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Protective effects of chitosan against the hazardous effects of zinc oxide nanoparticle in freshwater crustaceans *Ceriodaphnia cornuta* and *Moina micrura*

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ABSTRACT

Nanoparticle contamination in freshwater habitat leads to the drastic reduction in the population of freshwater micro crustaceans. Both *Ceriodaphnia cornuta* and *Moina micrura* were considered as the potential biological bio-tracers of freshwater ecosystem. This study describes the chemical synthesis of zinc oxide nanoparticles (ZnO NPs) using zinc nitrate as the starting material. The physico-chemical characterization of ZnO NPs was made by UV–Vis spectroscopy, X-Ray Diffraction (XRD), Fourier Transform Infra Red (FTIR) and Transmission Electron Microscopy (TEM). ZnO NPs elicited 100% and 76% mortality of freshwater crustaceans, *Ceriodaphnia cornuta* and *Moina micrura* at 160 $\mu g\,L^{-1}$ respectively. The accumulation of ZnO NPs in the intestine and loss of antennae and carapace were clearly visualized through light microscopy. The exposure of *C. cornuta* and *M. micrura* neonates at 160 $\mu g\,L^{-1}$ of ZnO NPs showed abnormal swimming behaviour after 12 h. However, chitosan significantly reduced the mortality and enhanced the survival of *C. cornuta* and *M. micrura* at 100 $\mu g\,ml^{-1}$. This study concludes the protective effect of chitosan against the hazardous effect of ZnO NPs in *C. cornuta* and *M. micrura*.

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1. Introduction

Nanoparticles can enter environment and transmit through air, soil and underground water, potentially affecting the health of organisms living in these environments. In addition, nanoparticles can enter the food chain and the presence of nanoparticles in living organisms may have a destructive effects leading to irreversible damage to them (Monica and Cremonini, 2009). Metal oxide nanoparticles (NPs) (e.g., ZnO, TiO2) are among the most studied NPs in ecotoxicology as a consequence of their wide application in novel technologies (Wu et al., 2013; Montes et al., 2012), and their production rate is expected to continue to rise in the coming years (Miller et al., 2010). Therefore, it is necessary to evaluate the hazard associated with engineered nanomaterials by evaluating their effects on organisms. Previously, the toxic effects of ZnO NPs to several organisms such as bacteria (Premanathan et al., 2011), algae (Ji et al., 2011), fish (Yu et al., 2011), and human cells (Paszek et al., 2012) have been reported.

Invertebrates are consumers in aquatic ecosystems and certain invertebrate species have been used as ecotoxicity test organisms. The freshwater crustaceans like *Daphnia* and *Ceriodaphnia* are bioindicators of environmental pollution and has been used to determine the toxicity of chemicals or nanoparticles (Blaise and Ferrard, 2005). They are commonly found in ponds and lakes and widely used as live fish food. They are also a very important component of the food chain of the aquatic ecosystem. They feed by filtering minute particles, such as bacteria and algae, from the fresh water in which they live. They interact with large portions of the environment and therefore have a greater potential to be affected by ingestion of pollutants compared to that of other aquatic organisms. It is reported that the exposure of aquatic organisms like *Daphnia* sp to metallic NPs such as Fe-NPs TiO_2, CuO/Cu_2O and

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Ag-NPs as well as carbon nanomaterial such as fullerene; and also silica NPs has been correlated to an increase in oxidative damages and to a modification of the antioxidant system (Fan et al., 2012; Ferreira et al., 2012). Previously, Vaseeharan et al. (2010) reported the protective efficacy of silver nanoparticles synthesized using tea leaf extracts in juvenile *Feneropenaeus indicus* against *Vibrio harveyi* infection. The silver nanoparticles synthesized using *Calotropis gigantea* leaf extracts enhanced the survival rate of *Artemia* naupli from *Vibrio alginolyticus* infection (Vaseeharan et al., 2012). Recently, Manju et al. (2016) reported that essential oil of *Nigella sativa* protects *Artemia* from the pathogenic effects of *Vibrio parahaemolyticus* Dahv2. Based on these perspectives, the present study is attempted to investigate the protective function of chitosan in aquatic species.

Chitin ($C_8H_{13}O_5N$) is a mucopolysaccharide polymer consisting of N-acetyl-D-glucosamine residues that are abundant in the shells of crustaceans and insects (Kaya and Baran, 2015; Kaya et al., 2015). Chitosan is derived from chitin by deacetylation and is manufactured commercially on a large scale for various biomedical applications. Chitosan have been reported to protect salmonids against bacterial disease (Anderson and Siwicki, 1994; Siwicki et al., 1994), enhance phagocytic activity in the gilthead sea bream (Esteban et al., 2001; Ortuno et al., 2000; Cuesta et al., 2003), immersion and dietary supplements (Kono et al., 1987; Kawakami et al., 1998) and burn wound healing in rats (Alemdaroglu et al., 2006). Therefore, the objective of this study was to determine the effects of ZnO NPs to the freshwater crustaceans, *C. cornuta* and *M. micrura*. In addition, the protective effect of chitosan on the survival and repair of lost parts of *C. cornuta* and *M. micrura* was investigated.

2. Materials and methods

2.1. Synthesis of ZnO nanoparticles

ZnO NPs were prepared by chemical methods using zinc nitrate and sodium hydroxide as precursors and soluble starch as a stabilizing agent (Yadav et al., 2006). 0.1 M zinc nitrate was mixed with 500 ml of 0.1% starch solution. The solution was kept under constant stirring using a magnetic stirrer to completely dissolve the zinc nitrate. After complete dissolution of zinc nitrate, 0.2 M sodium hydroxide solution was added drop wise under vigorous stirring. The reaction was allowed to proceed for 2 h. After the completion of the reaction, the solution was allowed to settle overnight, and the supernatant solution was discarded carefully. The remaining solution was centrifuged at 10,000*g* for 10 min and the supernatant was discarded. The obtained nanoparticles were washed thrice with distilled water to remove the impurities and excess starch that were bound with the nanoparticles. After thorough washing, the nanoparticles were dried at 80 °C overnight.

2.2. Physico-chemical characterization of ZnO nanoparticles

2.2.1. UV–Visible spectroscopic analysis

The bioreduction of Zn^+ ions in solutions was monitored by periodic sampling of aliquots (1 ml) of the aqueous component and measuring the UV–Vis spectra of the solution. UV–Vis spectra of these aliquots were monitored as a function of time of reaction on a spectrophotometer (UV-1800, Shimadzu, Japan) in 200–800 nm range operated at a resolution of 1 nm (Ankamwar et al., 2005).

2.2.2. X-ray diffraction (XRD) analysis

The particle size and crystalline nature of the zinc oxide nanoparticles were determined using XRD. This was carried out using XRD—6000/6100 (Shimadzu, Japan) models with 40 kv, 30 mA with Cu k α radians at 2 θ angles. X-ray powder diffraction is a rapid analytical technique primarily used for phase identification of a crystalline material and can provide information on unit cell dimensions. The analyzed material was finely ground, and the average bulk composition was determined. The particle size of zinc oxide nanoparticles was determined using Debye Scherrer's equation (DS).

$$D = 0.94\lambda/B\cos\theta$$

where λ is the wavelength (Cu Kα), β is the full width half- maximum (FWHM) of the ZnO (101) line and θ is the diffraction angle (Shankar et al., 2003).

2.2.3. Fourier transform infrared (FTIR) spectroscopy of ZnO NPs

Two milligram of ZnO NPs was mixed with 200 mg of potassium bromide (FT-IR grade) and pressed into a pellet. The sample pellet was placed into the sample holder and FT-IR spectra were recorded in FT-IR spectroscopy (Nicolet™ iS™5, Thermo Scientific, Madison, WI, USA) at a resolution of 4 cm^{-1} (Naheed et al., 2011).

2.2.4. Transmission electron microscopy

TEM analysis was performed by placing a small volume of ZnO NPs on carbon-coated copper grids and solvent were allowed to evaporate for 30 min. TEM measurements were performed on JOEL, Japan model instrument 1200 EX instrument on carbon coated copper grids with an accelerating voltage of 80 kV (Deepak et al., 2011).

2.2.5. Zeta potential

Zeta potential was determined using Zetasizer Nano Z (Malvern Instruments, UK) by dispersing synthesized ZnO NPs in water (Kim et al., 2014).

2.3. Ecotoxicity of ZnO NPs

2.3.1. Culture and maintenance of Ceriodaphnia cornuta and Moina micrura

C. cornuta and M. micrura used in this study were derived from the stock culture maintained in our laboratory for approximately two years prior to the beginning of the study. Stocks of *C. cornuta and M. micrura* were cultured in standard synthetic freshwater as per United States Environmental Protection Agency (USEPA) protocol (USEPA, 2002), (total hardness 80–100 mg L^{-1} as $CaCO_3$; pH 7.9 ± 0.2; alkalinity 57–64 as CaCO3; temperature 23 ± 2 °C), 16:8 h light: dark photoperiod and a density of below 50 animals per litre (Ferrando et al., 1995). Water was renewed twice each week, and they were fed daily with the unicellular algae, *Chlorella vulgaris*. Algae were cultured in nutrient medium (Bischoff and Bold, 1983). The above conditions were maintained throughout the experiment.

2.3.2. Effect of ZnO NPs to Ceriodaphnia cornuta and Moina micrura

A preliminary toxicity test of ZnO NPs was conducted on *C. cornuta* and *M. micrura* using the concentrations ranging from 2 $\mu g\,L^{-1}$ to 10 $\mu g\,L^{-1}$. However, these concentrations were observed to be non–toxic to *C. cornuta* and *M. micrura*. Hence, the concentrations greater than 10 $\mu g\,L^{-1}$ were used for acute toxicity studies. Based on the preliminary test, the effect of ZnO NPs to *C. cornuta and M. micrura* was studied using five different concentrations, i.e. 10, 20, 40, 80 and160 $\mu g\,L^{-1}$, plus a negative control (without ZnO NPs). Each ZnO NPs concentration was interacted with ten neonates collected from a selected brood for a period of 24 h. Both *C. cornuta and M. micrura* was not fed during the experimental period. The experiments were carried out under photoperiod conditions (light: dark: 16: 8 h). The glass beakers were not covered fully to allow proper passage of air. After the experiment, test animal that has not shown

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