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Interactions of cationic polystyrene nanoparticles with marine bivalve hemocytes in a physiological environment: Role of soluble hemolymph proteins

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ABSTRACT

The bivalve *Mytilus galloprovincialis* has proven as a suitable model invertebrate for evaluating the potential impact of nanoparticles (NPs) in the marine environment. In particular, in mussels, the immune system represents a sensitive target for different types of NPs. In environmental conditions, both NP intrinsic properties and those of the receiving medium will affect particle behavior and consequent bioavailability/uptake/toxicity. However, the evaluation of the biological effects of NPs requires additional understanding of how, once within the organism, NPs interact at the molecular level with cells in a physiological environment. In mammalian systems, different NPs associate with serum soluble components, organized into a "protein corona", which affects particle interactions with target cells. However, no information is available so far on the interactions of NPs with biological fluids of aquatic organisms.

In this work, the influence of hemolymph serum (HS) on the *in vitro* effects of amino modified polystyrene NPs (PS-NH_2) on *Mytilus* hemocytes was investigated. Hemocytes were incubated with PS-NH_2 suspensions in HS (1, 5 and 50 μg/mL) and the results were compared with those obtained in ASW medium. Cell functional parameters (lysosomal membrane stability, oxyradical production, phagocytosis) were evaluated, and morphological changes were investigated by TEM. The activation state of the signalling components involved in *Mytilus* immune response (p38 MAPK and PKC) was determined. The results show that in the presence of HS, PS-NH_2 increased cellular damage and ROS production with respect to ASW medium. The effects were apparently mediated by disregulation of p38 MAPK signalling. The formation of a PS-NH_2-protein corona in HS was investigated by centrifugation, and 1D- gel electrophoresis and nano-HPLC-ESI-MS/MS. The results identified the Putative C1q domain containing protein (MgC1q6) as the only component of the PS-NH_2 hard protein corona in *Mytilus* hemolymph. These data represent the first evidence for the formation of a NP bio-corona in aquatic organisms and underline the importance of the recognizable biological identity of NPs in physiological exposure medium when testing their potential impact environmental model organisms. Although the results obtained *in vitro* do not entirely reflect a realistic exposure scenario and the more complex formation of a biocorona that is likely to occur *in vivo*, these data will contribute to a better understanding of the effects of NPs in marine invertebrates.

1. Introduction

The development of nanotechnology will inevitably lead to the release of consistent amounts of nanoparticles (NPs) into aquatic environments, in particular in marine ecosystems, with potential

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adverse effects for aquatic organisms (Baker et al., 2014; Corsi et al., 2014). Invertebrates are emerging as suitable models for evaluating the impact of NPs in marine organisms (Matranga et al., 2012; Corsi et al., 2014; Canesi et al., 2016). The bivalve mollusc *Mytilus* spp. represents so far the most utilized invertebrate model (Canesi et al., 2012; Rocha et al., 2015; Canesi et al., 2016). The application of a battery of functional tests on *Mytilus* immune cells, the hemocytes, has been proven as a powerful tool for the rapid screening of the immunomodulatory effects of different types of NPs in cell models of marine organisms. They also represent robust alternative methods for testing the toxicity of NPs and a possible basis for designing *ecosafe* NP for marine ecosystem sustainability (reviewed in Canesi et al., 2012; Canesi and Procházková, 2013; Corsi et al., 2014).

The effects of NPs on mussel hemocytes were observed at concentrations ranging from 1 to 50 μg/mL in standard conditions utilizing artificial sea water (ASW) as exposure medium (Canesi et al., 2012; Canesi and Procházková, 2013; Canesi et al., 2016). The immunomodulatory effects of NPs were confirmed *in vivo*, in mussels exposed to different types of NPs, in particular using n-TiO_2 as a model NP type, although at much lower concentrations (μg/L). With regards to the *in vivo* exposure conditions, evidence is accumulating that in the aquatic environment NPs can undergo considerable transformation before reaching the target organism (Delay et al., 2012). Not only NP intrinsic properties (core composition, surface charge, size, shape, functionalization, *etc.*), but also those of the receiving medium (pH, ionic strength, natural organic matter) will affect agglomeration/aggregation/settling and consequent bioavailability, uptake and toxicity, in different environments (reviewed in Baker et al., 2014; Corsi et al., 2014; Canesi et al., 2015; Schaumann et al., 2015; Canesi et al., 2016).

However, the evaluation of the biological effects of NPs requires additional understanding of how, once within the organism, NPs interact at the molecular level with cells in a physiological environment, *i.e.* in biological fluids. In mammalian cells, different types of NPs associate with serum soluble components, organized into a "protein corona", which affects particle interactions with target cells (internalization and effects) (Cedervall et al., 2007; Lundqvist et al., 2008; Nel et al., 2009; Fubini et al., 2010; Monopoli et al., 2012; Wang et al., 2013; Fleischer et al., 2014; Treuel et al., 2015; Tenzer et al., 2013). The corona proteins control the specific cellular receptors used by protein-NP complex, the cellular internalization pathways, and the immune response (Wan et al., 2015). Cells recognize the biomolecular corona around a NP, but the biological identity of the complex may be considerably different among mammalian species (Monopoli et al., 2012; Wang et al., 2013; Fedeli et al., 2015).

No information is currently available on NP interactions with cells of aquatic organisms in the presence of biological fluids. The formation of a NP protein corona has been demonstrated so far only in a terrestrial invertebrate, the earthworm *Eisenia fetida*, where soluble coelomic proteins (EfCP) secreted *in vitro* by immune cells, the coelomocytes, form a long-lived corona around AgNPs (Hayashi et al., 2013). Recent data obtained in *Mytilus* hemocytes exposed to cationic polystyrene NPs (PS-NH_2) in the presence of hemolymph serum, suggested that also in marine invertebrates components of biological fluids may affect NP interactions with immune cells (Canesi et al., 2015a). Mussels have an open circulatory system, where the blood (hemolymph) is in direct contact with cells and tissues; therefore, no distinction exists between plasma serum and extracellular medium. The protein composition of *Mytilus* hemolymph serum has been recently characterized (Oliveri et al., 2014; Campos et al., 2015). In this work, the influence of hemolymph serum (HS) on the *in vitro* effects of PS-NH_2 on *Mytilus* hemocytes and the possible formation of NP-protein complexes in HS were investigated.

2. Materials and methods

2.1. Particle characterization

Primary 50 nm amino polystyrene NPs (PS-NH_2), purchased from Bangs Laboratories at 50 μg/mL were previously characterized (Della Torre et al., 2014; Canesi et al., 2015a) in MilliQ water and artificial sea water (ASW). Transmission Electron Microscope-TEM analysis confirmed primary particle nominal size of 50 nm. Dynamic Light Scattering-DLS analysis indicated no agglomeration, and a ζ-potential of $+43 \pm 1$ mV in MilliQ water suspensions. In contrast, in ASW small aggregates were observed (Z-average=200.3 nm, PDI=0.302) and a lower ζ-potential ($+14.2$ mV). For experiments carried out in mussel HS, PS-NH_2 suspensions (50 μg/mL) were freshly prepared in filter sterilized HS and vortexed prior to use. Particle size (Z-average and polydispersity index, PDI) was determined at different times (T0, T 1 h, T 2 h) by DLS (Malvern instruments LTD), using a Zetasizer Nano Series software, version 7.11 (Particular Sciences, UK). Measurements were performed in triplicate samples, each containing 10–14 runs of 10 s for determining Z-average. Samples were also observed by TEM.

2.2. Animals, hemolymph collection, preparation of hemocyte monolayers and hemocyte treatment

Mussels (*Mytilus galloprovincialis* Lam.) 4–5 cm long, sampled from an unpolluted area at Cattolica (RN) were obtained from SEA (Gabicce Mare, PU) and kept for 1–3 days in static tanks containing artificial sea water (ASW) (1 L/mussel) at 16 °C. Sea water was changed daily. Hemolymph was extracted from the posterior adductor muscle of 8–20 mussels, filtered and pooled in 50 mL Falcon tubes at 4 °C and hemocyte monolayers were prepared as previously described (Canesi et al., 2008). Hemocytes were incubated at 16 °C with different concentrations of PS-NH_2 in ASW or filter sterilized hemolymph serum (HS), for different periods of time, depending on the endpoint measured. PS-NH_2 were used at concentrations of 1, 5 and 50 μg/mL (corresponding to 1.46×10^{10}, 7.31×10^{10}, and 7.31×10^{11} particles/mL, respectively), as previously described (Canesi et al., 2015a, 2015b) and in analogy with studies carried out with functionalized PS NPs in human cells (Lunov et al., 2011; Wang et al., 2013). Untreated hemocyte samples (control in ASW or HS) were run in parallel.

2.3. Hemocyte functional assays

Lysosomal membrane stability-LMS, extracellular oxyradical production and phagocytosis were evaluated as previously described (Canesi et al., 2015a, 2015b). LMS in control hemocytes and hemocytes pre-incubated with different concentrations of PS-NH_2 for 30 min was evaluated by the Neutral Red (NR) Retention time assay. 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). Phagocytosis of neutral red-stained zymosan in 0.05 M Tris–HCl buffer (TBS), pH 7.8, containing 2% NaCl was added to each monolayer at a concentration of about 1:30 hemocytes: zymosan in the presence or absence of PS-NH_2, and allowed to incubate for 1 h. Monolayers were then washed three times with TBS, fixed with Baker's formol calcium (4%, v/v, formaldehyde, 2% NaCl, 1% calcium acetate) for 30 min and mounted in Kaiser's medium for microscopical examination with a Vanox optical microscope. Extracellular oxyradical production was measured by cytochrome *c* reduction. Hemolymph was extracted into an equal volume of TBS (0.05 M Tris–HCl buffer, pH 7.6, containing 2% NaCl). Aliquots (500 μl) of hemocyte suspensions were incubated with 500 μl of cytochrome *c* solution (75 μM