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TiO_2 nanoparticles reduce the effects of ZnO nanoparticles and Zn ions on zebrafish embryos (*Danio rerio*)

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ABSTRACT

We investigated the toxicity of a mixture of stick-shaped ZnO nanoparticles (NPs) and spherical-shaped TiO_2 NPs to zebrafish embryos (*Danio rerio*). The EC_{50} value of hatching inhibition of embryos exposed to suspensions of ZnO NPs was 1.3 mg/L. Upon addition of TiO_2 NPs, the EC_{50} value increased by up to a maximum of a factor of 6.3. A similar reduction of toxicity was found for mortality of embryos, as the LC_{50} values were reduced significantly ($p < 0.05$) with the addition of TiO_2 NPs at higher concentrations. A $Zn(NO_3)_2$ solution was used as a positive reference to assess the toxicity of Zn^{2+}. The toxicity of Zn^{2+} was reduced when TiO_2 NPs were added, as compared to the effects found when embryos were exposed to $Zn(NO_3)_2$ solutions without TiO_2 NPs. The contribution to toxicity of the particulate form of ZnO NPs was found to dominate the contribution of the released ions from ZnO NPs when explaining the toxicity of the suspensions of ZnO NPs in the absence and presence of TiO_2 NPs to zebrafish embryos.

1. Introduction

The application of nanomaterials presents a scientific breakthrough in industry and in consumer products. Some of the most commonly applied nanoparticles (NPs) are metallic oxides NPs. Both zinc oxide nanoparticles (ZnO NPs) and titanium dioxide nanoparticles (TiO_2 NPs) are widely used in products such as pigments for paints and cosmetics, and also as a UV filter in sunscreens (Zallen and Moret, 2006; Xia et al., 2008). These NPs promise remarkable benefits, but they will inevitably be released into the aquatic and the terrestrial environments. Attention is therefore required with regard to their combined ecotoxicological effects.

Zn is an important essential metal to all cells in all known organisms (Hogstrand, 2011). However, Zn can cause adverse effects when present in excess and is regarded as an important pollutant for fish and other aquatic biota. ZnO NPs have been classified as "extremely toxic" (Kahru and Dubourguier, 2010), and can be absorbed by the intestine as well as the gills of fish (Hogstrand, 2011). TiO_2 on the other hand is generally considered to be an inert material. The acute toxicity of TiO_2 NPs to aquatic organisms is low (Griffitt et al., 2008). Besides their low direct toxic effects, the presence of TiO_2 NPs may cause indirect adverse effects, for example by adsorbing metals and subsequently influencing their toxicity and bioaccumulation in the aquatic environment (Hartmann and Baun, 2010a; Hartmann et al., 2010b). The uptake and retention of Zn to *Daphnia magna* may be enhanced when the zinc ions are adsorbed on TiO_2 NPs (Tan et al., 2012). In addition, Tang et al. (2013) found that low concentrations of TiO_2 NPs (<1.0 mg/L) significantly enhanced the toxicity of Zn^{2+}, whereas the toxicity of Zn^{2+} decreased with increasing TiO_2 NPs concentration (10 mg/L). They reported that the possible reason is the substantial adsorption of Zn^{2+} by TiO_2 NPs (Tang et al., 2013). TiO_2 NPs have a relatively large surface area, thereby providing a great potential for sorption of metal ions and environmental contaminants (Hartmann and Baun, 2010a; Hartmann et al., 2010b).

The toxicity of metal-based NPs has received considerable attention in recent years, especially with regard to effects induced by individual NPs. Mixtures of NPs or composite NPs are nevertheless very common in the environment, and also in various NP-based products. Therefore, organisms are typically exposed to mixtures of various types of contaminants. Li et al. (2015) investigated the toxic effect of a mixture of ZnO NPs and Cu NPs, and found that ZnO NPs at non-toxic concentrations enhance Cu NPs toxicity in vitro. Few studies have been published on the toxicity of binary mixtures of metal-based NPs. Zou et al. (2014) found that TiO_2 NPs reduce the risks of Ag NPs in natural light to ciliated protozoa, but enhance the risks of Ag NPs in continuous light. Tong et al. (Tong et al., 2015) reported that inhibitory effect of ZnO NPs on bacterial cell membrane is reduced by TiO_2 NPs due to the adsorption of Zn^{2+}. Other studies were focused on the toxicity of mixtures of NPs with other metal components.

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Although dissolved metal ions play an important role in inducing acute or chronic toxicity of metal-based NPs to aquatic organisms, it has been reported in various studies that the particulate form of metal-based NPs also contributes significantly to the toxic effects observed (Hua et al., 2014a; Hua et al., 2014b; Song et al., 2014; Xiao et al., 2015). The first aim of this study is the assessment of the fate of NPs suspensions and assessment of the interactive effect of the particulate form of NPs and metal ions. The second aim is to investigate the interaction of ZnO NPs and TiO_2 NPs with regard to the toxicity of these NPs to zebrafish embryos in vivo.

2. Material and methods

2.1. Physicochemical characterization of NPs

The preparation of stock solutions and suspensions of nanoparticles is shown in the Supplementary material 1. The particle size and shape of NPs present in the suspensions were characterized after around 1 h of incubation in egg water using transmission electron microscopy (TEM) (JEOL 1010, JEOL Ltd., Japan). The particle size distribution was analyzed using the software Nano Measurer version 1.2 (Fudan University, China). The actual total concentrations of metal (C_A, including the particulate form and the ionic form of NPs) and the dissolved metal ions from NPs (C_I) in egg water (mg metal/L) were analyzed using inductively coupled plasma optical emission spectrometry (ICP-OES, iCAP 5100, Thermo Scientific, Cambridge, UK) after 24 h of equilibration of the samples. To determine concentrations of C_I in egg water, 5 mL of the suspensions was sampled after 24 h of incubation and centrifuged at 13,300*g* for 20 min following Fernandez-Cruz et al. (2013)). The centrifugation of the samples removed ZnO NPs, TiO_2 NPs and ions which might be sorbed to the TiO_2 NPs. The preparation of digested samples of ZnO NPs is described in our previous study (Hua et al., 2014b). For TiO_2 NPs, liquid and suspended solid samples were acid digested using the HNO_3/H_2SO_4 digestion method for Ti as described by Standard Method 3030 G and 2540 D for water and wastewater analysis (APHA-AWWA-WEF, 2005). The digested samples were then analyzed by ICP-OES.

2.2. Toxicity tests

Zebrafish embryos were obtained from AB wild type zebrafish. Embryos were assessed and distributed into 96 well-plates with one embryo per well from 24 h post-fertilization (hpf) to 120 hpf following the method previously described.(Hua et al., 2014b) The embryos were exposed to 250 μL/well of ZnO NPs together with or without TiO_2 NPs in egg water. As summarized by Hua et al. (2014b) it is difficult to experimentally quantify the concentration of $NP_{(ion)}$ shed from NPs and to prove the clear differentiation between the effects of $NP_{(particle)}$ and of $NP_{(ion)}$ in the observed toxicity. In our study, we used $Zn(NO_3)_2$ solutions and assumed that the concentration of Zn^{2+} in the mixture of $Zn(NO_3)_2$ and TiO_2 is the same with that $ZnO_{(ion)}$ in suspension in the mixture of ZnO NPs and TiO_2 NPs, if they have same concentrations of TiO_2 NPs and dissolved Zn^{2+} from ZnO NPs. Therefore, $Zn(NO_3)_2$ solutions were applied with or without TiO_2 NPs to investigate the interaction of Zn^{2+} and TiO_2 NPs. Various concentrations were selected, namely, 0, 2, 4, 8, 16, 32 mg Zn/L for ZnO NPs, 0, 2.5, 5, 10, 20, 40 mg Zn/L for $Zn(NO_3)_2$ solutions, and 0, 1.5, 3, 6, 12, 24 mg Ti/L for TiO_2 NPs. Throughout all procedures, the embryos and solutions were kept at 28.5 ± 0.5 °C. Zebrafish embryos were exposed in triplicate to either a control or to different concentrations of ZnO NPs, $Zn(NO_3)_2$, TiO_2 NPs, or binary combination of ZnO NPs with TiO_2 NPs, or $Zn(NO_3)_2$ with TiO_2 NPs. Each replicate contained 16 embryos for each treatment (48 embryos in total). Mortality and hatching rate were assessed every 24 h. Embryos were scored as dead according to OECD guideline 157 (OECD, 2011). In addition, we also investigated the impact of the particle-specific ZnO NPs on the toxicity of Zn ions ($Zn(NO_3)_2$) as a reference, the methods used are shown in the Supplementary material 2.

The median lethal concentration (LC_{50}) and the median effect concentration (EC_{50}) of hatching inhibition of zebrafish caused by stock solutions of NPs were calculated using GraphPad Prism 5 (California, USA) according to the following equation to describe the dose-response curve:

$$E = \frac{1}{1 + 10^{(\mathrm{LogEC_{50}} - \mathrm{LogC_a})\rho}} \quad (1)$$

where E is the effect (mortality, hatching inhibition) on zebrafish embryos caused by stock solutions of NPs (scaled from 0 to 1). C_a describes the initial actual exposure concentration of NPs and ρ represents the slope of the curve.

2.3. Contribution to toxicity of the particulate form and ionic form of ZnO NPs

As described in our previous study, we assumed that ZnO NPs suspensions contain both the particulate form of ZnO NPs and released ions from the ZnO NPs (Hua et al., 2014b). TiO_2 NPs suspensions are expected to be present solely in their solid phase and not in ionic forms (Antignano et al., 2008). The combined effects of two compounds can be calculated by the response addition model (RA) and concentration addition model (CA) (Altenburger et al., 2003). Based on our previous study, we found that more toxic effects by using CA than by using RA and therefore the increased relative contribution of the particle form of NPs (Hua et al., 2014b). Both results from CA and RA allowed us to conclude that the particle form of NPs was the main factor in inducing toxicity to zebrafish embryos for all NPs suspensions tested (Hua et al., 2014b). In addition, the mechanisms of action of the particulate form of ZnO NPs and released ions from the ZnO NPs are likely to be dissimilar, so the RA model was selected to explicitly account for the toxic impacts of the particulate form of ZnO NPs ($ZnO\ NP_{(particle)}$) and the ionic form shed from ZnO NPs ($ZnO_{(ion)}$) (Hua et al., 2014b). In the RA model, the toxic effects (mortality and hatching inhibition) on embryos induced by the $ZnO\ NP_{(particle)}$ ($E_{(particle)}$) can be computed as follows:

$$E_{(total)} = 1 - \left[(1 - E_{(ion)})(1 - E_{(particle)})\right] \quad (2)$$

where $E_{(total)}$ and $E_{(ion)}$ represent the effect of zebrafish embryos caused by $ZnO\ NP_{(total)}$ and $ZnO\ NP_{(ion)}$ (scaled from 0 to 1), respectively. This leaves $E_{(particle)}$ as the only unknown, allowing for direct calculation of the effect caused by the particles at any specific initial actual particle concentration.

The actual concentrations of $ZnO\ NP_{(total)}$ and of $ZnO\ NP_{(ion)}$ were measured by ICP-OES after 24 h of incubation in egg water. The actual concentrations of $ZnO\ NP_{(particle)}$ were calculated as the difference of the actual concentrations of $ZnO\ NP_{(total)}$ and the actual concentrations of $ZnO\ NP_{(ion)}$. The toxicity of $ZnO\ NP_{(ion)}$ was experimentally quantified on the basis of the concentration-response curve of $Zn(NO_3)_2$ alone or together with TiO_2 NPs. The total toxicity of $ZnO\ NP_{(total)}$ was assessed experimentally. An example of the calculation of the relative contribution to toxicity of $ZnO\ NP_{(particle)}$ and $ZnO\ NP_{(ion)}$ at the LC_{50} value of suspensions is shown in Supplementary material 3.

2.4. Statistical analysis

The experiments were repeated three times independently (with 16 embryos per treatment group, totally 48 embryos). Data are presented as mean with 95% confidence intervals (95% CI) or standard error of the mean (SEM). Mortality data obtained at 120 hpf were used to calculate LC_{50} values. The LC_{50} values, EC_{50} values of hatching rate, and 95% CI were calculated (Eq. (1)) using GraphPad Prism 5. The 95% CI of LC_{50}