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Synthesis methods influence characteristics, behaviour and toxicity of bare CuO NPs compared to bulk CuO and ionic Cu after *in vitro* exposure of *Ruditapes philippinarum* hemocytes

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ABSTRACT

Copper oxide (CuO) nanoparticles (NPs) are increasingly investigated, developed and produced for a wide range of industrial and consumer products. Notwithstanding their promising novel applications, concern has been raised that their increased use and disposal could consequently increase their release into marine systems and potentially affect species within. To date the understanding of factors and mechanisms of CuO (nano-) toxicity to marine invertebrates is still limited. Hence, we studied the characteristics and behaviour of two commercially available CuO NPs of similar size, but produced employing distinct synthesis methods, under various environmentally and experimentally relevant conditions. In addition, cell viability and DNA damage, as well as gene expression of detoxification, oxidative stress, inflammatory response, DNA damage repair and cell death mediator markers were studied in primary cultures of hemocytes from the marine clam *Ruditapes philippinarum* and, where applicable, compared to bulk CuO and ionic Cu (as $CuSO_4$) behaviour and effects. We found that the synthesis method can influence particle characteristics and behaviour, as well as the toxicity of CuO NPs to *Ruditapes philippinarum* hemocytes. Our results further indicate that under the tested conditions aggregating behaviour influences the toxicity of CuO NPs by influencing their rate of extra- and intracellular dissolution. In addition, gene expression analysis identified similar transcriptional de-regulation for all tested copper treatments for the here measured suite of genes. Finally, our work highlights various differences in the aggregation and dissolution kinetics of CuO particles under environmental (marine) and cell culture exposure conditions that need consideration when extrapolating *in vitro* findings.

1. Introduction

Engineered nanoparticles (NPs) have been increasingly developed over the last two decades, as their small size – three dimensions ≤100 nm – and the resulting large surface area to volume ratio can give rise to promising novel physicochemical properties (Nel et al., 2006; Oberdörster et al., 2005). Copper oxide (CuO) NPs are researched, developed and produced for a wide variety of industrial and consumer applications (Bondarenko et al., 2013; The Project on Emerging Nanotechnologies, 2016) and their increased production, use and disposal is expected to lead to their increased release into environmental systems, potentially affecting species within (Baker et al., 2014; Boxall et al., 2007).

Naturally occurring copper (Cu) is widely present and is an essential trace metal for many biological functions. Consequently, its homeostasis is effectively controlled under normal conditions (Gaetke et al., 2014; Stohs and Bagchi, 1995). Excess Cu, however, is known to provoke reactive oxygen species (ROS) formation, potentially causing damage to cellular membranes and DNA, leading to inflammation, degenerative diseases, neurological disorders and eventually cell death (Gaetke et al., 2014; Stohs and Bagchi, 1995). CuO NPs are therefore of particular interest in (eco-)toxicology, as their enhanced relative

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surface area does not only bear the potential for particle-specific effects (e.g. ROS formation on the particle surface and Trojan-horse type mechanisms), but also for an increased interaction with biological systems (Nel et al., 2006; Oberdörster et al., 2005), potentially enhancing the intrinsic toxicity of its parent (bulk) material. In agreement with this, the nano form of CuO has been found to be substantially more toxic than its bulk form, both *in vitro* (Bao et al., 2015; Gunawan et al., 2011; Karlsson et al., 2009; Mortimer et al., 2010; Thit et al., 2015) and *in vivo* (Abdel-Khalek et al., 2015; Chen et al., 2006; Heinlaan et al., 2008). Similar to Cu toxicity, CuO NP toxicity appears to be mediated by ROS formation (Bao et al., 2015; Gomes et al., 2013; Gomes et al., 2011; Gunawan et al., 2011; Hedberg et al., 2016; Piret et al., 2012; Thit et al., 2015; Wang et al., 2009), provoking DNA damage (Cronholm et al., 2013; Gomes et al., 2013; Isani et al., 2013; Karlsson et al., 2009; Piret et al., 2012; Thit et al., 2015; Triboulet et al., 2015) and inflammatory responses (Piret et al., 2012).

Cu ion leaching from particles has been identified as a potential major driver of CuO toxicity (Bao et al., 2015; Gunawan et al., 2011; Heinlaan et al., 2008; Mortimer et al., 2010; Semisch et al., 2014; Studer et al., 2010), although discussion remains in the case of CuO NP-induced toxicity in marine bivalves (Buffet et al., 2011; Gomes et al., 2014).

It has been demonstrated that CuO NPs undergo chemical and physical transformations influenced by the composition of the exposure media (predominantly pH, ionic strength and presence of organic matter) (Conway et al., 2015; Misra et al., 2012; Wang et al., 2012) and the particle characteristics (predominantly size, shape, coating and surface charge) (Misra et al., 2012; Piret et al., 2012). While previous work by Katsumiti et al. (2015) demonstrated that mode of synthesis, crystalline structure, size and the presence of stabilizing agents can influence the toxicity of TiO_2 NPs, to date little is known on the potential influences of synthesis methods on the characteristics of bare CuO NPs, their subsequent chemical and physical transformations in environmentally and experimentally relevant aquatic media, and consequently their toxicity.

The aquatic environment has long been considered as an eventual sink for various pollutants and NPs are expected to enter marine systems. The potential adverse effects provoked by CuO NPs warrant an extensive investigation into their behaviour, effects, and mechanisms of toxicity within those environments; however, to date only limited research has been done. Marine bivalves have been identified to be a potentially affected non-target species, as well as a valuable indicator species for trace metal (Goldberg et al., 1983) and metal nanoparticle exposure (Canesi et al., 2012; Rocha et al., 2015).

We therefore studied the characteristics and behaviour of two similarly sized, commercially available bare CuO NPs with distinct manufacturing processes (combustion synthesis and wet chemistry) in a variety of environmentally and experimentally relevant media. We further assessed their toxicity on primary cultures of hemocytes of the marine bivalve clam *Ruditapes philippinarum* by combining both measurements of cytotoxicity and of sub-lethal effects on DNA damage and on gene expression of detoxification, oxidative stress, inflammatory response, DNA damage repair and cell death mediator markers. NP behaviour and toxicity were further compared, if applicable, to those of bulk CuO and ionic Cu (as $CuSO_4$). This study aims to provide additional insight into the potential influence of the synthesis method on the characteristics and behaviour of CuO NPs in marine systems, as well as the resulting toxicity.

2. Materials and methods

2.1. Materials and particle characterization

CuO NPs with nominally similar size distributions of 25–55 nm (product number US3063) and ~40 nm (product number US3070), and similar purity (> 99.95% and > 99% respectively), but produced employing distinct synthesis methods were purchased from US Research Nanomaterials (Houston, USA). According to the manufacturer US3063 particles were produced by solution combustion synthesis, while the US3070 particles stem from wet chemistry synthesis (from here on called CuO_{COMB} and CuO_{WC}, respectively). Bulk copper oxide (bCuO) and ionic copper (as $CuSO_4$) of equal purity (99.99%) were purchased from Sigma-Aldrich (Madrid, Spain) (product numbers 450812 and 451657, respectively). All copper forms were purchased as powders.

The structural nature of CuO_{COMB}, CuO_{WC} and bCuO was measured using X-ray diffraction (XRD), using a powder diffractometer (Bruker D8 Advance 500) with Cu Kα radiation (40 KV, 40 mA). The chemical composition of the copper oxides was analysed by X-ray fluorescence spectroscopy (XRF) using a Bruker M4 tornado apparatus with a voltage of 50 KV and an intensity of 200 μA.

Particle size distributions and images revealing the particle shape and the micro- and nano-structure of the samples were obtained by Transmission Electron microscopy (TEM), using a JEOL2010F at 200 kV, with a structural resolution of 0.19 nm. More than 100 particles were analysed.

Textural characterization was carried out by measuring the N_2 physisorption at −196 °C, employing a Quantachrome Autosorb iQautomatic device. Before measurement, samples were submitted to a surface cleaning pre-treatment under high vacuum at 200 °C for 2 h. The isotherms obtained were used to calculate the specific surface area according to the standard Brunauer–Emmett–Teller (BET) equation.

Measurements of raw nanoparticle hydrodynamic size distributions and surface charge (zeta potential) were made by Dynamic Light Scattering (DLS) at 1 mg L^{-1} and Laser Doppler Micro-electrophoresis (LDE) (Malvern Zetasizer Nano ZS, Malvern Instruments, Malvern, UK) in ultrapure water (Milli-Q grade; Merck Millipore, Billerica, USA) and Eagle basal medium (BME) (1040 mOsm/kg, pH 7.4) at 250 mg L^{-1}. In addition, aggregation behaviour was studied over 48 h by Laser Diffraction Analysis (LDA) (Malvern Mastersizer 2000, Malvern Instruments) in ultrapure water, artificial seawater (ASW, ASTM D1141-75, pH 8.2), BME and the phosphate-buffered saline (PBS, pH 7.4) that was used as the basis for BME. Detailed medium compositions have been included in the Supplementary materials Table 1. Suspensions prepared for DLS and LDA were ultrasonicated for 10 min prior to initial measurements. For subsequent LDA measurements – after 0.5, 1, 3, 6, 24 and 48 h – the suspensions were gently inverted 10 times in order to resuspend sedimented aggregates.

Solubility of CuO particles was measured over 24 h at 10 ppm Cu in a variety of environmentally and experimentally relevant model fluids: ultrapure water, ASW, PBS, BME and artificial lysosomal fluid (ALF, pH 4.5). Dilutions were kept at 19 °C under constant gentle agitation at 50 rpm (Unimax 1010, Heidolph Instruments, Schwabach, Germany). Samples were taken after 0, 3 and 24 h, and Vivaspin® 5000 MWCO spinfilter columns (Sartorius Stedim Biotech, Goettingen, Germany) were used to separate particulate from ionic Cu at 3000 g for 15 min at 19 °C. Quantification of ionic Cu content was performed by ICP-OES (Optima 2000DV, PerkinElmer, Waltham, USA) and ICP-MS (iCAP Q, Thermo Fischer Scientific, Waltham, USA). The accuracy of the measurements was established through blanks and spike recovery experiments.

2.2. In vitro Assays

2.2.1. Isolation of hemocytes for in vitro exposure

Adult clams (*R. philippinarum*) (size: 3–4.5 cm height, mean weight ± sd: 13.6 ± 3.0 g) were supplied by a commercial clam fishery (Mariscos Ria de Vigo S.L., Vigo-Pontevedra, Spain). Prior to hemocyte extraction clams were acclimatized in filtered seawater at two individuals per litre (pH 8.1–8.2, temperature: 16–18 °C) for a minimum of two days, under constant aeration and daily feeding (marin Coraliquid sera GmbH, Heinsberg, Germany). Clams were