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Research article

Toxicity of ZnSe nanoparticles to *Lemna minor*: Evaluation of biological responses

Roshanak Tarrahi[a,b], Alireza Khataee[b,c,*], Ali Movafeghi[a], Farkhondeh Rezanejad[d]

[a] Department of Plant Biology, Faculty of Natural Sciences, University of Tabriz, 51666-16471 Tabriz, Iran
[b] Research Laboratory of Advanced Water and Wastewater Treatment Processes, Department of Applied Chemistry, Faculty of Chemistry, University of Tabriz, 51666-16471 Tabriz, Iran
[c] Institute of Environment, University of Tabriz, 51666-16471 Tabriz, Iran
[d] Department of Biology, Faculty of Sciences, Shahid Bahonar University of Kerman, Kerman, Iran

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ABSTRACT

A clear consequence of the increasing application of nanotechnology is its adverse effect on the environment. Semiconductor nanoparticles are among engineered nanomaterials that have been considered recently for their specific characteristics. In the present work, zinc selenide nanoparticles (ZnSe NPs) were synthesized and characterized by XRD, TEM, DLS and SEM. Biological aspects related to the impact of nanoparticles and Zn^{2+} ions were analyzed on the aquatic higher plant *Lemna minor*. The localization of ZnSe NPs in the root cells of *L. minor* was determined by TEM and fluorescence microscopy. Then, the entrance of ZnSe NPs into the plant cells was evaluated by a range of biological tests. The outcomes revealed that both the NPs and the ionic forms noticeably poisoned *L. minor*. In one hand, growth parameters and physiological indices such as photosynthetic pigments content were decreased. On the other hand, the activities of some antioxidant enzymes (superoxide dismutase (SOD) and catalase (CAT)), as well as the contents of nonenzymatic antioxidants (phenols and flavonoids) were elevated. Taken together, high concentration of ZnSe NPs and Zn^{2+} triggered phytotoxicity which in turn provoked the plants' defense system. The changes in antioxidant activities confirmed a higher toxicity by Zn^{2+} ions in comparison with ZnSe NPs. It means that the considered ions are more hazardous to the living organisms than the nanoparticles.

1. Introduction

Nanomaterials have unique chemical and physical properties and are widely used in various fields of science and technology. They have high motility and reactivity that is caused by a large fraction of the atoms on the surface. Many negative environmental effects of nanomaterials have emerged a challenging issue for environmental management (Conti et al., 2008; Zaka et al., 2016).

Semiconductors including zinc selenide (ZnSe) are engineered nanomaterials, which have been poorly studied. ZnSe is a member of $A^{II}B^{VI}$ binary semiconductors group and is used in high power CO_2 laser systems and short-wavelength photoelectronic devices due to its particular characteristics (e.g. blue laser, light emitting diodes, biomedical sensors, etc.) (Cao et al., 2008; Wang et al., 2007).

The synthesis of inorganic materials embraces a variety of procedures and techniques. Essentially, an eco-friendly synthesis of a single or heterogenous phase reaction with inorganic materials is a key target. Thus, in the current study the hydrothermal method has been introduced for advanced materials processing. Its process under high temperature and pressure, dissolves relatively insoluble materials and produce highly pure and crystalline products with low aggregation (Supothina et al., 2012). Thus, semiconductor nanocrystals can be dispersible in aqueous media. Features of the synthesized nanoparticles were commonly defined by XRD, SEM, DLS, and FT-IR methods. In addition, AAS has been performed to assess the ionic release of nanoparticles. NPs access to biological systems is controlled by their physical and chemical features which finally causes toxicity. Also, ionic release (from their composition), leads to formation of reactive oxygen species (ROS) inside cells (Hardman, 2006). So far, the toxic effects of zinc selenide nanoparticles (ZnSe NPs) has not been investigated on plants in aquatic ecosystems.

Members of the genus *Lemna* are aquatic vascular plants, which are widely used in ecotoxicological research. They are sensitive to a wide range of pollutants (Singh, 2015) and small volumes of sample toxicants

* Corresponding author. Research Laboratory of Advanced Water and Wastewater Treatment Processes, Department of Applied Chemistry, Faculty of Chemistry, University of Tabriz, 51666-16471 Tabriz, Iran
E-mail address: a_khataee@tabrizu.ac.ir (A. Khataee).

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are needed to be applied for their toxicity experiments (Kumar and Han, 2010; Van Hoeck et al., 2015) Moreover, *Lemna* plants have attracted attention due to their key roles in primary production, nutrient cycling and formation of aquatic ecosystems by supplying food for organisms of higher trophic levels beside their wide geographical distribution. *L. gibba* and *L. minor* are the most used species in phytotoxicity tests (Drost et al., 2007). *L. minor* has been regarded as the standard for toxicity measurements in the presence of environmental contaminants by the United States Environmental Protection Agency (1996) and OECD (2002). Small size, simple structure, facile manipulating and culture, high growth rate, small genome size and being initial generators in the environment are the special properties of this plant (O ros and Toma, 2012).

In the present study, ZnSe NPs were synthesized by hydrothermal method and their effects on *Lemna minor* were investigated. The influence of ZnSe NPs on growth parameters and physiological indices was evaluated. It is hypothesized that Zn^{2+} ions is a crucial reason for the toxicity caused by ZnSe NPs.

2. Materials and methods

Culture and exposure conditions are explained in the supplementary file.

2.1. Hydrothermal synthesis and characterization of ZnSe NPs

Chemicals and Materials used in the present work were of analytical grade and provided by Merck Co. (Germany).

$ZnSO_4{\cdot}7H_2O$ (99.5%) and Na_2SeO_3 (99%) precursors were applied to synthesize ZnSe NPs hydrothermally by adding hydrazine hydrate ($N_2H_4{\cdot}H_2O$) as the reducing agent. In a typical synthesis procedure for ZnSe NPs, $ZnSO_4{\cdot}7H_2O$ (0.9 mmol), Na_2SeO_3 powder (1 mmol) and NaOH (12.5 mmol) were mixed in 50 ml of distilled water. Hydrazine hydrate ($N_2H_4{\cdot}H_2O$) was poured dropwise to the first solution. The mixture was transferred into a 100 ml Teflon-lined stainless-steel autoclave and heated up to 180 °C for 24 h; afterwards, it was cooled to reach the room temperature. The precipitate was washed several times with distilled water and absolute ethanol. Finally, it was vacuum-dried at 60 °C for 5 h. As a result, the black powder was obtained (Khataee et al., 2014).

To confirm the crystalline form, purity and mean size of ZnSe NPs, XRD measurements were carried out at room temperature using D8 Advance diffractometer device (Bruker, Germany) with Cu Kα radiation (λ = 1.5406 A°). The accelerating voltage of 40 kV and the emission current of 30 mA were used. The average crystalline size of NPs was calculated by Debye–Scherrer formula (Patterson, 1939). Also, energy dispersive X-ray (EDX) spectroscopy was carried out using an EDX spectrometer (Hitachi S-4200, Japan). TEM images were taken by a Cs-corrected high-resolution TEM (JEM-2200FS; JEOL, Japan) operating at 200 kV. SEM analysis was carried out by a Hitachi SEM (S-4200, Japan) to study the morphology of NPs. In addition, the average hydrodynamic diameter of ZnSe NPs in medium was measured using DLS Nanotrac (Czeck) Wave. An analysis for determination of Zn and Se ions release from ZnSe NPs was performed in the emulsion solution at different concentrations (5, 20 and 80 mg L^{-1}). Both media, without and with *L. minor* plants, (after 4 days of treatment) were analyzed compared to the control using atomic absorption spectrophotometer (AAS) (Analytik Jena, NovaAA400, Germany). The solutions were centrifuged and the supernatants were sonicated prior to the analysis using an ultrasonic bath (Sonica 2200 EP S3, Italy). The assay was done for the detection of ions concentration in the solution.

2.2. Microscopic observation of ZnSe NPs

2.2.1. Fluorescent microscopy

Fluorescent images were taken by an Olympus BX51 fluorescence microscopy equipped by UMPlanFL-BDP and the BX-RFA fluorescence illuminator at 480–510 nm wavelengths (Olympus Optical Co., Ltd. Tokyo, Japan) in order to trace ZnSe NPs inside the treated root cells of *L. minor* (after a week) compared to control. For preparing the specimen for observation, the roots of *L. minor* were excised and soaked in 0.1% Auramine O for 10 min. Then, they were washed several times with distilled water to wash away any residue on the surface of the root cells. Stack z-projection could give the best images for the eventual setting of images (Movafeghi et al., 2010).

2.2.2. Transmission electron microscopy

In order to assess the entrance and localization of ZnSe NPs inside the root cells of *L. minor,* ultrastructural analysis was performed using transmission electron microscopy (TEM, Zeiss LEO 912 Omega, Germany). In this respect, sectioned roots of the 8 day-treated plants with ZnSe NPs were fixed initially by 2% (v/v) glutaraldehyde in 100 mM phosphate buffer, pH 7.4, for 24 h at 4 °C. The samples were then washed 4 times with phosphate buffer and the second fixation phase was done by 2% (w/v) osmium tetroxide for 2 h. After that, the samples were dehydrated by ethanol series followed by immersing in araldite epoxy resin. Leica ultramicrotome with a diamond knife (Leica Mikrosysteme, A-1170, Austria) was applied for excising the specimen at 60 nm thickness. At the final stage, micro slides were stained with methanolic uranyl acetate (2%) and Reynold's lead citrate (Ritzenthaler et al., 2002). A LEO 906 TEM was run at 80 kV electron voltage for observation.

2.3. Measurements of plant growth

Four morphological parameters, namely relative frond number (RFN), frond size, fresh weight and dry weight were analyzed to find out the impacts of various concentrations of ZnSe NPs on the plants (1–80 mg L^{-1}). RFN and frond size measurements were performed by using 20 isometric fronds for treatments while for fresh and dry weight analyses, 40 relative isometric fronds were used. All tests were executed at room conditions in the laboratory.

A stereomicroscope (Olympus, Japan) and a digital scale (Adam equipment, AAA 250L, USA) were employed for growth rate calculations every 2 days for a duration of 8 days. RFN was calculated through Eq. (1) (Mitsou et al., 2006).

$$RFN = (\text{frond } N_1 - \text{frond } N_0)/\text{frond } N_0, \quad (1)$$

N_0 and N_1 stand for frond number at day 0 and day n, respectively.

2.4. Quantification of enzymatic and nonenzymatic antioxidants

2.4.1. Enzymatic antioxidants assay

L. minor was treated by 1–80 mg L^{-1} of ZnSe NPs (equal to 0.007 mM–0.55 mM) as well as ionic forms of zinc (Zn^{2+}) including zinc acetate ($C_4H_6O_4Zn$) and zinc sulfate ($ZnSO_4$) in the equal range of 0.007 mM–0.55 mM and enzymes' activities were recorded versus the control during one week. A quantity of 0.250 g of the plant was extracted in the extraction buffer (0.1 mol, pH = 7.0) comprising 2% PVP (w/v) followed by centrifugation at 6000 × g for 15 min at 4 °C. The supernatant was utilized for the tests (Bradford, 1976). Each measurement was performed in three repeats.

The activity of SOD (EC 1.15.1.1) was acquired based on the method that has already been reported (Beyer and Fridovich, 1987). One unit of this enzyme inhibits 50% of photochemical reduction of NBT at 560 nm (Winterbourn et al., 1976).

The activities of POD (EC 1.11.1.7) and CAT (E.C. 1.11.1.6) were recorded by the methods of Chance and Maehly. One unit of POD generates 1 μmol L^{-1} tetraguaiacol min^{-1} [ε = 26.6 (mmol $L^{-1})^{-1}$ cm^{-1}] at 470 nm and a unit of CAT splits 1 μmol of H_2O_2 per minute at 240 nm [ε = 39.4 (mol $L^{-1})^{-1}$ cm^{-1}]. The quantity of enzymes were determined based on the amount of one mg of protein