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Toxicity of copper oxide nanoparticles in the blue mussel, *Mytilus edulis*: A redox proteomic investigation

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HIGHLIGHTS

- *Mytilus edulis* was exposed to doses of copper oxide nanoparticles (CuO NP).
- Toxicity was evident at the biochemical, cellular and histological levels.
- Gill is the main site of CuO NP accumulation.
- Redox proteomics identified proteins which are targets of oxidative stress.

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ABSTRACT

Relatively little is known about the fate and effects of nanomaterials even in relatively simple organisms such as *Mytilus edulis*. Here, copper oxide nanoparticles (CuO NP) are shown to induce dose-dependent toxic effects at the biochemical, physiological and tissue levels in the blue mussel. Stable CuO NP suspensions were sized by differential light scattering and nanoparticle tracking analysis to yield average particle diameters of approximately 100 nm. These were administered to *M. edulis*, at doses of 400, 700 and 1000 ppb. Ingested copper was predominantly located in the gill tissue with small amounts in digestive gland. Fifteen coomassie-stained spots were excised from two dimensional gel electrophoresis separations of gill tissue extacts and identified by peptide mass fingerprinting. These contained six unique proteins (alpha- and beta-tubulin, actin, tropomyosin, triosephosphate isomerase and Cu–Zn superoxide dismutase). Of these, two spots (actin and triosephosphate isomerase) showed decreased protein thiols while three (alpha-tubulin, tropomyosin and Cu–Zn superoxide dismutase) showed increased carbonylation which is indicative of protein oxidation of cytoskeleton and enzymes in response to CuO NP. The neutral red retention time (NRRT) assay revealed toxicity due to the CuO NPs which was comparable with toxic metal oxide nanoparticles such as chromium and cobalt. In contrast, non-toxic titanium and gold metal oxide nanoparticles gave no NRRT effects at similar NP concentrations. Histology revealed deposition of pigmented brown cells in response to CuO NP, located predominantly along the mantle and gill margin but also lining digestive tubules and some of the sinuses and distributed throughout the connective tissue and in the adductor muscle.

1. Introduction

Nanomaterials, which include nanoparticles, nanotubes, nanocomposites, and nanostructured materials/coatings are defined as materials with at least one dimension <100 nm (Martin, 1994). In comparison with macromaterials of the same chemical composition, nanomaterials often display unusual chemical characteristics (Smith et al., 2008; Nel et al., 2009). This has led to widespread research into their use in a host of applications including electronics, medical devices and cosmetics (Smith et al., 2008; Tiede et al., 2009). The commercial market for nanotechnology is projected to increase to $2.4 trillion by 2014 as ever-more applications are

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identified for novel materials (Holman and Lackner, 2006). However, research into the possible toxicological implications of nanomaterials is considerably less developed than that into their applications (Oberdörster et al., 2005; Unfried et al., 2007; Oberdörster et al., 2007; Zhu et al., 2010;Thomas et al. 2011; Elsaesser and Howard, 2012; Kahru and Ivask, 2013). This has led to emerging concerns that nanomaterials may pose an environmental risk, with possible implications for environmental and human health (Moore, 2006; Klaine et al., 2008; Barrena et al., 2009; Tedesco and Sheehan, 2010; Warheit, 2010; Thomas et al. 2011; Kahru and Ivask, 2013).

There is a range of ways in which nanomaterials may pose a toxicity threat to biological systems (Unfried et al., 2007; Oberdörster et al., 2007; Elsaesser and Howard, 2012; Kahru and Ivask, 2013). Nanoparticles (NPs) can produce reactive oxygen species (ROS) as a consequence of their disproportionately large surface area compared to bulk materials (Stoeger et al. 2006). Overproduction of ROS, to a point exceeding the antioxidant defence capacity of cells, causes oxidative stress which can directly modify membrane lipids, proteins and DNA (Finkel and Holbrook, 2000; Winterbourn, 2008). Even where NPs do not directly produce ROS, they can cause physical disruption to important cell structures such as mitochondria leading to oxidative stress (Xia et al. 2006; Hsin et al., 2008). This type of stress has been identified in several studies with cell and animal models exposed to defined nanomaterials (Tedesco et al., 2008; Nel et al., 2009; Gomes et al., 2011). Secondly, nanomaterials may become coated with a "corona" of proteins which could confer dynamic biological functionality on them when in contact with cells or tissues (Lynch et al., 2007; Cedervall et al., 2007; Sund et al., 2011). Nanomaterials can reach vital organs (Elsaesser and Howard, 2012), cross biological barriers and cause physical damage inside the cell, including effects on lysosomes, and mitochondria (Davda and Labhasetwar, 2002; Xia et al. 2006; Hsin et al., 2008; Bexiga et al., 2011; Elsaesser and Howard, 2012). Since proteins are the main quantitative targets of ROS (Davies, 2005), they are an attractive target for detection of redox effects arising from NP exposure (Tedesco et al., 2008, 2010a,b; Sund et al., 2011).

Several physicochemical attributes of NPs seem to influence their potential toxicity (Nel et al., 2009). These include the average particle diameter (smaller particles being more toxic than larger ones), chemical composition, coating and high aspect ratio (ratio of length to width; Poland et al., 2008). Variation in these and other properties makes it difficult to predict the likely toxicity of a given nanomaterial (Elsaesser and Howard, 2012; Kahru and Ivask, 2013). We have previously explored possible toxic effects of gold nanoparticles on the blue mussel, *Mytilus edulis*, a popular sentinel species in ecotoxicology (Bayne, 1985; Tedesco et al., 2008, 2010a,b). These studies suggested that smaller-sized nanomaterials caused more oxidative stress than larger particles of similar composition, and confirmed that the NPs were internalised into the tissues of the animal. Mussels have an open circulatory system which is constantly exposed to changes in environmental factors, including exposure to contaminants (Pipe et al., 1999) and can concentrate contaminants to a factor of 10^5 within their tissues (Widdows and Donkin, 1992). One of the main components of the bivalve defence system is haemocytes and these play a role in nodule formation, phagocytosis, melanisation and production of ROS (Wishkovsky, 1988; Lin et al., 2011). In marine mussels, oxyradicals are generated both internally and externally to haemocyte lysosomes (Winston et al., 1996), important sites of pollutant sequestration and detoxification in mussels (Moore, 1985; Viarengo et al., 1987; Domouhtsidou et al., 2004). Lysosomal membrane stability is widely used as a biomarker in environmental biomonitoring (Regoli, 1992; Lowe et al., 1995; Domouhtsidou et al., 2004), and reduction of lysosomal stability is directly linked with impaired cellular immunity (Rickwood and Galloway, 2004; Moore, 2009).

For risk-assessment of nanomaterials it will be necessary to understand more about the fate of these materials both in the aquatic environment and also within complex multi-organ organisms (Oberdörster et al., 2005; Klaine et al., 2008; Tedesco and Sheehan, 2010; Elsaesser and Howard, 2012; Sharifi et al., 2012; Kahru and Ivask, 2013). As filter-feeders, mussels are especially selective in the size-range of NPs which they ingest (Defossez and Hawkins, 1997; Ward and Kach, 2009) and filter-feeders are therefore especially attractive targets for probing the environmental fate of nanomaterials (Moore, 2006; Canesi et al., 2012). We previously exposed *M. edulis* to gold nanoparticles which revealed evidence for size-dependent modest oxidative stress including oxidation of lipids and protein thiols (Tedesco et al., 2008, 2010a,b). These particles resulted in physiological stress as evidenced by NRRT assay (Tedesco et al., 2008, 2010a,b). Exposure of mussels to nanosized carbon black resulted in no toxicity to haemocytes by the criterion of NRRT assay, but did cause an increase in inflammatory processes at highest doses (Canesi et al., 2008). These researchers later extended this approach to a panel of commercially-available NPs including fullerenes, TiO_2 and SiO_2 NPs (Canesi et al., 2010). A further study with *M. edulis* involved nanomaterials derived from glass-wool (a proposed adsorbant material for floating oil spill containment) (Koehler et al., 2008). This showed that diffusion and endocytosis resulted in entry of glass-wool NPs to gill and digestive gland cells and their concentration in mitochondria, lysosomes and nuclei. The glass-wool NPs caused decreased NRRT and accumulation of lipofuscin as biomarkers of general pathology and oxidative stress, respectively, (Koehler et al., 2008).

Study of the toxic threat potentially posed by nanomaterials ideally calls for inputs from several disciplines to facilitate nanomaterial characterisation and study of effects at the whole-organism, physiological, cellular and biochemical levels. In this study, we have characterised CuO NPs of average hydrodynamic diameter 100 nm, exposed *M. edulis* to a dose-range of this nanomaterial and studied distribution, physiological and biochemical effects. A schematic overview is provided in Fig. 1. Our study revealed physiological, histological and proteome-level toxic effects of CuO NPs and confirms oxidative stress arising from this treatment.

2. Materials and methods

2.1. Chemicals

5-Iodoacetamido-fluorescein (IAF), 5-fluorescein thiosemicarbazide (FTSC), dithiothreitol (DTT), *N*-ethylmaleimide (NEM), urea, and all other general reagents suitable for electrophoresis were purchased from Sigma–Aldrich (Dorset, UK).

2.2. Preparation of the nanoparticle suspensions

CuO NPs were purchased from Sigma–Aldrich (product number 544868). The nominal size was 50 nm according to the manufacturer by TEM. Nanopowder (10 mg) was suspended in 10 mL of 20 mM citric acid adjusted to pH 7, and sonicated for 1 h using a tip sonicator. A stepped microtip was used and the total power transferred to the suspension was 2.4W (determined by the calorimetric method, Taurozzi et al., 2012). Ultrasound was applied as 15s pulses with 15s breaks between them (Taurozzi et al., 2012). The suspension was left at 60 °C overnight and it was then filtered using a 220 nm pore size cellulose acetate filter (Millipore, Watford UK). The size of the particles in the suspension was characterised using dynamic light scattering (DLS, using a Malvern Zetasizer 3000HSa), nanoparticle tracking analysis, (NTA, Nanosight,

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