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Comparative assessment of toxicity of ZnO and amine-functionalized ZnO nanorods toward *Daphnia magna* in acute and chronic multigenerational tests

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ABSTRACT

Zinc oxide nanomaterials (ZnO NM) have been used in a large number of applications due to their interesting physicochemical properties. However, the increasing use of ZnO NM has led to concerns regarding their environmental impacts. In this study, the acute and chronic toxicity of ZnO nanorods (NR) bare (ZnONR) and amine-functionalized (ZnONR@AF) toward the freshwater microcrustacean *Daphnia magna* was evaluated. The ZnO NR were characterized by transmission electron microscopy (TEM), X–Ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), and the zeta potential and hydrodynamic diameter (HD). The acute $EC50_{(48h)}$ values for *D. magna* revealed that the ZnONR@AF were more toxic than the ZnONR. The generation of reactive oxygen species (ROS) was observed in both NM. Regarding the chronic toxicity, the ZnONR@AF were again found to be more toxic than the ZnONR toward *D. magna*. An effect on longevity was observed for ZnONR, while ZnONR@AF affected the reproduction, growth and longevity. In the multigenerational recovery test, we observed that maternal exposure can affect the offspring even when these organisms are not directly exposed to the ZnO NR.

1. Introduction

Due to the fast growth of the nanotechnology domain, nanomaterials (NM) are being used in a wide variety of applications in the industrial, domestic and health sectors (Nel et al., 2006). These NM can be applied in their pure form or following surface functionalization with NM, polymers, surfactants or ligands, which modify the physicochemical properties and enhance the performance of the material (Kango et al., 2013). However, the surface modification of NM can also alter their toxicological properties, resulting in an increase of the toxicity (Perreault et al., 2012; Vicentini et al., 2017), reduction of the toxicity (Božič et al., 2017; Rossetto et al., 2014b) or in some cases, it does not influence in the toxicity of the NM (Wallin et al., 2017).

At the nanoscale, the properties of materials may differ significantly from those of the respective bulk materials (Rossetto et al., 2014a). The same particular characteristics of NM that permit their application in many products, such as small size, high surface area and capacity for agglomeration or dispersion, can facilitate their translocation between environmental compartments, biological transport, and cellular interactions, highlighting the importance of researching the bioavailability, degradability, reactivity and toxicity of NM (Khanna et al., 2015).

The toxicity effects of NM are associated with several factors, including: (a) the NM themselves, which can be toxic when taken up by organisms (Griffitt et al., 2009); (b) the enhanced solubility of NM into toxic metal ions in the case of metal and metal oxide NM (Blinova et al., 2010; Melegari et al., 2013; Rossetto et al., 2014a); (c) the combined effects of the NM and the released ions, enhancing the toxicity (Poynton et al., 2011); and (d) a "Trojan horse effect", due to the possibility of the nanoparticles (NP) entering the cell through mechanisms of internalization and causing damage inside the cell (Limbach et al., 2007; Sabella et al., 2014).

Due to its specific optical, magnetic, semiconducting, piezoelectric, antibacterial properties and diverse growth morphologies (Vaseem et al., 2010), ZnO is one of the most commonly used metal oxide NM in industry and in everyday products, such as personal care products, polymeric membranes for water treatment, coatings and antifouling paints (Vaseem et al., 2010), with an estimated production of 30,000

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metric tons per year (BCC Research, 2015). The rapid increase in the application of ZnO NM has led to this material being released and dispersed in the environment and bioavailable forms are thus present in ever greater amounts (Nel et al., 2006; Selck et al., 2016). Nevertheless, the environmental effects of these NM are still not fully understood (Selck et al., 2016).

The toxicity of ZnO NM has been widely studied and reported in the literature toward different types of test organisms. Ann et al. (2015) observed that ZnO NP with size between 20 and 40 nm were more toxic to the L929 fibroblast mouse cell lines than the particles with size between 50 and 80 nm. According to Kumar et al. (2017) the surface functionalization of ZnO NP with polar groups can enhance the interaction between the NM and the bacterial cell wall, improving the antibacterial property of ZnO. In larval *Danio rerio*, ZnO NP were more toxic than $ZnSO_4$, however, ZnO NP did not involve transcript alterations but occurred a slight induced cell differentiation and pathways related with the immune system and activated divers key genes involved in cancer cell signaling compared to the zinc salt (Kim et al., 2017).

Regarding to the acute toxicity of ZnO NP toward the freshwater microcrustacean *D. magna*, Blinova et al. (2010) reported an $EC50_{(48h)}$ value of 2.6 mg/L testing NP with 70 nm. Santo et al. (2014) obtained an $EC50_{(48h)}$ value of 3.1 mg/L for NP > 100 nm and $EC50_{(48h)}$ of 1.9 mg/L for NP < 50 nm. In a study by Heinlaan et al. (2008) the $EC50_{(48h)}$ of ZnO NP in the range of 50–70 nm toward *D. magna* was of 3.2 mg/L. Mwaanga et al. (2014) observed the oxidative stress associated to biochemical changes due to the exposition of *D. magna* to ZnO NP for 72 h. Bacchetta et al. (2016), observed morphological alterations caused by ZnO NP in *D. magna*, however, the cytotoxicity of ZnO NP was mainly attributed to the dissolved ions from the NP.

In contrast to the acute toxicity, the chronic effects of ZnO NP toward *D. magna* are poorly investigated. Only a few studies reported the chronic toxicity to this NM. Zhao et al. (2012) observed effects on growth and reproduction of *D. magna* with a very low no observed effect (NOEC) and lowest observed concentration (LOEC) values to the reproduction parameter (0.0008 and 0.004 mg Zn/L, respectively), comparing to the study of Adam et al. (2014) (0.0058 and 0.131 mg/L) and Lopes et al. (2014) (0.125 mg Zn/L). Bacchetta et al. (2017) reported a significand inhibition of the reproduction and ultrastructural damages on *D. magna* after the chronic exposition to ZnO NP.

Studies involving the multigenerational and recovery phase approach of NM toward *D. magna* are less investigated than the chronic toxicity. Thus, despite the efforts of the scientific community to understand the mechanisms and impacts of these NM to the environment, there is still a gap on the understanding in how the NM can affect the organisms after the exposure was removed and if the effects of the maternal exposure will be evident in the future generations; and how the continuous exposure for several generations can affect the organisms. Bacchetta et al. (2017), analyzed the recovery of *D. magna* after chronic exposure to NP ZnO (21 days) until the end of the life cycle of the daphnids (around 60 days) and observed a significant reduction in longevity while the reproduction did not show significant difference from the control. Concerning to the multigenerational toxicity tests with NM, Arndt et al. (2014) observed that some carbon NM did not induce multigenerational toxicity on *D. magna* while others can affect the survival and reproduction of the organisms. These studies demonstrate that the ZnO NP can affect the whole life cycle of *D. magna* and their future generations, highlining the importance of conducting multigenerational toxicity tests.

Considering that most of the studies about ZnO NM are carried out with NP, thus, we studied the ZnO in a rod shape. In the present study, we evaluated the acute, chronic and multigenerational effects of ZnONR and ZnONR@AF in comparison with zinc sulfate ($ZnSO_4$) by exposing the freshwater microcrustacean *Daphnia magna* to these materials. Daphnia are widely used as bioindicators in toxicology studies with NM due to their parthenogenetic reproductive strategy, good responses to environmental stressors and their well know biology. How *D. magna* are genetic clones, these organisms are ideal for multigenerational tests. The consequences of the maternal exposures (F0) to future generations of daphnids (F1 and F12) were evaluated using the effects in the life cycle parameters. The main questions of this study were: (a) Does the samples cause acute effects and the acute concentrations can induce the generation of reactive oxygen species? (b) Does the chronic exposure affect the life cycle parameters of *D. magna*? (c) Can the samples affect the future generations of *D. magna* even when the offspring was not directly exposure to the samples (after the maternal exposure)? And (d) are the life cycle parameters able to detect these effects on a multigenerational recovery test?

2. Material and methods

2.1. Chemicals

All chemical products employed in the experiments were obtained from Sigma-Aldrich, Acros Organics, ThermoFischer Scientific and Vetec Química. They were of A.C.S reagent grade and used without prior treatment. Ultrapure (UP) water was generated by a Mili-Q Advantage A10 water purification system (Milipore, USA).

2.2. Synthesis of ZnONR

ZnONR were synthesized as described by Yang and Liu, (2011) with minor modifications. Briefly, zinc acetate (60 mmol) and potassium hydroxide (130 mmol) were solubilized in 40 and 60 mL of methanol, respectively, and the solutions was stirred under reflux for 72 h at 60 °C. The solid precipitate was isolated from the solution by centrifugation and washed three times with water and then ethanol. It was then dried for 48 h at 50 °C.

2.2.1. Amine-functionalization of ZnO NR

The ZnONR surface was amine-functionalized according to Vicentini et al. (2017) with minor modifications. Briefly, ZnONR (1 g) and 3-[2-(2-aminoethylamino)ethylamino]propyltrimethoxysilane (AEAEAPTMS) (25 mmol) were added to 80 mL of anhydrous toluene and the solution was stirred under an argon atmosphere at room temperature for 24 h. ZnONR@AF was isolated from the solution by centrifugation, rinsed twice with ethanol and dried for 2 h at 100 °C. The ZnONR@AF were then washed with water and dried for 48 h at 50 °C.

2.3. Characterization of ZnO NR

The crystalline structure of ZnO NR was determined by X-ray diffraction (XRD). The diffraction angle was recorded in the angular range of $2\theta = 0°–80°$ at room temperature using a Philips X'Pert diffractometer equipped with a copper tube (CuKα, $\lambda = 1.54056$ Å). The crystallite diameter (D) of both ZnO NR was estimated using the Scherrer equation according to Rossetto et al. (2014a). The amine-functionalization of ZnO NR was verified by Fourier transform infrared spectroscopy (FTIR) (Bruker FT-IR alfa spectrophotometer). Samples were blended with KBr and then pressed into disks for analysis in the range of 4000 and 400 cm^{-1}. The size and shape of the ZnO NR were determined by transmission electron microscopy (TEM) (JEOL, JEM-1011 TEM, 100 kV). The samples for TEM analysis were prepared by dropping the solutions on a C-Cu grid (300 mesh) and dried in a desiccator under vacuum for 24 h. The surface area was determined by the Brunauer–Emmett–Teller (BET) method using a NOVA® surface area analyzer (Quantachrome Instruments). The zeta potential (Pζ) was determined by the electrophoretic mobility approach using a ZetaPlus system (NanoBrook 90 Plus Zeta, Brookhaven Instruments Corporation, USA). The stability of the particles, in the different media, was determined by the hydrodimic diameter (HD) using a dynamic light scattering system (NanoBrook 90Plus Zeta, Brookhaven Instruments

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