Environmental Pollution 185 (2014) 219–227

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Different toxicity mechanisms between bare and polymer-coated copper oxide nanoparticles in *Lemna gibba*

François Perreault, Radovan Popovic [1], David Dewez*

Department of Chemistry, University of Quebec in Montreal, Case Postal 8888, Succursale Centre-Ville, Montreal, QC H3C 3P8, Canada

ARTICLE INFO

Article history:
Received 3 April 2013
Received in revised form
4 October 2013
Accepted 24 October 2013

Keywords:
Copper oxide nanoparticle
Lemna gibba
Nanotoxicology
Reactive oxygen species
Polymer coating

ABSTRACT

In this report, we investigated how the presence of a polymer shell (poly(styrene-co-butyl acrylate) alters the toxicity of CuO NPs in *Lemna gibba*. Based on total Cu concentration, core–shell CuO NPs were 10 times more toxic than CuO NPs, inducing a 50% decrease of growth rate at 0.4 g l^{-1} after 48-h of exposure while a concentration of 4.5 g l^{-1} was required for CuO NPs for a similar effect. Toxicity of CuO NPs was mainly due to NPs solubilization in the media. Based on the accumulated copper content in the plants, core–shell CuO NPs induced 4 times more reactive oxygen species compared to CuO NPs and copper sulfate, indicating that the presence of the polymer shell changed the toxic effect induced in *L. gibba*. This effect could not be attributed to the polymer alone and reveals that surface modification may change the nature of NPs toxicity.

1. Introduction

Copper oxide nanoparticles (CuO NPs) are used to provide antimicrobial properties to coatings, plastics, textiles and food packaging (Abramova et al., 2013; Llorens et al., 2012). CuO NPs are known to be very toxic compared to other types of nanomaterials (Hsieh et al., 2013; Wang et al., 2011a) however the risks associated with their application are still unclear due to the limited knowledge of NPs interactions with biological systems (Luyts et al., 2013). With the rapid development of nanotechnology and the increasing risk of ecosystem contamination (Klaine et al., 2008; Nowack et al., 2012), there is an urgent need to understand the unique aspects of NPs toxicology.

Metal NPs may induce toxicity by direct interactions of NPs with cells or by the release of toxic metal ions. The contribution of metal ions to total toxicity is difficult to determine and previous studies have led to several contradictory findings. In the green alga *Pseudokirchneriella subcapitata*, CuO NPs effect was found to be solely due to the release of Cu ions (Aruoja et al., 2009; Franklin et al., 2007). However, in both algae and aquatic macrophytes, other studies have found that NPs solubilization could not explain CuO NPs toxicity and therefore indicated a contribution of the nanoparticulate form in CuO NPs effect (Manusadzianas et al., 2012; Shi et al., 2011). The specific nature of these NPs effects is still unclear. Direct NPs effects may be associated with the induction of oxidative stress (Moos and Slaveykova, in press), membrane deterioration (Wang et al., 2011a, 2011b) and decreased cellular exchanges with the media due to particles binding on cell membrane (Lin et al., 2009; Perreault et al., 2012a). Moreover, direct uptake of CuO NPs was found for microalgae (Perreault et al., 2012b), cyanobacteria (Wang et al., 2011b) and higher plants (Dimkpa et al., 2012, 2013). Intracellular interactions can therefore have an important role in CuO NPs toxicity.

Metal NPs are often coated with organic compounds in order to increase NPs dispersion and stability in aqueous media. Such surface modification, by changing the physico-chemical properties of NPs, may however change their cellular toxicity (Verma and Stellacci, 2010). Recently, we showed that core–shell CuO NPs (CS-CuO NPs), consisting of an inorganic CuO core and an organic (poly(styrene-co-butyl acrylate)) polymer shell, were more toxic to the green alga *Chlamydomonas reinhardtii* than bare CuO NPs (Perreault et al., 2012b; Saison et al., 2010). This higher toxicity could not be attributed to the effect of the polymer on *C. reinhardtii* (Saison et al., 2010), which suggests that CS-CuO NPs may induce a different effect than bare CuO NPs. The mechanisms involved in this

Abbreviations: Φ'_MII, Photosystem II operational quantum yield; CS-CuO NPs, core–shell copper oxide nanoparticles; DCF, 2′,7′-Dichlorofluorescein; FDA, fluorescein diacetate; H_2DCFDA, 2′,7′-Dichlorodihydrofluorescein diacetate; NPs, nanoparticles; P.I., performance index of Photosystem II; PSII, Photosystem II; ROS, reactive oxygen species.

* Corresponding author.
E-mail address: dewez.david@uqam.ca (D. Dewez).

[1] In memory of Pr. Radovan Popovic (1940–2012).

0269-7491/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.envpol.2013.10.027

effect were not fully understood, although higher NPs uptake and oxidative stress were hypothesized as participating in CS-CuO NPs toxicity.

The objective of this study was to determine how polymer coating alters the mechanism of toxicity of CuO NPs. The effects of bare CuO NPs, CS-CuO NPs and ionic copper were compared in the aquatic macrophyte *Lemna gibba*, a model organism widely used, along with *L. minor*, for ecotoxicological assays due to its small size, simple culture techniques and high sensitivity (ASTM, 2004; Greenberg et al., 1992). The results of this report allowed the identification of cellular effects specifically associated with CS-CuO NPs, which indicate that surface modification of nanomaterials may alter their mechanism of cellular toxicity. Modified nanomaterials should therefore be carefully evaluated for any new toxicological effects.

2. Material and methods

2.1. Biological material

Lemna gibba (CPCC #310) was obtained from the Canadian Phycological Culture Centre and grown in a freshwater culture medium (pH 6.5, ionic strength 0.0127 M) according to Frankart et al. (2002). See Table S1 for complete medium composition. Plants were grown in a CONVIRON growing chamber (Controlled Environments Limited, Winnipeg, MA, Canada), with a 16 h/8 h light/dark photoperiod. Illumination (100 $\mu E\ m^{-2}\ s^{-1}$) was provided by white fluorescent lamps (Sylvania GRO-LUX F40/GS/WS, Drummondville, QC, Canada) and temperature was kept at 24 °C.

2.2. NP synthesis

Copper sulfate salt ($CuSO_4 \cdot 5H_2O$, 99% purity) was obtained from Sigma–Aldrich (Sigma–Aldrich Canada Ltd., Oakville, ON, Canada) while CuO nanopowder was obtained from MTI Corporation (MTI corporation, Richmond, CA, USA). CuO NPs had an average particle diameter of 30–40 nm according to the manufacturer, with purity >99%. Particle suspensions were prepared in culture media and sonicated before use during 3 min at 40% intensity (VibraCell 400W, Sonics &. Materials Inc, USA). CS-CuO NPs, where the shell was composed of poly(styrene-co-butyl acrylate), were kindly provided by the Claverie research group. Briefly, polyacrylic acid was first synthesized by reversible addition-fragmentation chain transfer polymerization mediated with trithiobenzyl carbonate. In 20 ml of nanopure water, 4 g of CuO NPs and 110 mg of polyacrylic acid were stirred and sonicated for 3 min. In a 250 ml round-bottom flask, 4.0 ml of styrene, 51.5 mg of sodium dodecyl sulfate and 20.1 ml of the CuO dispersion were slowly stirred and degassed for 30 min by sparging with argon. The mixture was heated at 80 °C. A solution containing 40 mg of 4,4-azobis-4-cyanovaleric acid, three drops of sodium hydroxide (30%) and 20.2 ml of nanopure water was added continuously over 4 h. The mixture was kept at 80 °C for 1 h in order to complete the reaction. Thermogravimetric analysis of the CS structure indicated a weight composition of 33% core (CuO) and 67% shell (polymer). For more details concerning synthesis and characterization data, see Daigle and Claverie (2008). To obtain the polymer emulsion used for comparison with CS-CuO NPs treatment, the same synthesis was performed without adding CuO NPs in the reaction.

2.3. NP treatment

L. gibba plants were exposed to CuO NPs, CS-CuO NPs or copper sulfate in 24-well microplates. 2 ml of culture media supplemented with either no added copper (Ctrl), bare CuO NPs or CS-CuO NPs was added to each well and one 3-fronded *L. gibba* colony was placed per well. *L. gibba* colonies were exposed for 24-h and 48-h. Exposure was not prolonged beyond 48-h to avoid total coverage of the well surface.

Treatment concentrations that induced a similar accumulated copper content in the colonies were selected for this study. For the determination of total Cu concentration, 1 ml of treatment media was added to an acid-washed glass tube and the solution was evaporated. 1 ml of HNO_3 was added and heated at 120 °C overnight. Total Cu concentrations in the treatments were measured using a SpectrAA 220 FS atomic absorption spectrometer (Varian, Palo Alto, CA, USA). Standard curve was done using a 1000 ppm Specpure analytical standard copper solution (Alfa Aesar, Ward Hill, MA, USA). Each sample was measured three times and measurements were done in three independent replicates. Total Cu concentrations were respectively 0.68 ± 0.06, 1.04 ± 0.12, 2.08 ± 0.34 and 4.51 ± 0.95 g l^{-1} for CuO NPs, 0.25 ± 0.02, 0.42 ± 0.04, 0.72 ± 0.06 and 1.24 ± 0.12 g l^{-1} for CS-CuO NPs and 0.004 ± 0.00004, 0.008 ± 0.00007, 0.016 ± 0.00007 and 0.032 ± 0.0002 g l^{-1} for copper sulfate. For clarity, these concentrations were rounded in figures and in the text.

2.4. Characterization of NP suspensions

Characterization of NP suspensions was done in the culture medium. Particle size distribution was determined by dynamic light scattering with a ZetaPlus particle sizer (Brookhaven Instruments Corporation, USA). Electrophoretic mobility was determined with the ZetaPlus system. NP size and shape was verified by transmission electronic microscopy (TEM). A drop of NP suspension was placed on a carbon-coated copper grid and, after 1 min, the media was removed with a Whatman filter paper. Samples were visualized using a FEI Tecnai 12 120 kV microscope and pictures taken with a Gatan 792 Bioscan 1k × 1k Wide Angle Multiscan CCD camera.

Atomic absorption spectrometry was used to determine the soluble Cu fraction in the medium after the particles were removed by centrifugation (Manusadzianas et al., 2012). Particle suspensions were centrifuged at 12 000 g for 30 min, the supernatant was collected and samples were further filtered (0.22 μm) before analysis to remove any remaining particulate matter (*e.g.* polymer residue found in the CS-CuO NPs' supernatant). Soluble Cu concentrations were measured by atomic absorption spectrometry as described in Section 2.3.

2.5. Esterase enzymatic activity

The activity of esterase enzymes, known to be sensitive to copper effect (Franklin et al., 2001) was evaluated using the fluorescein diacetate (FDA) fluorescent probe (Invitrogen Molecular Probe, USA). FDA is a non-polar compound and diffuses passively inside the plant, where it is hydrolyzed by esterase enzymes into the fluorescent compound fluorescein. FDA stock solution (10 mM) was prepared in methanol. The esterase enzymatic activity was evaluated in the same microplate used for treatment by adding 5 μl of FDA solution in each well. Plates were incubated 30 min in the dark and FDA fluorescence was measured at 530 nm with a SpectraMax M2 microplate fluorometer (Molecular Devices Inc, CA, USA), using an excitation wavelength of 485 nm. FDA fluorescence emission was expressed in function of the biomass content (dry weight).

2.6. ROS formation

2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA) (Invitrogen Molecular Probe, USA) was used as a probe for ROS