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Biomarkers in *Mytilus galloprovincialis* exposed to suspensions of selected nanoparticles (Nano carbon black, C60 fullerene, Nano-TiO_2, Nano-SiO_2)

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ABSTRACT

The potential for ecological toxicity associated with nanomaterials is a growing area of investigation, in particular in the aquatic environment. In suspension feeding invertebrates, the cellular immune system and digestive gland are likely to be targeted, due to their highly developed processes for the cellular internalisation of nano- and micro-scale particles that are integral to key physiological functions such as cellular immunity and intracellular digestion.

We have recently demonstrated that suspensions of selected commercial nanomaterials, namely Nano carbon black (NCB), C60 fullerene (C60), Nano-titanium dioxide (n-TiO_2) and Nanosilica (n-SiO_2) induce oxyradical production and lysosomal enzyme release in the hemocytes of the marine mussel *Mytilus in vitro*. In this work the possible effects of *in vivo* exposure to these NPs were investigated. Mussels were exposed to different concentrations (0.05–0.2–1–5 mg/l) of NP suspensions for 24 h and different biomarkers were evaluated in hemocytes, digestive gland and gills. Characterisation of NP suspensions in artificial sea water (ASW) was performed, indicating the formation of agglomerates of different sizes in the nano-micromolar range for different types of NPs. Formation of larger agglomerates was observed at the end of exposure. The results show that all NP suspensions induced significant lysosomal membrane destabilisation in both the hemocytes and the digestive gland, with NCB $\gg$ C60 > n-TiO_2, >n-SiO_2. In the digestive gland, all NPs induced lysosomal lipofuscin accumulation only at the highest concentrations tested to a different extent depending on the NP type. NCB, TiO_2 and SiO_2 also induced lysosomal neutral lipid accumulation. Moreover, all NPs increased the activity of the antioxidant enzyme catalase, with n-SiO_2 > NCB $\cong$ TiO_2 > C60; NCB and n-TiO_2 also stimulated glutathione transferase (GST). Changes in catalase and GST activities were also observed in gills, with both increases and decreases depending on NP type and concentration. The reported results demonstrate that in mussels responses to exposure to NP suspensions involve changes in lysosomal and oxidative stress biomarkers in the digestive gland, suggesting uptake of NP aggregates/agglomerates mainly through the digestive system. Overall, these data further support the hypothesis that suspension feeding invertebrates represent a significant target for NPs in the aquatic environment.

1. Introduction

The potential for human and ecological toxicity associated with nanomaterials is a growing area of investigation as more nanomaterials and products are developed and brought into commercial use (Oberdörster et al., 2005a,b; Borm et al., 2006). Although nanoparticle (NP) toxicity has been widely investigated in mammalian systems, both *in vitro* and *in vivo*, few studies were addressed to the effects of nanomaterials in a variety of organisms and environments. Industrial products and wastes tend to end up in waterways: in this light, uptake and effects of NPs in the aquatic biota can represent a major concern (Moore, 2006; Baun et al., 2008). Data from ecotoxicity tests in crustaceans, fish and algae indicated low (mg/l range) hazard potential of NPs on aquatic species (Oberdörster, 2004; Lovern and Klaper, 2006; Federici et al., 2007; Warheit et al., 2007; Lovern et al., 2007; Smith et al., 2007). However, when sublethal effects and mechanisms of action were investigated, C60 fullerenes were shown to produce oxidative damage in brain of the juvenile largemouth bass, where they are probably taken up *via* the olfactory nerve (Oberdörster, 2004); in adult male fathead minnow, water-stirred-nC60 increased lipid peroxidation in brain and gills, as well as expression of liver CYP2 family isozymes (Zhu et al., 2006). In the freshwater bivalve *Elliptio complanata*, Cd–Te quantum dots induced oxidative stress in gills and digestive gland (Gagnè et al., 2008). These data indicate that further research is needed to

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gain information on the biological impact of nanoparticles on the aquatic biota.

In aquatic organisms, potential routes include direct ingestion or entry across epithelial boundaries such as gills, olfactory organs or body wall; moreover, aquatic organisms have highly developed processes for the cellular internalisation of nanoscale (≤100 nm) and microscale (100–100,000 nm) particles, namely endocytosis and phagocytosis, respectively (Moore, 2006). These processes are integral to key physiological functions such as intracellular digestion and cellular immunity. In invertebrates, the cellular immune system, gut epithelium and digestive gland are likely to be targeted. In particular, suspension-feeding organisms represent a unique target group for nanoparticle toxicology (Baun et al., 2008).

Most studies assessing the effects of NPs in aquatic invertebrates have focused on freshwater species, invertebrates (mainly crustaceans, and *Daphnia* in particular) and vertebrates (fish) (Handy et al., 2008; Klaine et al., 2008). Less information is available in species from estuarine and marine environments, where the chemical behaviour of NPs and consequent fate may be different and may differently affect the organisms. Although actual concentrations of NPs have not been routinely measured in these environments, coastal waters are expected to represent the ultimate sink for many of the manufactured NPs on a large-scale production.

We have recently demonstrated that in the hemocytes of the marine mussel *Mytilus galloprovincialis* Lam. *in vitro* exposure to suspensions of selected commercial nanomaterials (NCB, C60, n-TiO_2, n-SiO_2) stimulated different immune parameters, such as the oxidative burst and the release of hydrolytic enzymes, in the 1–10 mg/l concentration range (Canesi et al., 2008, 2010). The effects of NP suspensions were mediated by rapid activation of the stress-activated mitogen-activated protein kinases (MAPKs) that play a key role in immune and inflammatory responses (Canesi et al., 2008, 2010).

In this work, we evaluated the possible *in vivo* short-term effects of the same NPs (NCB, C60 fullerene, n-TiO_2, n-SiO_2) in *Mytilus galloprovincialis*. As previously described (Canesi et al., 2008, 2010), the NPs were chosen as model NPs whose toxicity has been widely investigated in both vertebrate and invertebrate systems, as well as for their elevated production and widespread commercial use. Mussels were exposed to different concentrations (0.05–0.2–1–5 mg/l) of different NP suspensions for 24 h and different biomarkers were evaluated. Lysosomal membrane stability (LMS) was evaluated in the hemocytes by the NR retention time assay. In the digestive gland lysosomal biomarkers (LMS, lipofuscin and neutral lipid accumulation), antioxidant and Phase II enzyme activities (catalase and glutathione transferase – GST) were determined. Catalase and GST activities were also evaluated in the gills that represent the site of sorting of NPs suspended in sea water (Koehler et al., 2008; Ward and Kach, 2009).

2. Materials and methods

2.1. Preparation and characterisation of NPs

Commercial nanoparticles were characterised by a combination of analytical techniques as previously described (Canesi et al., 2008, 2010). Nanosized fullerene (C60) was provided from Sigma–Aldrich (Milan, Italy) with nominal purity >99.8%. Purity of C60 fullerene (>99.9%) was confirmed by HPLC-MS (High Pressure Liquid Chromatography Mass Spectrometry) analysis (see Table 1). Nanosized NCB Printex90, Nanosized Titanium Dioxide P25 (declared purity of >99.5%) and Nanosized Nanosilica Aerosil200 (declared purity of >99%) were provided from Degussa Evonik (Essen, Germany). The mean average size of primary particles was determined by TEM (Transmission Electron Microscope) analysis with a Jeol 3010 Transmission Electron Microscope operating at 300 kV. Surface area was obtained by the method of Brunauer, Emmett and Teller (Brunauer et al., 1938) (BET) by nitrogen adsorption on a Micrometrics ASAP 2000 instrument at an adsorption temperature of −196 °C, after pretreating the sample under high vacuum at 300 °C for 2 h. Size distributions of NPs were evaluated by Dynamic Light Scattering (DLS) analysis. Stock suspensions of NPs were freshly prepared in artificial sea water (ASW) at a concentration of 100 μg/ml, sonicated for 15 min at 100 W, 50% on/off cycle while cooling the preparing dispersion in an ice bath, with a UP200S Hielscher Ultrasonic Technology (Teltow, Germany). The ASW (36‰ salinity) was prepared according to the ASTM protocol E 724-98 (ASTM, 2004) and filtered through a 0.45 μm Teflon filter. DLS analysis was performed with a Submicron Particle Sizer Nicomp 380 (Santa Monica, CA, USA) equipped with a 35 mW He–Ne laser, 632.8 nm laser diode and photodiode detector set at 90 °C.

2.2. Animals and treatments

Mussels (*Mytilus galloprovincialis* Lam.), 4–5 cm long, were purchased from an aquaculture farm (Arborea-OR, Italy) and kept for 3 days in static tanks containing 11 ASW/mussel at 16 °C. Sea water was changed daily. Stock suspensions of NPs in ASW (100 μg/ml) were prepared by sonication as for DLS analysis and immediately added to tanks in order to reach the desired concentrations. Mussels (10 mussels for each condition) were exposed to NCB, C60, n-TiO_2 or n-SiO_2 for 24 h at 0.05–0.2–1–5 mg/l/mussel nominal concentration levels. For each treatment, a parallel control group of mussels (untreated) was kept in clean ASW for 24 h. These concentrations were chosen to correlate with our previous studies carried out *in vitro* on *Mytilus* hemocytes (Canesi et al., 2008, 2010). Experiments were repeated 4 times. Exposure to different types of NPs did not result in significant mortality. Animals were not fed during the experiments.

Hemolymph was extracted from the posterior adductor muscle of 10 mussels, using a sterile 1 ml syringe with a 18 G 1/2 in. needle. With the needle removed, hemolymph was filtered through a sterile gauze and pooled in 50 ml Falcon tubes at 4 °C. Tissues (digestive glands and gills) were rapidly dissected, pooled, frozen in liquid nitrogen and maintained at −80 °C for determination of enzyme activities. For histological analyses, small pieces of digestive glands were placed on aluminium chucks, immersed in hexane pre-cooled to −70 °C in liquid nitrogen and maintained at −80 °C (Moore, 1976).

2.3. Lysosomal membrane stability in mussel hemocytes

In vivo lysosomal membrane stability (LMS) in the hemocytes of control and exposed mussels was evaluated by the Neutral Red (NR) Retention time assay as previously described (Canesi et al., 2008, 2010) according to Lowe et al. (1995). Hemocyte monolayers on glass slides were incubated with 30 μl of a NR solution (final concentration 40 μg/ml from a stock solution of NR 40 mg/ml DMSO); after 15 min excess dye was washed out, 30 μl of ASW, and slides were sealed with a coverslip. Every 15 min slides were examined under an optical microscope and the percentage of cells showing loss of the dye from lysosomes in each field was evaluated. For each time point 10 fields were randomly observed, each containing 8–10 cells. The endpoint of the assay was defined as the time at which 50% of the cells showed sign of lysosomal leaking (the cytosol becoming red and the cells rounded) (Lowe et al., 1995). Triplicate preparations were made for each sample. All incubations were carried out at 16 °C.