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Evaluation of zinc oxide nanoparticles toxicity on marine algae *chlorella vulgaris* through flow cytometric, cytotoxicity and oxidative stress analysis

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ABSTRACT

The increasing industrial use of nanomaterials during the last decades poses a potential threat to the environment and in particular to organisms living in the aquatic environment. In the present study, the toxicity of zinc oxide nanoparticles (ZnO NPs) was investigated in Marine algae *Chlorella vulgaris* (*C. vulgaris*). High zinc dissociation from ZnONPs, releasing ionic zinc in seawater, is a potential route for zinc assimilation and ZnONPs toxicity. To examine the mechanism of toxicity, *C. vulgaris* were treated with 50 mg/L, 100 mg/L, 200 mg/L and 300 mg/L ZnO NPs for 24 h and 72 h. The detailed cytotoxicity assay showed a substantial reduction in the viability dependent on dose and exposure. Further, flow cytometry revealed the significant reduction in *C. vulgaris* viable cells to higher ZnO NPs. Significant reductions in LDH level were noted for ZnO NPs at 300 mg/L concentration. The activity of antioxidant enzyme superoxide dismutase (SOD) significantly increased in the *C. vulgaris* exposed to 200 mg/L and 300 mg/L ZnO NPs. The content of non-enzymatic antioxidant glutathione (GSH) significantly decreased in the groups with a ZnO NPs concentration of higher than 100 mg/L. The level of lipid peroxidation (LPO) was found to increase as the ZnO NPs dose increased. The FT-IR analyses suggested surface chemical interaction between nanoparticles and algal cells. The substantial morphological changes and cell wall damage were confirmed through microscopic analyses (FESEM and CM).

1. Introduction

Nanotechnology has come to the forefront of research in the past decade. With the advent of this technology, wide varieties of nanoparticles (NPs) with a variety of unique characteristics are manufactured and are used for a broad range of applications (Zhao and Castranova., 2011). Metal oxide NPs are among the most used engineered NPs in various commercial products, leading to concerns of their potential toxicity to human and environmental health (Aschbergeretal., 2011). Increasing use of metaloxide NPs is likely to result in the release of these particles into the aquatic environment. Adverse effects of NPs on the aquatic environment and organisms recently have drawn much special attention (Blaise etal., 2008; Farre´ et al., 2009).

Among metal oxide nanomaterials, zinc oxide nanoparticles (ZnO NPs) are noted for their chemical stability and strong adsorption ability and have been extensively used in commercial products like sunscreens, coatings, and paints (Osmond and McCall., 2010). ZnO has a high inherent risk of water contamination, and can reach high concentrations in surface waters posing significant threat to aquatic ecosystems (Gottschalk et al., 2009). Previous studies have demonstrated that ZnO NPs are toxic to microorganisms, cells, plants, aquatic biota and rodents (Brayner et al., 2006; Lin and Xing., 2007; Premanathan et al., 2011; Reddy et al., 2007; Wang et al., 2008a; Zhu et al., 2009). Zhu et al. (2008) found that ZnO NPs had higher acute toxicity to zebrafish embryos than nano-TiO_2 and nano-Al_2O_3. The toxicity of ZnO NPs (96hLC_{50}, 4.9 mg/L)to zebra fish was also much higher than that of nano-TiO_2 (96 hLC_{50}, 124.5 mg/L) (Xiong et al., 2011). Increased production of antioxidant enzymes in organisms is regarded as an early warning indicator of pollution in the environment (Song et al., 2008). Antioxidant enzyme activity can protect cells from the adverse effects of reactive oxygen species (ROS). Superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) enzymes help to check cellular levels of ROS (Weckx and Clijsters, 1996). Studies of the biotoxicity of ZnO NPs suggest several mechanisms of action of NPs, one of which is their ability to release free zinc ions which

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can synergistically enhance the production of ROS and cause oxidative damage in cells. Overproduction of ROS is believed to be a major mechanism of the toxicity of NPs (Sharifi et al., 2012). During this process, chemical reactions occur and result in increased formation of the superoxide radical (O^{2-}), which leads to ROS accumulation and oxidative stress (De Berardis et al., 2010). It has been reported that ZnONPs disturb the balance between oxidation and anti-oxidation processes and cause oxygen stress responses (Hao and Chen, 2012).

As primary producers, phytoplankton plays a key role in aquatic ecosystems. Microalgae, being sensitive to pollutants, are excellent aquatic models and being widely prevalent in lakes and seas is easy to culture and propagate (Chen et al., 2012). The evaluation of NP effects upon marine phytoplankton is a necessary step to predict their potential impact on coastal marine food webs and on the whole ecosystems they support (Manzo et al., 2013). ZnO NPs, on entering the aquatic environment, release free metal ions in solution state. The dissolution speed of the particles mainly depends on the particle size, surface area, rough degree, etc. ZnO is slightly soluble, and can release zinc ions into the solution. Some researchers consider that dissolved zinc ions play an important role in the toxicity of ZnO NPs (Song et al., 2010). Some studies report that the toxicity of ZnO NPs was closely linked with its dissolved free ions (Franklin et al., 2007; Heinlaan et al., 2008; Wienchet al., 2009), but others have also shown that the toxicity of ZnO NPs was much higher than its ion toxicity (Nair et al., 2009; Wong et al., 2010). Both *Escherichia coli* and *Pseudomonas fluorescens* exhibited a high tolerance to zinc ions at the tested ZnO concentration (20 mg/L). The attachment of ZnO NPs to the surface of bacteria was the main cause of the ZnO toxicity of these bacterial species (Jiang et al., 2009).The point is that, at current knowledge, the observed ZnO toxicity cannot be attributed univocally to the release of zinc ions or to the nanosize as the peculiar surface interactions.

Hence, the aim of the current study was to elucidate the different mechanistic modes of cytotoxicity of ZnO NPs towards marine algae, *Chlorella vulgaris*. The mechanistic end points included oxidative stress analyses, membrane permeability assessment, and ionic dissolution studies. This study is first of its kind in the usage of flow cytometry techniques to investigate the toxicity of ZnO NPs to cell viability in *C. vulgaris* and to offer a more theoretical foundation on which to evaluate the toxic effects of ZnO NPs.

2. Material and methods

2.1. Synthesis of ZnO NPs

The preparation of ZnO NPs was done by the solid state pyrolytic method by mixing zinc acetate dihydrate (2.2 g, 10 mmol) and sodium bicarbonate (2.0 g, 23.8 mmol) at room temperature. The mixture was then pyrolysed at 300 °C for 3 h. Zinc acetate dihydrate gets reduced to ZnO NPs by thermal decomposition. Sodium acetate formed in the mixture is washed off with deionized water, to obtain pure ZnO NPs.

2.2. Characterization of ZnO NPs

ZnO NPs were characterized by X-ray diffraction method (XRD), field emission scanning electron microscopy (FESEM) and energy-dispersive X-ray spectrometry (EDX). The XRD measurement of ZnO NPs was carried out on films of the respective solutions drop-coated onto a glass substrate on a Rigaku SmartLab instrument operated at a voltage of 9 kW and a current of 30 mA with CuKa radiations. The ZnO NPs were mounted on the copper stubs, and the images were studied using FESEM with a Supra 55 (Carl Zeiss, Germany) with secondary electron detectors at an operating voltage of 5 kV. The elemental analysis was done by energy dispersive X-ray analysis (EDX) coupled to FESEM.

2.3. Dissolution of ZnO NPs

The suspensions of ZnO NPs were prepared by dispersing ZnO NPs in sea water with a bath sonicator for 30 min to break aggregates as much as possible. To determine whether Zn^{2+} dissolved from ZnO NPs suspensions might play a role in the observed toxicity, ZnO NPs (50–300 mg/L) were centrifuged at 10,000 rpm for 15 min and clear supernatants were carefully collected. The released Zn^{2+} concentration in the supernatants were measured by Atomic Absorption Spectrometer.

2.4. Algae culture

C. vulgaris was obtained from Marine Biotechnology Division, Earth System Sciences Organization – National Institute of Ocean Technology, Chennai, Tamil Nadu,India and cultured in autoclaved 1 L flask, filled with 400 ml of sea water and F/2 nutrients without trace metals and ethylenediaminetetraacetic acid (EDTA).The flasks were autoclaved at 121 °C for 20 min and allowed to cool for 24 h prior to use. The sterilized media were inoculated with cells of *C. vulgaris*, and the algae were cultured for 5–7 d with continuous aeration. Algae was incubated under cool white fluorescent lights (12 h light: 12 h dark) at a temperature of 20 °C.

2.5. Toxicological studies

The exponential phase of *C. vulgaris* was used. Cells were treated with different concentrations (50, 100, 200, 300 mg/L) of the ZnO NP dispersion. An initial cell count of 4×10^5 cells was used for all experiments. The samples were kept in the static conditions, and intermittent mixing was carried out to avoid sticking of the cells to the walls of the container. Incubation was done under cool white fluorescent light (12 h light: 12 h dark) at a temperature of 20 °C. The samples were analyzed after an interaction period (with NPs) of 24 h and 72 h respectively.

2.6. Flow cytometric analysis

In this study, fluorescence of cells stained with propidium iodide (PI) was measured to examine the cell viability. PI is a fluorescent dye that intercalates with double-stranded nucleic acids to produce red fluorescence when excited by blue light. But PI will not be able to pass through intact cell membranes of live cells. However, when the cell dies the integrity of the cell membrane fails and PI would be able to enter and stain the nucleic acids (Ormerod, 1990). PI can be used in this manner to discriminate between live nonfluorescent cells and dead fluorescent cells. Initially, cells were collected by centrifugation (3500 rpm, 10 min), washed with phosphate buffer solution (PBS, pH 7.0), and then stained with PI during an incubation period of 20 min. The final concentration of PI in the cell suspensions was 60 μM. The fluorescent emission of this compound was collected in the FACS Calibur- Becton Dickinson FL3 channel.

2.7. Cytotoxicity assay

2.7.1. MTT test

In order to determine the cytotoxicity effect of ZnO NPs, 3-(4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide (MTT) dye reduction assay was performed on *C. vulgaris* using increasing concentrations (10–300 mg/L). The result of the assay depends on