Toxicology 269 (2010) 182–189

Contents lists available at ScienceDirect

Toxicology

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

Toxicity of ZnO and CuO nanoparticles to ciliated protozoa *Tetrahymena thermophila*

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ARTICLE INFO

Article history:
Received 1 May 2009
Received in revised form 8 July 2009
Accepted 13 July 2009
Available online 19 July 2009

Keywords:
Tetrahymena thermophila
Protozoa
Metal oxide nanoparticles
Copper
Zinc
Toxicity

ABSTRACT

The toxic effects of nanoparticles (NPs) of ZnO and CuO to particle-ingesting model organism protozoa *Tetrahymena thermophila* were evaluated. Nano-ZnO was remarkably more toxic than nano-CuO (EC_{50} values ~5 mg metal/l *versus* 128 mg metal/l). Toxic effect of CuO depended on particle size: nano-CuO was about 10–20 times more toxic than bulk CuO. However, when calculated on basis of bioavailable copper (quantified using recombinant Cu-sensor bacteria) the 4-h EC_{50} values of nano- and bulk formulations were comparable (2.7 and 1.9 mg bioavailable Cu/l, respectively), and statistically different from the EC_{50} value of Cu^{2+} (1.1 mg/l). Differently from CuO particles, bulk and nanosized ZnO as well as Zn^{2+} were of similar toxicity (4-h EC_{50} values 3.7 and 3.9 mg bioavailable Zn/l, respectively, and 4.9 mg Zn^{2+}/l). Thus, the toxic effect of both, CuO and ZnO (nano)particles to protozoa was caused by their solubilised fraction. The toxic effects of the copper compounds were not dependent on exposure time (4 and 24 h), whereas the toxicity of zinc compounds was about 1.5 times lower after 24 h of exposure than after 4 h, probably due to adaptation. In summary, we recommend *T. thermophila* as a simple eukaryotic particle-ingesting model organism for the toxicity screening of NPs. For the high throughput testing we suggest to use the 4-h assay on microplates using ATP and/or propidium iodide for the evaluation of cell viability.

1. Introduction

Metal oxide-based nanoparticles (NPs) are increasingly used in applications such as fillers, opacifiers, catalysts, semiconductors, cosmetics, and microelectronics (Nel et al., 2006; Reijnders, 2006). ZnO NPs are included in personal care products—toothpaste, beauty products and sunscreens (Serpone et al., 2007), as well as in textiles (Becheri et al., 2008). Nano-CuO has potential wide industrial use in applications such as gas sensors and catalytic processes (Carnes and Klabunde, 2003; Dutta et al., 2003).

Although some NPs are already produced in industrial amounts, and thus, may pose hazard to humans and environment, ecotoxicity data for NPs are just emerging (see review by Kahru et al., 2008). Concerning ZnO NPs, there are ecotoxicity data available for bacteria (Adams et al., 2006; Heinlaan et al., 2008; Huang et al., 2008; Mortimer et al., 2008), algae (Franklin et al., 2007; Aruoja et al., 2009), crustaceans (Heinlaan et al., 2008) and nematodes (Wang et al., 2009). In some studies the bacteria have been studied comparatively with eukaryotic cell lines (Reddy et al., 2007; Nair et al., 2008). The effects of ZnO nano- and bulk formulations have also been studied on yeast *Saccharomyces cerevisiae* (Kasemets et al., 2009). Differently from nano-ZnO, the toxicity data regarding nanosized CuO are rare and restricted mostly to ecotoxicological effects towards bacteria and crustaceans (Heinlaan et al., 2008), and algae (Aruoja et al., 2009).

The ciliated protozoa *Tetrahymena* sp. has been used in toxicology for decades as a useful model organism for cellular and molecular biologists as well as for environmental research (Sauvant et al., 1999; Gutiérrez et al., 2003). It is also an advantageous eukaryotic model system for mechanistic studies, as it contains many genes conserved in several eukaryotes (including humans), differently from other widely used unicellular model organisms. For example, more than 800 human genes have orthologs in *Tetrahymena thermophila* but not in *S. cerevisiae*, and 58 of these genes are associated with human diseases (Eisen et al., 2006). Lastly, as protists have highly developed systems for internalisation of nanoscale (100 nm or less) and microscale (100–100,000 nm) particles (Frankel, 2000), they are very good model organisms for nanotoxicology (Holbrook et al., 2008; Kahru et al., 2008).

Both, zinc and copper are essential trace elements for the living organisms, but in high concentrations can produce cellular damage (Goyer and Clarkson, 2001). The influence of copper and zinc on *Tetrahymena* has been formerly studied using several endpoints of physiological response: mortality, cell proliferation, rate of endocytosis, cell membrane integrity, grazing capacity, metabolic activity, lysosomal function (Nicolau et al., 1999; Dias and Lima,

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doi:10.1016/j.tox.2009.07.007

2002; Nilsson, 2003; Dayeh et al., 2005a,b; Gallego et al., 2007). In general, it has been shown that copper is more toxic than zinc for protozoa (Gallego et al., 2007), although in some studies the opposite effect has been demonstrated (Nicolau et al., 1999, 2004). Rico et al. (2009) have shown that there exists remarkable variability in biological responses to heavy metals among different protozoa species, which is further increased by the diversity of experimental conditions used (Gallego et al., 2007). Indeed, it is known that the toxic effects of metals on protozoa are influenced by cell density and factors that modify speciation and bioavailability of metals (pH, content of organic matter, etc.) (Gutiérrez et al., 2003). In addition, metal solubility and speciation may be changed also by organisms; for instance, in case of bacteria it has been shown that initially insoluble forms of heavy metals in soil–water suspensions may become bioavailable due to the direct contact between bacteria and soil particles (Kahru et al., 2005). Variation of the test results may also be related to different toxicity endpoints and assay protocols. One of the assays commonly used for measuring the viability of *Tetrahymena* involves various fluorescent indicator dyes, for instance, neutral red, alamar blue, 5-carboxyfluorescein diacetate acetoxymethyl ester, propidium iodide (Dayeh et al., 2005a,b; Gallego et al., 2007). In addition, quantification of adenosine-5′-triphosphate (ATP) has been used as a marker for general energetic state of protozoa after exposure to toxicants, including zinc and copper (Nicolau et al., 2004).

The aim of the present study was to evaluate the toxicity of two types of metal oxide NPs (ZnO and CuO) to ciliated protozoa *T. thermophila*. It has been shown previously that in case of unicellular organisms (algae, bacteria, yeasts), which *a priori* do not internalise particles, the toxicity of above mentioned NPs was mostly explained by solubilised metal ions and thus correlated with the toxicity of Cu^{2+} and Zn^{2+} to those organisms (Heinlaan et al., 2008; Aruoja et al., 2009; Kasemets et al., 2009). *Tetrahymena*, however, is a particle-ingesting organism and thus, the toxicity mechanism of metal oxide NPs may be different. In addition, it is important to investigate the effects of NPs on protozoa as they are crucial members of the aquatic food chain as well as an important part of activated sludge biological consortium involved in the treatment of wastewaters. Indeed, sooner or later wastewater treatment plants face NPs as many of them (nano-ZnO, nano-TiO_2 and nano-silver) are already produced in high tonnages and used in various consumer products.

Dose–response effects of ZnO and CuO nanoparticles to protozoa were evaluated at two different exposure times (4 and 24 h). To study the effect of particle size, respective bulk formulations were studied in parallel. $ZnSO_4$ and $CuSO_4$ served as ionic controls for evaluating the toxic effect of solubilised metals. Two different endpoints were used to evaluate the toxic effects of nanoparticles: (i) propidium iodide (PI) staining that gives information on cells with disrupted membranes and (ii) amount of cellular adenosine-5′-triphosphate (ATP) that reflects the number of viable cells. PI is a DNA intercalating dye, which is generally excluded from viable cells, thus the fluorescence detected is proportional to the number of membrane-damaged or dead cells (Dayeh et al., 2004). ATP is the major energy currency molecule of the cell that can be formed either in photosynthesis, fermentation or aerobic respiration, depending on organism, and consumed by many enzymes and cellular processes including biosynthetic reactions, motility and cell division (Prescott et al., 1999). ATP is ubiquitously distributed in any biological material and can be easily extracted from the cells and assayed (Lundin and Thore, 1975). To quantify the bioavailable fraction of metals released from the metal oxide particles during the test, the recombinant metal-sensing bacteria were used. To our knowledge, this is the first study on effects of metal oxide NPs to ciliated protozoa *T. thermophila*.

2. Materials and methods

2.1. Cell culture

T. thermophila (strain BIII) was grown axenically in modified SSP medium (Gorovsky et al., 1975) containing 2% proteose peptone (Fluka), 0.1% yeast extract (Lab M) and 0.2% glucose, supplemented with 250 μg/ml each of streptomycin sulphate (Sigma–Aldrich) and penicillin G (Gibco). To prepare the cultures for toxicity testing, 1 ml of the stock culture was transferred to 9 ml of sterile modified SSP medium and grown for 24 h. 10 ml of the 24-h culture was transferred to 40 ml of sterile medium in a 250 ml Erlenmeyer flask and further cultivated for 18–24 h. The cultures were grown on an orbital shaker at 100 rpm, 30 °C. During the exponential growth phase (at the cell density of 5×10^5 cells/ml) the cells were harvested by centrifugation at $300 \times g$ for 5 min and washed twice with Osterhout's medium (0.01% NaCl, 0.0008% $MgCl_2$, 0.0004% $MgSO_4$, 0.0002% KCl, 0.0001% $CaCl_2$ in MilliQ water, pH 6.6; Osterhout, 1906; Society of Protozoologists, 1958). Cell density was determined by counting the cells in haemocytometer (Neubauer Improved, bright line; Germany). To allow the counting, cells were first immobilised in 5% formalin. For toxicity analysis, the density of cells in Osterhout's medium was adjusted to 10^6 cells/ml (twice the final cell density used in the testing).

2.2. Nanoparticles, reference compounds and exposure of T. thermophila

Nano-ZnO (advertised particle size 50–70 nm) and nano-CuO (30 nm) were purchased from Sigma–Aldrich and analysed in parallel with bulk ZnO (Fluka) and CuO (Alfa Aesar). $ZnSO_4 \times 7H_2O$ and $CuSO_4$ (both from Alfa Aesar) served as ionic controls for ZnO and CuO, respectively. The stock suspensions/solutions of the tested chemicals were prepared in deionised water (MilliQ, Millipore). The stock suspensions of metal oxides (40 g/l) were sonicated for 30 min, stored in the dark at +4 °C and used for testing within 2 months. Stock solutions of $ZnSO_4 \times 7H_2O$ and $CuSO_4$ were prepared analogously, but were not sonicated. Before toxicity testing, stocks were vigorously vortexed. The aqueous suspensions of the studied metal oxides (both nano- and bulk formulations) have been previously characterized by scanning electron microscopy (SEM): despite of agglomeration, individual nanoscale particles were present in nano-ZnO and nano-CuO suspensions (Kahru et al., 2008). Osterhout's medium was used throughout the experiments as a diluent and a control. The following nominal concentrations (chosen according to pre-screening results) were used for the toxicity testing: 0.31, 0.62, 1.24, 2.48, 4.97 mg Cu^{2+}/l of $CuSO_4$; 31.25, 62.5, 125, 250, 500 mg/l of nano-CuO; 500, 1000, 2000, 4000, 8000 mg/l of bulk CuO; 2.84, 5.69, 11.37, 22.75, 45.49 mg Zn^{2+}/l of $ZnSO_4 \times 7H_2O$; 1.85, 5.55, 8.33, 12.5, 25 mg/l of nano- and bulk ZnO. The toxicity testing was conducted as follows: 500 μl of the toxicant in Osterhout's medium was pipetted into the wells of 24-well polystyrene culture plates (Falcon), each concentration in two replicates, and 500 μl of *T. thermophila* cells in Osterhout's medium (10^6 cells/ml) was added to the wells (final cell density in the test medium was 5×10^5 cells/ml). Osterhout's medium served as a control. In addition, a cell-free control was made, where 500 μl of Osterhout's medium was added to 500 μl of toxicant suspension/solution. The test plates with protozoa were incubated for 4 and 24 h at 25 °C in the dark, without shaking. The pH of *T. thermophila* control culture in Osterhout's medium was 6.5. The pH of nano- and bulk ZnO solutions containing *T. thermophila* increased slightly with increasing concentration of metal oxide, being 6.9 at the highest concentration tested, while the pH of nano- and bulk CuO suspensions was 6.6 at all the tested concentrations. The pH of the exposure medium increased on average by 0.4 units during the 24-h exposure time in all the experiments.

2.3. Cell viability assays

After 4 and 24 h of incubation of *T. thermophila* with or without toxicants, 100 μl was transferred from each well to 96-well black polypropylene microplate (Greiner Bio-One, Germany) for the viability testing with the fluorescent dye propidium iodide (PI, Fluka) and another 100 μl into a microcentrifuge tube for the ATP assay. The stock solution of PI was prepared in deionised water at a concentration of 1 mg/ml. This was further diluted with deionised water to obtain the working solution of 100 μg/ml, which was 10 times the final concentration in the viability assay. 10 μl of the PI working solution was pipetted directly into each well of 96-well microplate containing 100 μl of exposure medium and the microplates were further incubated for 15 min at 25 °C in the dark. The fluorescence was quantified using the Fluoroskan Ascent FL microplate reader (Thermo Labsystems, Helsinki, Finland) at excitation and emission wavelengths of 530 and 590 nm, respectively.

ATP content of the cellular suspensions was measured using the luciferin–luciferase method essentially as described in Kahru et al. (1982). Briefly, for ATP extraction 100 μl of protozoa culture samples were rapidly mixed with an equal volume of ice-cold 10% trichloroacetic acid containing 4 mM ethylenediamine-tetraacetic acid (EDTA) in microcentrifuge tubes. The fixed samples were stored at −18 °C till analysis. Prior to analysis the samples were thawed and diluted 50-fold with Tris–EDTA buffer (0.1 M Tris, 2 mM EDTA, adjusted to pH 7.75 with acetic acid). 200 μl of diluted sample was pipetted into the luminometer cuvette and first the background light emission ($RLU_{background}$) was measured. Then 20 μl of reconstituted ATP Monitoring Reagent from the ViaLight®HS Bioassay Kit (Lonza Rockland, USA) was added and the light emission of the sample (RLU_{sample})