Marine Environmental Research 111 (2015) 34–40

Contents lists available at ScienceDirect

Marine Environmental Research

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

Evidence for immunomodulation and apoptotic processes induced by cationic polystyrene nanoparticles in the hemocytes of the marine bivalve *Mytilus*

L. Canesi [a, *], C. Ciacci [b], E. Bergami [c], M.P. Monopoli [d], K.A. Dawson [d], S. Papa [e], B. Canonico [b], I. Corsi [c]

[a] Dept.of Earth, Environmental and Life Sciences-DISTAV, University of Genoa, Italy
[b] Dept.of Earth, Life and Environmental Sciences-DISTEVA, University of Urbino, Italy
[c] Dept. of Physical, Earth and Environmental Sciences, University of Siena, Italy
[d] Centre for BioNanoInteractions, School of Chemistry and Chemical Biology, University College Dublin, Ireland
[e] Department of Biomolecular Sciences-DISB, University of Urbino, Italy

ARTICLE INFO

Article history:
Received 26 January 2015
Received in revised form
30 April 2015
Accepted 15 June 2015
Available online 16 June 2015

Keywords:
Nanoplastics
Marine invertebrates
Immunity
Apoptosis

ABSTRACT

Polymeric nanoparticles can reach the marine environment from different sources as weathering of plastic debris and nanowaste. Nevertheless, few data are available on their fate and impact on marine biota. Polystyrene nanoparticles (PS NPs) can be considered as a model for studying the effects of nanoplastics in marine organisms: recent data on amino-modified PS NPs (PS-NH_2) toxicity in sea urchin embryos underlined that marine invertebrates can be biological targets of nanoplastics. Cationic PS NPs have been shown to be toxic to mammalian cells, where they can induce apoptotic processes; however, no information is available on their effects and mechanisms of action in the cells of marine organisms. In this work, the effects of 50 nm PS-NH_2 were investigated in the hemocytes of the marine bivalve *Mytilus galloprovincialis*. Hemocytes were exposed to different concentrations (1, 5, 50 μg/ml) of PS-NH_2 suspension in ASW. Clear signs of cytoxicity were evident only at the highest concentrations (50 μg/ml). On the other hand, a dose dependent decrease in phagocytic activity and increase in lysozyme activity were observed. PS-NH_2 NPs also stimulated increase in extracellular ROS (reactive oxygen species) and NO (nitric oxide) production, with maximal effects at lower concentrations. Moreover, at the highest concentration tested, PS-NH_2 NPs induced apoptotic process, as evaluated by Flow cytometry (Annexin V binding and mitochondrial parameters). The results demonstrate that in marine invertebrates the immune function can represent a significant target for PS-NPs. Moreover, in *Mytilus* hemocytes, PS-NH_2 NPs can act through mechanisms similar to those observed in mammalian cells. Further research is necessary on specific mechanisms of toxicity and cellular uptake of nanoplastics in order to assess their impact on marine biota.

1. Introduction

Plastic debris and their degradation products into smaller fragments, at the micro-, and potentially also the nano-scale level, as well as microplastics present in different items, such as cosmetics, are widespread in the marine environment, both in oceans and sediments (Cole et al., 2011; Hidalgo-Ruz et al., 2012; Wright et al., 2013). Occurrence of nanoplastics in the sea and their possible impact on marine organisms is obviously part of the growing concern for the continuous and increasing release of plastic wastes in the aquatic compartment, including estuarine and coastal areas (Wegner et al., 2012; Corsi et al., 2014). In general, bioavailability of micro- and nano-plastics may depend on their size, density, shape, and surface charges which will affect their behavior in sea water, leading to agglomeration, resuspension and settling; moreover, their uptake, disposal and bioaccumulation by marine organisms is influenced by their feeding behavior, with benthic detritivores and suspension feeders representing more susceptible target species (Wright et al., 2013). Microplastics have been detected in edible

* Corresponding author. DISTAV-Dipartimento di Scienze della Terra, dell'Ambiente e della Vita, Università di Genova, Corso Europa 26, 16132 Genova, Italy.
E-mail address: Laura.Canesi@unige.it (L. Canesi).

http://dx.doi.org/10.1016/j.marenvres.2015.06.008
0141-1136/

marine bivalves, indicating also a possible threat to seafood safety (Van Cauwenberghe and Janssen, 2014).

Polystyrene (PS) is one of the most largely used plastics worldwide, used in food and industrial packaging, disposable cutlery, compact disc cases, building insulation, medical products and toys (Andrady, 2011; PlasticsEurope, 2013). This versatile and non-biodegradable polymer is found in the oceans as micro- and nano-debris, accounting for 24% of the macroplastics in the estuarine habitat (Browne et al., 2008). One of the first evidences of PS impact on filter-feeders is reported by Ward and Kach (2009), showing that marine aggregates of nano-sized PS facilitate food ingestion and are translocated from the gut to the circulatory system, where they are retained for more than a month (Ward and Kach, 2009). Concerning nano-scale PS, only few studies have so far investigated the possible effects in marine species. In the blue mussel *Mytilus edulis*, feeding rate was affected and an increased production of pseudofaeces was observed (Wegner et al., 2012). In our previous study, with sea urchin embryo (*Paracentrotus lividus*), exposure to PS NPs, in particular amino-modified PS NPs (PS-NH_2) caused severe developmental defects (with an EC_{50} of 3.85 μg/ml) and induced *cas8* gene expression at 24 h post fertilization, suggesting the involvement of apoptotic pathways (Della Torre et al., 2014). However, no information is yet available on the effects of PS NPs at cellular level in marine organisms. The importance of the immune system as a target of NPs toxicity has been already described in two model marine organisms, the bivalve *Mytilus* spp. and the sea urchin *P. lividus* (Canesi et al., 2012; Matranga and Corsi, 2012; Canesi and Procházková, 2013; Corsi et al., 2014). These studies underlined that NPs affect lysosomal function, stimulate the production of reactive oxygen species, and decrease the phagocytic activity in *Mytilus* immune cells, the hemocytes (reviewed in Canesi and Procházková, 2013).

PS NPs are well established in a variety of biological and medical applications including fast synthesis, low costs, easy separation and surface modification. The importance of particle surface functionalization for targeted biomedical application, as well as particle charge as potential determinant factor of cytotoxicity has been underlined in a number of studies on the interactions between functionalized cationic and anionic PS NPs and mammalian cells (Fleischer and Payne, 2014). In particular, cationic particles, such as PS-NH_2, have shown more adverse effects than anionic particles (Liu et al., 2011). A cationic surface would enable the particle to interact with the cell membrane more easily due to their similar molecular structure to proteins, hence, promoting the cell uptake of the NPs (Nel et al., 2009). For example, in human macrophages, PS-NH_2 trigger inflammasome activation and subsequent release of proinflammatory cytokines, induce lysosomal membrane destabilization and release of lysosomal enzymes, as well as production of reactive oxygen species (Lunov et al., 2011). In human astrocytoma cells, PS-NH_2 also induced lysosomal damage and apoptotic processes (Bexiga et al., 2011; Wang et al., 2013). In mammalian cells, different NPs, including PS-NH_2 associate with serum soluble components, organized into a "protein corona", which affects particle interactions (internalization and effects) (Fleischer and Payne, 2014 and refs. quoted therein). However, no information is available on the interactions of functionalized PS NPs in the cells of marine organisms.

In this work, a battery of functional immune assays was applied to investigate the short term in vitro effects of PS NPs on *Mytilus* immune cells. For this first study, PS-NH_2 were chosen as a model of functionalized PS NPs on the basis of their stronger toxicity demonstrated in both in both mammalian cells and the sea urchin. Several functional parameters were evaluated: lysosomal membrane stability and lysosomal enzyme release, extracellular oxyradical production and Nitric Oxide (NO) production, phagocytic activity, as well as pro-apoptotic processes at both plasma membrane and mitochondrial level.

2. Materials and methods

2.1. Characterization of PS-NH_2 nanoparticles

Primary characterization of unlabeled 50 nm amino polystyrene NPs (PS-NH_2), purchased from Bangs Laboratories, was performed as described in our previous paper (Della Torre et al., 2014). Primary particle diameter of PS-NH_2 was determined by transmission electron microscopy (Philips Morgagni 268D electronics, at 80 KV and equipped with a MegaView II CCd camera. Artificial sea water (ASW) was prepared according to ASTM protocol (pH 8, salinity 36‰) (ASTM, 2004) and filtered with 0.22 μm membrane. PS-NH_2 suspensions (50 μg/ml) were prepared in ASW, quickly vortexed prior to use but not sonicated. Size (Z-average and polydispersity index, PDI) and zeta potential (ζ-potential, mV) were determined by Dynamic Light Scattering (Malvern instruments), using a Zetasizer Nano Series software, version 7.02 (Particular Sciences, UK). Measurements were performed in triplicate, each containing 11 runs of 10 s for determining Z-average, 20 runs for the ζ-potential.

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, using a sterile 1 ml syringe with a 18 G1/2″ needle. With the needle removed, hemolymph was filtered through a sterile gauze and pooled in 50 ml Falcon tubes at 4 °C. 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, unless otherwise indicated, for different periods of time, depending on the time of endpoint measured. Short-term exposure conditions (from 30 min to 4 h) were chosen to evaluate the rapid in vitro responses to PS-NH_2 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), in analogy with those previously observed with other types of NPs in mussel hemocytes (Canesi et al., 2008, 2010; Ciacci et al., 2012) and with studies carried out with functionalized PS NPs in human cells (Lunov et al., 2011; Wang et al., 2013). Untreated (control in ASW) hemocyte samples were run in parallel.

2.3. Hemocyte functional assays

Hemocyte functional parameters (lysosomal membrane stability, lysosomal enzyme release, phagocytosis, extracellular oxyradical production, Nitric oxide production) were evaluated essentially as previously described (Canesi et al., 2008, 2010; Ciacci et al., 2012). Lysosomal membrane stability in control hemocytes and hemocytes pre-incubated with different concentrations of PS-NH_2 (1, 5, 50 μg/ml) for 30 min was evaluated by the Neutral Red Retention time assay. Hemocyte monolayers on glass slides were incubated with 30 μl of an 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. Control hemocytes were run in parallel. 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