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Comparative effects of zinc oxide nanoparticles and dissolved zinc on zebrafish embryos and eleuthero-embryos: Importance of zinc ions

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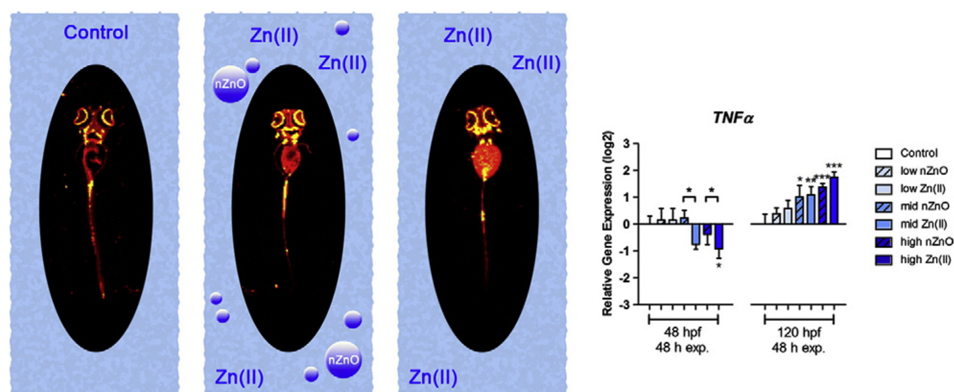
HIGHLIGHTS

- Effects of nZnO and Zn(II) on zebrafish early life stages are compared.
- We show transcriptional alterations and effects on hatching of embryos.
- Mechanism of action including pro-inflammatory action is hypothesized.
- Zinc originating from nZnO to Zn(II) is equally incorporated into tissues.
- Effects of nZnO are mainly based on the release of Zn(II).

GRAPHICAL ABSTRACT



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ABSTRACT

The increasing use of zinc oxide nanoparticles (nZnO) and their associated environmental occurrence make it necessary to assess their potential effects on aquatic organisms. Upon water contact, nZnO dissolve partially to zinc (Zn(II)). To date it is not yet completely understood, whether effects of nZnO are solely or partly due to dissolved Zn(II). Here we compare potential effects of 0.2, 1 and 5 mg/L nZnO and corresponding concentrations of released Zn(II) by water soluble $ZnCl_2$ to two development stages of zebrafish, embryos and eleuthero-embryos, by analysing expressional changes by RT-qPCR. Another objective was to assess uptake and tissue distribution of Zn(II). Laser ablation-ICP-MS analysis demonstrated that uptake and tissue distribution of Zn(II) were identical for nZnO and $ZnCl_2$ in eleuthero-embryos. Zn(II) was found particularly in the retina/pigment layer of eyes and brain. Both nZnO and dissolved Zn(II) derived from $ZnCl_2$ had similar inhibiting effects on hatching, and they induced similar expressional changes of target genes. At 72 hours post fertilization (hpf), both nZnO and Zn(II) delayed hatching at all doses, and inhibited hatching at 1 and 5 mg/L at 96 hpf. Both nZnO and Zn(II) lead to induction of metallothionein (*mt2*) in both embryos and eleuthero-embryos at all concentrations. Transcripts of oxidative stress related genes *cat* and *Cu/Zn sod* were also altered. Moreover, we show for the first time that nZnO exposure results in transcriptional changes of pro-inflammatory cytokines *IL-1β* and *TNFα*. Overall, transcriptional alterations were higher in embryos than eleuthero-embryos. The similarities of the effects lead to the conclusion that effects of nZnO are mainly related to the release of Zn(II).

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1. Introduction

Metal-based nanoparticles are increasingly implemented in materials, cosmetics and technical applications. Consequently, they may be ultimately released into the environment during production, use and disposal at the end of their life. Zinc oxide nanoparticles (nZnO) are widely used in semiconductors, (organic) solar panel devices (Zimmermann et al., 2012), paints, personal care products (sunscreens) and even in waste water treatment. Nanoparticles might have different physico-chemical properties or behaviours in contrast to bulk materials, making prediction of their fate challenging (Li et al., 2013, 2014). Their state of aggregation, and consequently their settling to sediments, depend on surface properties, abiotic factors and the presence of dissolved organic matter in surrounding media (Shaw and Handy, 2011; Zhou and Keller, 2010). Most (nano)particles in aquatic environments are covered by adsorbed layers of natural organic material, such as humic substances and polysaccharides, which can influence stability of inorganic nanoparticle suspensions (Hyung et al., 2007) and provide potential binding sites for trace elements (Buffle et al., 1990).

Zinc is an essential trace element for organisms but induces toxicity at elevated concentrations. Partially but relatively quickly, nZnO dissolve in water, and the release of free zinc ions has been previously shown to be the primary source of toxicity (Blinova et al., 2010; Buerki-Thurnherr et al., 2013; Franklin et al., 2007). However, in other studies nZnO showed higher toxicity (Bai et al., 2010; Hu et al., 2009; Fernández et al., 2013), or induced additional effects (Poynton et al., 2011) than dissolved Zn(II) alone. Thus, there are conflicting observations and therefore further research is needed to resolve these discrepancies.

In organisms, cellular zinc ion fluctuations are mainly regulated by zinc binding metallothioneins (MT). Both, Zn(II) and nZnO induce reactive oxygen species (ROS) formation (Dineley et al., 2003; Heng et al., 2010; Sensi et al., 1999). ROS induction triggering an oxidative stress response has become a widely accepted paradigm for cellular effects of nanoparticles (Nel et al., 2006; Sharma et al., 2012; Song et al., 2010; Zhu et al., 2009). Excessive production of ROS can induce pro-inflammatory and cytotoxic effects (Nel et al., 2006). nZnO and associated released Zn(II) may ultimately lead to apoptosis (Buerki-Thurnherr et al., 2013) and acute toxicity at high concentrations. In zebrafish, adverse effects of nZnO include reduced hatching of embryos and ROS production (Bai et al., 2010; George et al., 2011; Ong et al., 2013; Xia et al., 2011; Zhao et al., 2013; Zhu et al., 2009). However, induction of inflammatory responses has not yet been investigated in fish.

In our present study we compare effects of nZnO and equal concentrations of released Zn(II) (derived from $ZnCl_2$) in experimental exposures of embryos and hatched zebrafish eleuthero-embryos in environmentally relevant media. Naturally occurring alginate, a polysaccharide-based organic material, was chosen as a supplement to stabilize nanoparticles and reduce their aggregation rate (George et al., 2011, 2012). We hypothesize that effects in embryos and eleuthero-embryos are similar, because they are induced by released Zn(II). This is assumed because agglomerated nanoparticles might not pass the pores in the chorion (Fent et al., 2010), but rather act as “delivery vehicle” for zinc ions when attached to the chorion (Handy et al., 2008). The aims of our present work were to (1) evaluate the role of the protective egg chorion; (2) compare the influence of nZnO and released Zn(II); (3) determine the uptake of zinc by embryos and eleuthero-embryos using ICP-MS analysis, and its distribution into tissues by laser-ablation ICP-MS imaging; and (4) to assess the effects of nZnO and Zn(II) on the expression of target genes including pro-inflammatory genes. Our results indicate a fast dissolution of zinc from nZnO in water, making the free metal ions the primary source of the observed effects.

2. Materials and methods

2.1. Nanoparticle characterisation

Zinc oxide nanoparticles (nZnO) were supplied by Genes'Ink (Marseille, France). The specific surface area was analysed by BET method (Brunauer et al., 1938) on a Gemini model 2380 (Micromeritics Instrument Corp., Georgia, USA). In addition, the zeta potential of nZnO in the exposure medium was measured using a Malvern Zetasizer Nano ZS (Malvern Instruments ltd., Malvern, UK). In the electric field, the electrophoretic mobility of charged nanoparticles was determined at 25 °C. Automatic measurements (10–100 runs) were performed with triplicates and the Smoluchowski's model was applied for analysis.

For scanning electron microscopy (SEM; Carl Zeiss Supra 40 VP, Germany) nZnO were dispersed in H_2O, ultrasonicated for 5 min at a frequency of 37 kHz (Elmasonic S 30) and air dried. The working distance was set on 4 mm, and an acceleration voltage of 5 kV was used.

Mean size of single particles was determined by tracking the scattered light using the NanoSight LM10 (NanoSight Ltd., Amesbury, UK) followed by evaluation using the Nanoparticle Tracking Analysis (NTA) software. The system relies on the Brownian motion and records a multitude of particles over a period of time generating a subsequent size distribution and mean size. Each concentration of nZnO and $ZnCl_2$, as well as controls, were analysed three times independently by NTA at 0 h and 24 h after preparation. Each sample was injected three times and analysed in triplicate calculating the arithmetic average thereof. Standard deviations were determined from mean size obtained from replicate runs.

2.2. Inductively-coupled-plasma mass-spectrometry (ICP-MS)

The zinc ion dissolution of nZnO in Holtfreter's Media at 0 h and 24 h after sample preparation was quantified using ICP-MS. Samples were centrifuged for 30 min at 30,000 *g* and the concentrations of the zinc isotopes (using ^{64}Zn and ^{66}Zn) in the supernatant were determined using an ICP-MS system (Agilent 7500cx, Basel, Switzerland) equipped with an Octopole Reaction System, pressurized with an optimized helium flow of 5 mL min^{-1}. Supernatants were diluted 1:100 in 1% HNO_3. Rubidium was used as internal standard. ICP-MS measurements were performed to determine the corresponding concentration of dissolved zinc released by $ZnCl_2$ (Sigma-Aldrich, Switzerland). Here, strictly speaking, the dissolved Zn(II) species is not pure. The main dissolved species is $Zn^{2+}{}_{(aq)}$, followed by minor amounts of $ZnCl^{+}{}_{(aq)}$ and $Zn(OH)^{+}{}_{(aq)}$. The concentrations of soluble Zn(II) released by suspended nZnO at exposure concentrations of 0.2, 1 and 5 mg/L correspond to Zn(II) concentrations of 0.1, 0.5, and 2.2 mg/L, respectively.

At each sampling time point, 3 embryos of each of the 4 replicates were washed twice in 1 mM EDTA and individually dissolved in 300 μL aqua regia (3:1 HCl:HNO_3) for two days. If not hatched, additional 3 embryos per replicate were dechorionated, and chorion as well as dechorionated embryos dissolved in aqua regia separately. Samples were diluted 1:20 in HPLC-grade Nanopure water. Quality control measures included the use of procedural blanks.

2.3. Laser-ablation inductively-coupled-plasma mass-spectrometry (LA-ICP-MS) imaging

At each time point, embryos of the highest dose groups and controls were dehydrated and embedded in agarose according to the protocol of Sabaliauskas et al. (2006). A microtome (SM2010R, Leica) was used to prepare embryo cross sections with a flat surface. A NWR213 laser ablation system (ESI, Portland, USA) was coupled to the above ICP-MS system for bioimaging, yet without using helium as collision gas. This allowed the qualitative assessment of element distribution in zebrafish embryo cross sections. The laser was operated at 50% of its maximal

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