overview of their chemistry, interactions and potential environmental implications. *Sci Total Environ* 400: 396–414.
- Bystrzejewska-Piotrowska G, Golimowski J, Urban JP (2009) Nanoparticles: their potential toxicity, waste and environmental management. *Waste Manage* 29: 2587–2595.
- Kim JS, Yoon TJ, Yu KN, Kim BG, Park SJ, et al. (2006) Toxicity and tissue distribution of magnetic nanoparticles in mice. *Toxicol Sci* 89: 338–347.
- Hafeli UO, Pauer GJ (1999) In vitro and in vivo toxicity of magnetic microspheres. *J Magn Magn Mater* 194: 76–82.
- García MP, Parca RM, Chaves SB, Silva LP, Santos AD, et al. (2005) Morphological analysis of mouse lungs after treatment with magnetite-based magnetic fluid stabilized with DMSA. *J Magn Magn Mater* 293: 277–282.
- Shubayev VI, Pisanic TR, Jin S (2009) Magnetic nanoparticles for therapeutics. *Adv Drug Delivery Rev* 61: 467–477.
- Brunner TJ, Wick P, Manser P, Spohn P, Grass RN, et al. (2006) In vitro cytotoxicity of oxide nanoparticles: comparison to asbestos, silica and the effect of particle solubility. *Environ Sci Technol* 40: 4374–4381.
- Hu X, Cook S, Wang P, Hwang H (2009) In vitro evaluation of cytotoxicity of engineered metal oxide nanoparticles. *Sci Total Environ* 407: 3070–3072.
- Choi JY, Lee SH, Na HB, An K, Hyeon T, et al. (2010) In vitro cytotoxicity screening of water-dispersible metal oxide nanoparticles in human cell lines. *Bioprocess Biosyst Eng* 33: 21–30.
- Usenko CY, Harper SL, Tanguay RL (2007) In vivo evaluation of carbon fullerene toxicity using embryonic zebrafish. *Carbon* 45: 1891–1898.
- Zhu X, Zhou J, Cai Z (2011) TiO<sub>2</sub> nanoparticles in the marine environment: impact on the toxicity of tributyltin to abalone (*Haliotis diversicolor supertexta*) embryos. *Environ Sci Technol* 45: 3753–3758.
- He S, Feng Y, Gu N, Zhang Y, Lin X (2011) The effect of γ-Fe<sub>2</sub>O<sub>3</sub> nanoparticles on *Escherichia coli* genome. *Environ Pollut* 159: 3468–3473.
- Hassoun E, Kariya C, Williams FE (2005) Dichloroacetate-induced developmental toxicity and production of reactive oxygen species in zebrafish embryos. *J Biochem Mol Toxic* 19: 52–58.
- Singh N, Jenkins GJS, Asadi R, Doak SH (2010) Potential toxicity of superparamagnetic iron oxide nanoparticles (SPION). *Nano Rev* 1: 5358; DOI: 10.3402/nano.v1i0.5358.
- Keller AA, Wang H, Zhou D, Lenihan HS, Cherr G, et al. (2010). Stability and aggregation of metal oxide nanoparticles in natural aqueous matrices. *Environ Sci Technol* 44: 1962–1967.
- Kaegi R, Ulrich A, Sinnet B, Vonbank R, Wichser A, et al. (2008) Synthetic TiO<sub>2</sub> nanoparticle emission from exterior facades into the aquatic environment. *Environ. Pollut* 156: 233–239.
- Klaine SJ, Alvarez PJ, Batley GE, Fernandes TF, Handy RD, et al. (2008). Nanomaterials in the environment: behavior, fate, bioavailability, and effects. *Environ Toxicol Chem* 27: 1825–1851.
- Peralta-Videa JR, Zhao L, Lopez-Moreno ML, la Rosa de G, Hong J, et al. (2011) Nanomaterials and the environment: A review for the biennium 2008–2010. *J Hazard Mater* 186: 1–15.