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Title: 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 behaviour 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-NH₂) on *Mytilus* hemocytes was investigated. Hemocytes were incubated with PS-NH₂ 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-NH₂ increased cellular damage and ROS production with respect to ASW medium. The effects were apparently mediated by dysregulation of p38 MAPK signalling. The formation of a PS-NH₂-protein corona in HS was investigated by centrifugation, and 1D- gel electrophoresis and nano-HPLC-ESI-MS/MS. The results identified the Putative Clq domain containing protein (MgClq6) as the only component of the PS-NH₂ 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 bio-corona that is likely to occur in vivo, these data will contribute to a better understanding of the effects of NPs in marine invertebrates.

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Dear Prof. Domingo,

I send you the revised manuscript “Interactions of cationic polystyrene nanoparticles with marine bivalve hemocytes in a physiological environment: role of soluble hemolymph proteins” for publication in Environmental Research.

We thank all the Reviewers for their useful comments. All their suggestions have been taken into account and responses to their comments on a point by point basis are enclosed.

Thank you very much for your attention

Looking forward to hearing from you

Sincerely yours

Laura Canesi

Response to Reviewers

Reviewer #1: The ms fills a gap about the information about the interaction between NPs and biological fluid in aquatic organisms. In this case, polystyrene nanoparticles-amino modified and serum hemocytes were selected. The PS-NH₂ hard protein corona in *Mytilus* hemolymph was identified. The title reflects the content of the article. The abstract is clear and well written. The M&M are well described. The results are presented in clear way and the discussion is well structured. The Tables and Figures are of good quality and illustrative. The information of Supplementary Material is usefulness. The ms is very interesting in order to understand the mechanisms underlined the interaction between nanoparticles and aquatic organisms in physiological medium.

Only two minor comments:

a) the units should be written according to international system of units (e.g. to change ml by mL, ul by uL,...),

these corrections have been made throughout the ms.

b) What is the reason for the selected range of PS-NH₂ suspensions in HS 1-50 ug/mL?

The reason was clearly indicated in 2.2 (M&M section) of the original ms:

‘PS-NH₂ were utilized at concentrations of 1, 5 and 50 µg/ml (corresponding to 1.46 x 10¹⁰, 7.31 x 10¹⁰, and 7.31 x 10¹¹ particles/ml, respectively), as previously described (Canesi et al., 2015) and in analogy with studies carried out with functionalized PS NPs in human cells (Lunov et al., 2011; Wang et al., 2013).

Reviewer #3: This is a nice work on the physiological effects of nanoplastics on *Mytilus*. It presents a good combination of in vitro assays and biochemical data, although it lacks actual in vivo data (i.e., on complete, living animals). This somewhat reduces the environmental significance of the results. Similarly, the formation of the hard corona on plastic particles was only seen in concentrates (around 5x) of hemolymph serum, not in straight serum, again making doubtful whether the process occurs in vivo. However, these are developments that can be addressed in further research. The paper is well written, although I personally find the discussion too long, but I agree that is a matter of taste. The figures are correct. There is scarce information on the identification of the protein forming the corona, even in the supplementary material; for example, there is no information about the peptides used for identification of MgC1q6 (at least as supplementary material).

We fully agree with the referee that our in vitro data do not demonstrate the formation a protein corona in vivo. Our data simple represent the first attempt to show the presence of a protein identify the protein corona in a biological fluid from an aquatic organism and to identify the type of proteins involved. Clearly this goal has been reached since not only we showed the presence of a corona in NPs dispersed in the hemolymph of mussels but also we

identified the protein involved (MgC1q6). This is the first evidence demonstrating that the generation of protein corona around NPs is also taking place in biological fluids of marine organisms. However, it must be underlined that even in the wide literature on mammalian models, most information on the characterization of NP-protein corona was obtained in vitro, simply mixing NPs with plasma or serum samples.

A sentence on this point has been added in the discussion and in the abstract.

As to the use of concentrates, the experimental conditions were chosen to ensure full protein coverage around NPs, due to the low protein concentrations in mussel hemolymph. A similar procedure was also utilized in studies with earthworms (Hayashi et al., 2013), since this is a feature common to the hemolymph of invertebrate species.

A sentence has been added in 2.6 (M&M section).

Identification of MgC1q6: the referee is right, and a supplementary table (Table 1S) reporting the details of peptide characterization has been added.

Reviewer	#4:	Reviewer	comments
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Manuscript: "Interactions of cationic polystyrene nanoparticles with marine bivalve hemocytes in a physiological environment: role of soluble hemolymph proteins"

This manuscript reports a nano-ecotoxicological study on the formation of a bio-corona around PS nanomaterials treated with mussel haemolymph. The authors report the formation of a hard corona and identified the main protein of which it is comprised of. Due to the importance of the protein corona in the modulation of NP uptake and toxicity, this work is of interest to the peers and well within the scope of the journal. Overall, this is an elegant work, complete and competently executed. I commend the general experimental approach and the full physico-chemical characterization of the materials, which is rightfully considered critical in nanotoxicological research. The discussion is sound, even though there are one or two issues that could benefit from some clarifications. I note that the authors have recently published on the same subject (e.g. their recent work in Mar. Environ. Res) but I do not think there is significant redundancy, even though the two works might have been nicely combined. Below there are some few aspects that could improve the overall quality of the work.

1. The authors must keep in mind that the experiment, although elegant, does not entirely reflect a realistic exposure scenario. If the animals were exposed to PS NPs via water, the bio-corona would most likely be more complex, since the NPs would need to cross complex tissues and other types of biological barriers. The work on itself does not provide definitive proof that the NPs can actually reach the haemolymph in vivo, even though it is likely. This aspect should be mentioned in the Discussion and a note should be added to Conclusions (and perhaps the Abstract) as well.

The referee is absolutely right. However, the study of NP-protein corona in non mammalian models is still at its infancy, and also the only available data so far were those obtained in

earthworms in vitro. This point has been underlined in the discussion and a sentence has been added in the abstract.

2. The work is overall well-written and properly presented but quite often the reader feels that the text should be shortened and simplified. In general, the manuscript could be shortened by 20-30% without compromising its quality. Methods and materials are a good example. The techniques hitherto employed are almost entirely described elsewhere. There are parts of the text that are seemingly repetitive that should be checked. For instance, parts of Results and Discussion that recapitulate M&M. Another example, the type of information like that of P. 7, L. 61 ("were cut parallel") is not needed and should be removed throughout. Please keep descriptions to their bare need.

The ms. (in particular the M&M section) has been shortened by more than 10%, also taking into the account additions required by all the referees.

3. The work has no clear objectives, just a recapitulation of what was done. L. 1- 22 of P. 5 should be removed and replaced by the objectives of the study, e.g. "To study the formation of a protein bio-corona in PS NPs in mussel haemolymph and how it modulated NP parameters and affects haemocyte function" etc. The readers can understand what was done in M&M and so forth.

The suggested corrections have been made.

4. The literature thus seems a bit biased. The authors were certainly not the first to unravel the importance of changes in NP chemistry leading to agglomeration, for instance. Literature should be revised. Baseline Nanotoxicology works need to be allocated and debated regarding fundamental topics in the field, even if they are not directly "eco"-related, for there is much work already done in more biomedical-related research. Another example, the predominance of granular haemocytes in mussels is long known (P. 11).

We agree with the referee that much work has been already done on baseline nanotoxicology in biomedical-related research, and two more review papers have been added (Fubini, et al., 2010, Nanotoxicology; Docter et al., 2015, Nanomedicine (Lond)). However, for this reason the book chapter of Rahman et al. (2013) was already quoted in the reference list. With regards with the self-citations from members of Prof. Dawson's group, they were undoubtedly the first to demonstrate the formation of a protein corona in biomedical research.

With regards to the literature on NP behavior in environmental media (eco-related), the reference Schaumann, et al., Sci Tot. Environ., 2015 has been added .

As to the predominance of granular hemocytes in mussels, due to the wide audience of the journal , it is worth underlying for those not familiar with this model. The sentence has been however deleted following the Referee's suggestion.

5. One-dimensional electrophoresis does not provide much resolution for protein identification, especially considering the staining that was used. Even though it provided results, the authors must

state this limitation in the discussion, for there are certainly other proteins that were just not detected and/or identified. The "smeared" look of the gels (Fig. 4) is a clear evidence for this. This is the most important technical criticism that I hold to the work. This type of assessment should be performed with more adequate proteomics tools. Since it does not compromise the main motto of the work (i.e., the formation of a bio-corona) I do not think it is dismissive of the overall quality of the manuscript, however, the authors must acknowledge this flaw and alert the readers to it. Note also that this protein has just an unreviewed annotation and that there is no real "Bivalvia database" in Uniprot ("Bivalvia" is just a search term). The relatively modest coverage and score (although sufficient) indicate, in this case, poor quality of the band. 2DE, at least, would have been critically important, as separation method.

We agree with the referee's comments, and more detailed studies are needed to identify the corona proteins in our experimental model. A sentence on this point has been added in the discussion of the revised ms. However, previous studies on mussel hemolymph serum proteome, allowed to identify mussel proteins with good confidence with both 1- and 2D—gel electrophoresis (Oliveri et al., 2014). The proteomic approach, despite allowing the identification of more proteins in low amounts, did not show any qualitative differences with respect to 1D gels.

In this work, in a first attempt to investigate the possible formation of a protein corona in biological fluids of marine invertebrates, we applied the standard basic protocol utilized in mammalian models to isolate the NP-corona proteins (centrifugation, 1D gel, MS). Peptide analysis identified different peptides in the band, all with a low Mascot score (<25). In contrast, a total of seventeen MgC1q6 –related peptides with a Mascot score of 423 were identified. Details on peptide identification have been added in the revised ms. in a supplementary table (Table 1S).

Bivalvia: the sentence has been changed.

6. The work is overall well-written but needs some polishing. For instance, amend the repetition of "utilizing" by replacing this work (that does not sound all that good" every once in a while by appropriate synonyms, e.g., "used", "tested", etc. Other examples: P. 8., L. 1. "a G2 Tecnai". P. 10, L. 5. "Trypsinised peptidesâ€". P. 12, L. 29 "2.5. fold". P. 17, L. 5. "transport". Check abbreviation use and explanation at first use (quite often either obaent or misplaced). Check reference formats - e.g. Nel et al., 2009; unitalicized species names; journal title abbreviations, etc. These are just examples. The text should be checked entirely.

All the text has been checked, including references, and the appropriate corrections have been made.

7. P. 15, L. 39 and subsequent. This part of the text on kinases must be better explained and more objectively integrated with the work. Why JNK MAPKs and why is the data not shown? As it is, this text seems rather out-of-the blue.

The part on kinases has been re-written and better explained. The sentence on JNK has been deleted for sake of clarity, since no changes in phosphorylation were observed in response to PS-NH₂ in either medium.

Highlights

- The effects of PS-NH₂ NPs on *Mytilus* hemocytes were compared in serum-HS and ASW
- HS increased cellular/lysosomal damage, ROS production and p-p38MAPK levels
- NP-corona proteins in HS were isolated and identified by MS
- NP-protein coronas in biological fluids can affect NP impact in marine species

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

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Key words: *nanoplastics, marine invertebrates, immunity, hemocytes, hemolymph serum, protein corona.*

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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 ~~both *in vitro* and *in vivo*.~~ In environmental conditions, both NP intrinsic properties and those of the receiving medium will affect particle behaviour 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-NH₂) on *Mytilus* hemocytes was investigated. Hemocytes were incubated with PS-NH₂ 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-NH₂ increased ~~lysosomal destabilization, oxyradical production and~~ cellular damage and ROS production with respect to ASW medium. The effects were apparently mediated by dysregulation of p38 MAPK signalling. The formation of a PS-NH₂-protein complex corona in HS ~~were isolated~~ was investigated by centrifugation, and SDS 1D- gel electrophoresis. ~~The results of~~ and nano-HPLC-ESI-MS/MS. The results identified the Putative C1q domain containing protein (MgC1q6) as the only component of the PS-NH₂ hard protein corona in *Mytilus* hemolymph. ~~The results~~ These data

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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. ~~These~~Although the results obtained *in vitro* do not entirely reflect a realistic exposure scenario and the more complex formation of a bio-corona that is likely to occur *in vivo*, these data will contribute to a better understanding of the ~~*in vivo*~~ effects of NPs in marine invertebrates.

Funding sources

This work was partly supported by the Italian Ministry of Research (PRIN2009FHHP2W) Marine ecotoxicology of nanomaterials: toxicity and bioaccumulation of nanotitanium dioxide in edible species in the presence of metals and dioxin.

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 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 and Corsi, 2012; Corsi et al., 2014; Canesi and Corsi, 2016). The bivalve mollusc *Mytilus spp.* represents so far the most utilized invertebrate model (Canesi et al., 2012; Rocha et al., 2015, Canesi and Corsi, 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 and Corsi, 2016). The immunomodulatory effects of NPs were confirmed *in vivo*, in mussels exposed to different types of NPs, in particular using n-TiO₂ 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 and Frimmel, 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 \(reviewed in Baker et al., 2014; Corsi et al., 2014; Canesi et al., 2015a; Schaumann et al., 2015; Canesi and Corsi, 2016\).](#)

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) (Cederval et al., 2007; Nel et al. 2009; [Fubini et al., 2010](#); Monopoli et al. 2012; Wang et al., 2013; Fleischer and Payne, 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-NH₂) 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-NH₂ on *Mytilus* hemocytes was investigated. Cells were incubated with different concentrations (1, 5 and 50 µg/ml) of PS NH₂ suspensions in HS and the results were compared with those obtained in ASW medium as previously described (Canesi et al., 2015a). Functional parameters of hemocytes (lysosomal membrane stability, oxyradical production, phagocytic activity) were evaluated, and morphological changes were investigated by TEM analysis. Moreover, the activation state of the main signalling components involved in *Mytilus* immune response (p38 MAPK and PKC) (Canesi et al., 2009) was determined. The possible formation of NP protein complexes in PS NH₂ suspensions in HS was investigated by applying the procedure described by Monopoli et al (2013), adapted for mussel HS, for separation and identification of NP protein corona proteins by centrifugation, SDS gel electrophoresis and mass spectrometry analysis, 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-NH₂), purchased from Bangs Laboratories at 50 µg/mL were previously characterized (please refer to Della Torre et al., 2014 and Canesi et al., 2015) 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

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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 ~~hemolymph serum (HS)~~, PS-NH₂ suspensions (50 ~~$\mu\text{g}/\text{mL}$~~) 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 1h, T 2h) 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 seconds 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, ~~using a sterile 1 ml syringe with a 18 G1/2" needle. With the needle removed, hemolymph was filtered through a sterile gauze (0.22 μm)~~ and pooled in 50 ~~mL~~ Falcon tubes at 4°C. ~~Hemocyte and hemocyte~~ monolayers were prepared as previously described (Canesi et al., 2008). Hemocytes were incubated at 16°C with different concentrations of PS-NH₂ in ASW or filter sterilized hemolymph serum (HS), for different periods of time, depending on the endpoint measured. PS-NH₂ were ~~utilized~~ used at concentrations of 1, 5 and 50 ~~$\mu\text{g}/\text{mL}$~~ (corresponding to 1.46×10^{10} , 7.31×10^{10} , and 7.31×10^{11} particles/~~mL~~, respectively), as previously described (Canesi et al., 2015) 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., 2015). ~~Lysosomal membrane stability (LMS) in control hemocytes and hemocytes pre-incubated with different concentrations of PS-NH₂ for 30 min was evaluated by the Neutral Red (NR) Retention time assay. 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. 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 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). ~~For each experiment, control hemocyte samples were run in parallel. Triplicate preparations were made for each sample. All incubations were carried out at 16°C.~~

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Phagocytosis of neutral ~~red-stained zymosan by hemocyte monolayers was used to assess the phagocytic ability of hemocytes.~~ 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-NH₂ (1, 5 and 50 µg/ml)₂ and allowed to incubate for 1 h. Monolayers were then washed three times with TBS, fixed with Baker's formal 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. ~~For each slide, the percentage of phagocytic hemocytes was calculated from a minimum of 200 cells.~~

Extracellular oxyradical production ~~generation of superoxide~~ was measured by ~~the reduction of~~ cytochrome *c* reduction. Hemolymph was extracted into an equal volume of TBS (0.05M Tris-HCl buffer, pH 7.6, containing 2% NaCl). Aliquots (500 µl) of hemocyte ~~suspension in triplicate suspensions~~ were incubated with 500 µl of cytochrome *c* solution (75 µM ferricytochrome *c* in TBS), with or without PS-NH₂ (final concentration 1, 5, 50 µg/ml)₂. Cytochrome *c* in TBS was used as a blank. Samples were read at 550 nm at different times (from 0 to 30 min) and the results

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expressed as changes in OD per mg protein. For each experiment, control hemocyte samples were run in parallel. Triplicate preparations were made for each sample. All incubations were carried out at 16°C.

2.4 Transmission electron microscopy

Hemocytes were incubated with PS-NH₂ suspensions (5 µg/~~ml~~mL) in ASW or HS in glass chamber slides (Lab-Tek, Nunc, 177380) for 15 min at 16 °C. Untreated controls were run in parallel. Samples were treated as previously described (Ciacci et al., 2012; Balbi et al., 2013). Cells were washed out in 0.1 M cacodylate buffer in ASW and fixed in ~~0.1 M cacodylate~~the same buffer ~~in ASW~~ containing 2.5% glutaraldehyde, for 1 hour at room temperature. ~~The cells~~Cells were postfixed in 1% osmium tetroxide for 10 min and 1% uranyl acetate for 1 h. ~~Subsequently, samples were,~~ dehydrated through a graded ethanol series and embedded in epoxy resin (Poly-Bed; Polysciences, Inc., Warrington, PA) overnight at 60 °C. Ultrathin sections (50 nm) were ~~cut parallel to the substrate and~~ observed with ~~G2a~~Tecnai bio-twinG2 Spirit BioTWIN electron microscope (Philips, Eindhoven, The Netherlands) without additional staining. Digital images were taken with Megaview 3 CCD camera and iTEM software and processed with Adobe Photoshop CS2.

2.5 Electrophoresis and Western blotting

Hemocyte monolayers were ~~prepared as previously described (Canesi et al., 2008).~~ incubated with PS-NH₂ suspensions (1.5 ~~ml~~mL, final concentration 5 µg/~~ml~~mL) in either ASW or HS ~~were added to each hemocyte monolayer. Control hemocytes received only 1.5 ml of ASW or HS. Incubations were carried out~~ at 16 °C for different periods of time. Control hemocytes were run in parallel. Levels of phosphorylated ~~MAPK~~sp38 Mitogen Activated Protein Kinase (MAPK) and Proteinkinase C (PKC) in hemocyte protein extracts ~~from hemocyte monolayers~~ were determined by SDS-PAGE and Western blotting using phosphospecific antibodies ~~as previously described (Canesi et al., 2009; Ciacci et al., 2010).~~ ~~Supernatants from each culture dish were discarded and the hemocytes were lysed with 0.1 vol. of ice cold lysis buffer and sonicated for 45 s at 50W. Samples were boiled for 4 min and then centrifuged for 10 min at 14,000g to remove insoluble~~

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~~debris. Supernatants were mixed 1:1 (v:v) with sample buffer (0.5 M Tris HCl pH 6.8, 2% SDS, 10% glycerol, 4% 2mercaptoethanol, 0.05% Bromophenol blue) and samples (normalized to 30 µg of protein before loading) were resolved by 12% (for p38 MAPK) or by 10% (for PKC) SDS-polyacrylamide gel electrophoresis (Laemmli Laemli, 1970). Pre-stained molecular mass markers (Bio-Rad) were run on adjacent lanes. The gels were electroblotted and stained with Coomassie blue according to (Towbin et al., 1979). Blots were probed with human recombinant-specific, anti-phospho-p38 MAPK (Thr180/Tyr182, New England Biolabs Inc) and anti-phospho-PKC(pan) (ser660, New England Biolabs Inc.) diluted (1:1000) as primary antibodies, and horseradish peroxidase peroxidase- conjugated goat anti-rabbit IgG (Sigma) diluted (1:3000) as secondary antibody. Nitrocellulose membranes were stripped and re-probed with rabbit polyclonal anti-actin antibody (Sigma) diluted (1:1000) as loading controls. Immune complexes were visualized using an enhanced chemiluminescence Western blotting analysis system (Amersham-Pharmacia) following the manufacturer's specifications. Western blot films were digitized (Chemidoc-Biorad) and band optical densities were quantified using a computerized imaging system (QuantityOne). Relative optical densities (arbitrary units) at each time point were normalized against those of each control group.~~

2.6 Isolation of PS-NH₂-protein complexes

Hemolymph was drawn ~~by injection from the adductor muscle of~~ about 60 mussels and filter sterilized ~~hemolymph serum (HS)~~ was obtained ~~by filtration (0.22 µm) as previously described (Pezzati et al., 2015) above.~~ Due to the low protein content of native HS (about 2 mg/ml), ~~samples were concentrated as follows: HS mL), sample concentration was performed in order to ensure full particle coverage. Samples were~~ dialysed overnight at 16°C against MilliQ water ~~using with~~ 10 kDa cutoff tubes; ~~samples were then, subsequently~~ lyophilized ~~for 24 h using an Edwards Freeze Dryer Modulyo Apparatus~~ and kept at -80°C. Samples were resuspended in sterilized ASW and protein content, evaluated following Harthree (1972) was adjusted to 10 mg/ml.

PS-NH₂ were incubated with HS at the nominal particle concentration of 25 µg/mg ~~hemolymph~~ protein- for 24 h at 16°C on a rocking platform. All experiments were carried out at least twice in ~~duplicate samples-duplicates~~. Parallel samples ~~to be utilized~~ for field emission scanning ~~microscopy~~ ~~microscopy~~-FESEM analyses were also prepared (see ~~method~~-below). After incubation, particle-protein complexes were recovered by centrifugal isolation ~~following~~ (Monopoli et al. ~~(., 2013)~~), with some modifications. ~~Briefly, samples~~. ~~Samples~~ were centrifuged at 17,000 x g for 75 min at 4 °C. The supernatant was stored at -80 °C (SN) and the pellet was ~~resuspendedre-suspended~~ in ASW, transferred to a new vial, and centrifuged again ~~at 17,000 x g for 75 min~~ to pellet the particle- protein complexes. This washing procedure, ~~utilizedused~~ for removing unbound and loosely bound proteins from nanoparticles, was repeated three times, to obtain W1,W2 and W3 samples. The pellet, containing the hard corona (HC) proteins, was ~~resuspendedre-suspended~~ in 0.1 ~~mL~~ ASW and protein content was evaluated, as well as in SN, W1, W2, W3 samples. W3 samples did not contain any detectable amount of proteins. Samples were added with SDS-sample buffer and boiled for 5 min. Proteins (25 µg) were separated by 10% SDS/PAGE ~~as previously described~~ (Pezzati et al., 2015).

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2.7 Nano -HPLC-ESI-MS/MS

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Bands of interests were cut from the gel and destained, reduced, alkylated and digested with trypsin following Shevchenko *et al.* (1996) as previously described (Pezzati et al., 2015). ~~Tryptic~~Trypsinized peptides were analysed by ~~nano~~-HPLC-MS/MS using an Ultimate 3000 nano-HPLC system (managed by CHROMELEON software, version 6.70 SP2a, LC Packings, Amsterdam, NL) connected to a Hybrid Quadrupole-Orbitrap mass spectrometer (Q Exactive, Thermo Scientific). Data were submitted to the SEQUEST search engine against Uniprot ~~bivalvia~~sequence database ~~for Bivalvia~~ (See Supplementary Information).

2.8 Field Emission Scanning Electron Microscopy

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Samples of PS-NH₂ suspensions in ASW (25 µg/~~ml~~mL) were pelleted by centrifugation and resuspended in MilliQ water, in order to eliminate the excess NaCl. In parallel, samples of the pellet obtained by the centrifugation procedure applied to the PS-NH₂ suspension in HS (25 µg/mg protein/~~ml~~mL) to separate the NP corona proteins, containing PS-NH₂-protein corona complexes (HC), were resuspended in 1 ~~ml~~mL of MilliQ water. Both samples were vortexed and two drops of each suspension were placed on a lacey carbon holder and left to dry in air without coating. Samples were observed by field emission scanning electron microscopy (FESEM) on a Zeiss SUPRA 40 VP scanning electron microscope operating at 20 kV.

2.9 Statistical analysis

Data are the mean ± SD (triplicates) of at least 4 independent experiments. Statistical analysis was performed using ANOVA followed by Tukey's post hoc test. Data from Western blot analyses were analyzed by the Mann-Whitney U test with significance at $p < 0.05$.

3. Results

3.1 Characterization of PS-NH₂ suspensions in different media

DLS analyses were performed to follow agglomeration of PS-NH₂ suspensions (50 µg/~~ml~~mL) in mussel hemolymph serum (HS) at 0, 1 and 2 hrs (Table 1) and compared with those previously obtained in MilliQ and ASW (Della Torre et al., 2014; Canesi et al., 2015). The formation of small agglomerates was detected in HS, with Z-average and PDI values in the range of 178-186 nm and 0.34-0.37, respectively, from 0 to 2 h (Table 1, Fig. S1a). A representative TEM image of PS-NH₂ suspensions in HS is reported in Fig. S1b. The slightly smaller size of these agglomerates with respect to those formed in ASW (200 nm) (Table 1) may be due to the lower pH of HS (7.3) with respect to that of ASW (8.0), as well as to the presence of organic serum components in HS. No agglomeration was observed in MilliQ water.

3.2 Effects of HS on responses of mussel hemocytes to PS-NH₂

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The effects of PS-NH₂ suspensions in HS on hemocyte functional parameters were evaluated in comparison with ASW medium and the results are ~~reported~~shown in Fig. 1. As previously reported (Canesi et al., 2015), short term incubation (30 min) of hemocytes with PS-NH₂ suspensions in HS induced a large and dose-dependent decrease in LMS (from -30% with respect to controls) at 1 µg/~~m~~mL); much smaller effects were observed in ASW medium (Fig. 1a). In particular, at 5 µg/~~m~~mL the decrease in LMS was 15% in ASW and about 50% in HS. In addition, stimulation of extracellular ROS production was two-fold higher in HS than in ASW both at 5 and 50 µg/~~m~~mL (Fig. 1b). In both exposure media, PS-NH₂ induced a general decrease in phagocytic activity. However, only in HS a significant effect could be observed also at the lowest concentration tested (-20% at 1 µg/~~m~~mL) (Fig. 1c).

In Fig. 2 representative TEM images of control hemocytes and hemocytes incubated for 15 min with 5 µg/~~m~~mL PS-NH₂ in ASW or HS are reported. Control hemocytes with several intracellular granules and intact elongated filopodia were observed, both in HS (Fig. 2a) and in ASW (not shown). ~~Granular hemocytes represent the dominant cell type in *Mytilus galloprovincialis* (Ciacci et al., 2009).~~—Exposure to PS-NH₂ induced rapid morphological alterations in hemocytes: in ASW, loss of filopodia as well as appearance of plasma membrane blebs were observed, together with the presence of large vacuoles (Fig. 2b). Similar, but more evident alterations were observed in hemocytes incubated with PS-NH₂ in HS, characterized by the presence of swollen cytoplasmic extensions (blebbed pseudopodia) (Fig. 2c and 2d). In both conditions, no cellular uptake of individual PS-NH₂ or their agglomerates could be observed.

The effects of PS-NH₂ suspensions in HS and ASW on signaling components involved in hemocyte activation were evaluated, and the results are reported in Fig. 3. Hemocytes were incubated for different periods of time (from 10 to 60 min) with PS-NH₂ (5 µg/~~m~~mL) and the phosphorylation state of the stress activated p38 MAPK and of PKC was evaluated in cell protein extracts by electrophoresis and Western blotting with specific anti-phospho-MAPK or anti-phospho-PKC(pan) antibodies. Fig. 3a and Fig. 3b show representative blots of p-p38 and p-PKC

obtained after incubation with PS-NH₂ in ASW or HS. Densitometry analysis of phosphorylated protein bands showed a progressive decrease in p38 MAPK phosphorylation in ASW (-55% with respect to controls at 60 min). On the other hand, in the presence of HS, PS-NH₂ induced a time dependent increase in p-p38 levels, that was maximal (up to 2,5 folds with respect to controls) at 60 min (Fig. 3c). PKC phosphorylation was evaluated utilizing anti-pan-phospho-PKC antibodies, that recognize two phosphorylated protein bands of ~70 and 75 kDa, respectively, in mussel hemocytes as already reported in mammalian cells (Canesi et al., 2006; Ciacci et al., 2010). In ASW, PS-NH₂ induced a transient increase in phosphorylation of both PKC bands at 30 min (+75% with respect to control). On the contrary, in the presence of HS, significant decreases in the levels of p-PKC were observed at 30 and 60 min (-25% and -20% of controls, respectively) (Fig. 3d).

3.3. Isolation of PS-NH₂ protein complexes and protein ~~Identification~~ identification by nano-HPLC-ESI-MS/MS

~~In order to investigate the possible formation of a long lived (hard) protein corona around PS-NH₂, particles were incubated for 24 h at 16°C in filter sterilized HS following the protocol by Monopoli et al. (2013) with slight modifications. After incubation, samples were centrifuged to pellet the particle protein complexes. Samples were resuspended in filter sterilized ASW, transferred to a new vial, and centrifuged again to pellet the particle protein complexes; this procedure was repeated three times. Samples (SN, Wash 1-3 and NP pellet), containing the same amount of proteins, were run on 10% SDS gel under non-reducing conditions, and separated protein bands stained with Coomassie blue.~~ PS-NH₂ suspensions in HS were subjected to a basic protocol utilized to isolate the NP-corona proteins in mammalian serum (centrifugation, 1D gel electrophoresis, MS) (Monopoli et al. 2013) with slight modifications. A representative gel is reported in Fig. 4. In lane 2 the classical protein separation profile of whole *Mytilus* serum (WS) is reported, characterized by the absence of proteins of MW higher than 100 kDa and by a major protein band around 35-30 kDa (Oliveri et al., 2014; Pezzati et al., 2015). A similar pattern was observed in the supernatant obtained from the first centrifugation of the PS-NH₂ suspension in HS

(SN). The procedure resulted in the strong presence of 30-32 kDa protein band in wash 1 (W1) that became attenuated in W2, and was not present in W3, confirming the successful application of the protocol. After the third washing step the supernatant did not contain any detectable amount of proteins. In the final pellet, containing the hard protein corona (HC), a single protein band of the apparent MW of a 32 kDa was observed. Densitometric analysis of this band in all samples indicated that the protein content in the HC sample was about 40% of that present in whole hemolymph serum (Fig. S2).

The 32 kDa band was cut from the gel, trypsin digested and analyzed by nano-HPLC-ESI-MS/MS. ~~Data were submitted to the SEQUEST search engine against Uniprot bivalvia database.~~ The results allowed to specifically identify the PN-NH₂ corona protein as the Putative C1q domain containing protein MgC1q6 of *M. galloprovincialis* (FOV443), with ~~ten~~ high confidence peptides corresponding to a sequence coverage of 75.76% and high sequest score of 423. ~~This protein was previously isolated by different methods in *Mytilus galloprovincialis* hemolymph (Oliveri et al., 2014; Pezzati et al., 2015).~~ Details on all the identified peptides (seventeen in total) related with the MgC1q6 protein are reported in Table S1.

3.4 Field Emission Scanning Electron Microscopy (FESEM)

FESEM observations were carried out on PS-NH₂ suspensions in ASW (0.25 mg/~~mL~~mL) and in the NP pellet obtained by the centrifugation procedure applied to obtain the PS-NH₂-protein corona complexes. As shown in a representative image in Fig. 5 (a and c), in ASW, agglomerates made up of small clusters or chains of nanosized particles were observed. In samples containing PS-NH₂-protein corona complexes, individual particles were embedded in an amorphous material forming large agglomerates (about 1 μm in size) (Fig 5 b and 5d).

4. Discussion

~~We have recently demonstrated that in~~In *Mytilus* hemocytes PS-NH₂ suspensions in ASW medium (1, 5, 50 μg/~~ml~~mL) ~~induced~~have been recently shown to induce a dose-dependent

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decrease in phagocytic activity and increase in lysozyme activity; moreover, stimulation of ~~extracellular ROS (reactive oxygen species)~~oxyradical and nitric oxide production, was observed, with maximal effects at lower concentrations. Clear signs of cytotoxicity (lysosomal damage and apoptotic processes) were evident only at the highest concentration tested (Canesi et al., 2015a). The effects of PS-NH₂ in *Mytilus* hemocytes were comparable with those observed in mammalian cells (Wang et al., 2013). ~~However,~~ although the responses ~~of mussel hemocyte~~ were particularly rapid, ~~occurring within 1 h of exposure~~, in line with the physiological role of invertebrate immune cells as first line of defence against non-self material (Canesi and Procházková, 2013).

The results ~~obtained in~~of the present work demonstrate that the presence of biological fluids, i.e., hemolymph serum, significantly affects the responses of *Mytilus* hemocytes to PS-NH₂ ~~exposure~~. In order to evaluate particle behavior in exposure medium, DLS analysis of PS-NH₂ suspensions in HS was performed: the results showed the formation of agglomerates of about 180 nm in size that were stable throughout the time frame ~~utilized for~~of hemocyte exposure. This size was slightly smaller than that previously measured in PS-NH₂ suspensions in ASW (Canesi et al., 2015a). This slight difference may be due to differences in pH between ASW and HS, as well as to the presence of organic components in HS.

Exposure to PS-NH₂ ~~in~~ HS significantly increased lysosomal membrane destabilization and ~~ROS~~oxyradical production with respect to ASW exposure medium. TEM ~~observations~~analysis indicated that PS-NH₂ in ASW induced rapid cellular damage, in particular at the plasma membrane level (membrane blebbing and loss of filopodia). More extensive damage was observed in HS, with formation of short, blebbed pseudopodia. Large intracellular vacuoles were often ~~observed~~seen in both conditions. No uptake of PS-NH₂ agglomerates could be observed in ~~either~~ ASW or HS within the short exposure conditions of the experiment (15 min). These effects were in agreement with the large decrease in phagocytic activity of zymosan particles ~~observed~~induced in both conditions at longer times of incubation ~~(60 min) (-40% with respect to controls)~~, indicating an overall disruption of phagocytic/endocytic pathways.

The mechanisms involved in mediating the effects of PS-NH₂ in different exposure media were investigated by evaluating the phosphorylation (activation) state of the stress activated p38 MAPK and of PKC, the main cytosolic ~~kinase components~~kinases involved in the immune function of mussel hemocytes, ~~i.e. the stress activated p38 MAPK and PKC~~ (Canesi et al., 2006a; Ciacci et al., 2010). ~~Distinct effects were observed in response to PS-NH₂ in ASW with respect to HS.~~ In ASW ~~medium~~, PS-NH₂ induced a decrease in p-p38 MAPK levels, and a transient phosphorylation of PKC (both the 75 and 70 kDa isoforms). ~~In mussel hemocytes, transient phosphorylation of p38 MAPK represents a common response to~~ In contrast, different types of NPs (carbon black and metal oxides) ~~tested using ASW medium have been previously shown to induce a transient phosphorylation of p38 MAPK~~ (Canesi et al., 2008; Ciacci et al., 2012). ~~In contrast~~this light, the results ~~here obtained~~ show ~~that PS-NH₂ in ASW induced dephosphorylation of p38 MAPK, indicating~~ a distinct effect of cationic NPs. ~~These results on p38 MAPK and~~ give a further insight on the specificity of the responses of mussel hemocytes to different types of NPs, ~~and~~. Moreover, ~~these data~~ indicate that PKC, in addition to stress-activated MAPK, represents a potential target for NPs.

~~In contrast, in~~ Distinct effects were observed in the presence of HS, where a ~~progressive~~persistent increase in ~~the level~~phosphorylation of ~~p-p38-MAPK~~ was observed, whereas the levels of p-PKC phosphorylation ~~was~~were slightly decreased. ~~No significant differences in the phosphorylation state of stress activated JNK MAPKs were observed in either medium (not shown).~~ The extent and time course of activation (~~phosphorylation~~) of p38 MAPK and PKC are crucial in determining the outcome of the response to immune stimuli: in particular, in *Mytilus* hemocytes, transient phosphorylation is associated with efficient activation of the immune response, whereas persistent phosphorylation is generally related to lysosomal damage and immunotoxic effects (Betti et al., 2006; Canesi et al., 2008). The results indicate that, the stronger effects of PS-NH₂ on functional parameters and cellular damage observed in the presence of physiological medium, ~~the effects of PS-NH₂~~ are partially mediated by dysregulation of p38 MAPK signaling.

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Overall, these data suggest that the presence of HS components may affect surface interactions between PS-NH₂ and the hemocytes, and consequent activation state of membrane receptors and related signaling pathways, leading to cellular responses distinct from those observed in ASW medium.

In mammalian ~~systems~~biological fluids, attachment of proteins and lipids ~~in biological fluids~~ results in the formation of hard and soft coronas around NPs, with long and short typical exchange times, respectively (Rahman et al., 2013); Docter et al., 2015). The ~~typical~~ lifetime of hard corona has been shown to be of several hours, long enough for many biological and physiological phenomena to occur: therefore, the hard corona defines the biological identity of the particle. The competition between different proteins in mammalian serum for adsorption on the NP surface changes the composition of the corona over time. In most cases, proteins with high abundance in the plasma are first adsorbed on the surface, and over the time, they are replaced by proteins with lower concentration but higher affinity (Rahman et al., 2013). In a first attempt to characterize the possible formation of a hard protein corona in mussel serum, PS-NH₂ were incubated for 24 h in HS, and the suspension was subjected to the standard procedure described by utilized for mammalian serum (Monopoli et al (2013), adapted for mussel HS, for separation and identification of NP-protein corona complexes by centrifugation, ~~SDS gel electrophoresis and mass spectrometry~~. The results identified ID PAGE and MS. Although further studies are needed to better characterize and identify the corona proteins using more adequate proteomic tools, this procedure allowed for the identification of the only protein associated with PS-NH₂ as the Putative C1q domain containing protein MgC1q6 of *M. galloprovincialis* (FOV443).

A recent proteomics study demonstrated that in *M. galloprovincialis* MgC1q6 (or ~~extrapallial~~Extrapallial protein (~~EP~~) precursor) is the most abundant serum protein, with different bands detected by both ~~one~~1- and ~~two-dimensional~~2-D gel electrophoresis and MS analysis (Oliveri et al., 2014). Shotgun analysis of *Mytilus* hemolymph proteome showed that MgC1q6 has a relative abundance about three times higher in serum than in hemocytes (Campos et al., 2015). MgC1q6 or

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EP is the same protein previously identified by different methods and named as serum protein band 1-SPB1, histidine-rich glycoprotein-HRG, heavy metal-binding protein-HIP, keystonein (reviewed in Oliveri et al., 2014). It is an acidic glycoprotein with a high histidine content that can bind Ca^{2+} and heavy metals. Recently, by a MS-based approach, a complex and anomalous N-glycan structure was determined in *M. edulis* EP (Zhou et al., 2013). Such unique structure and calcium and heavy metal-binding properties indicate that this protein plays a key role in multiple biological functions including shell formation, metal ion transportation and detoxification (Zhou et al., 2013). Moreover, we have recently demonstrated that MgC1q6 has a key role in immunity, acting as a specific serum opsonin that mediates adhesion and killing ~~of invading~~ of invading bacteria carrying D-mannose-sensitive ligands (Pezzati et al., 2015). These data supported the role of this protein in innate immune response, due to the presence of conserved complement C1q domains, ~~a complement component that in vertebrates enhances phagocytosis~~ (Gerdol et al., 2011).

The results of the present study identify MgC1q6 as the only protein component of the hard corona formed in *M. galloprovincialis* HS around PS-NH₂. Binding of MgC1q6 to PS-NH₂ could be partly due to the positive charges retained by PS-NH₂ in high ionic strength media, as shown by the ~~positive~~ values of ζ potential recorded in ASW (Canesi et al., 2015a). Protein glycosylation may also contribute to interactions with PS-NH₂; glycosylation of the protein corona has been shown to play an important role in maintaining the colloidal stability of NPs in human plasma (Wan et al. 2015).

~~A recent study reported that a single protein, namely histidine rich glycoprotein-HRG, present in human (and mouse) plasma, but not in fetal calf serum, is potentially capable of completely changing the biological fate of SiO₂-NPs by strongly reducing their capture by macrophages (Fedeli et al., 2015).—HRG appears to have a high affinity for the silica surface, likely arising from its histidine rich motifs, and is able to compete for NP binding with proteins present in plasma at much higher concentrations (albumins, fibrinogen, etc.). However, in human serum, the limited plasma concentration of HRG compared to more abundant proteins allows for the formation of a~~

~~homogeneous and stable corona only at low NP concentrations ($\mu\text{g/ml}$), resembling the *in vivo* situation. In mussels serum, EP, or histidine rich MgCl₆ protein, accounts for 60–70% of the total protein content (Oliveri et al., 2014); moreover, our data of densitometric band analysis showed that the amount of proteins stably bound to PS-NH₂ represented about 40% of its total serum content. Therefore, is not surprising that in these conditions MgCl₆ represents the only hard corona protein.~~

Overall, the results obtained so far underline the need of understanding how the formation of a NP protein corona may affect the biological outcome of NP exposure₂ in marine organisms as in mammalian systems. In general, the presence of proteins reduces NP surface energy by nonspecific adsorption, leading to lowered membrane adhesion and uptake efficiency (Lesniak et al., 2013). Therefore, the formation of the protein corona in mammalian serum is considered as a general protective effect from the potential cytotoxicity of NPs. Similarly, in earthworms, incubation of AgNPs with coelomic proteins lead to formation over time of AgNP-EfCP corona complexes that induced significantly greater NP accumulation in coelomocytes. Therefore, it was hypothesized that NP hard corona proteins function as recognizable molecular patterns, making the NP-protein complexes “visible” for clearance by phagocytic cells (Hayashi et al., 2013). The results obtained in this work indicate that in the marine mussel *Mytilus* the formation of a serum protein corona increases, rather than decrease, the short term *in vitro* toxicity of PS-NH₂ towards immunocytes. Similarly, a recent study reported that a single histidine rich glycoprotein-HRG, present in human serum, HRG (and mouse) plasma, but not in foetal calf serum, forms a stable hard corona around SiO₂-NPs that confers to these particles the ability to evade uptake their capture by macrophages, thus potentially changing their biological fate (Fedeli et al., 2015). In different model organisms, the outcome of the response may depend on the type of NPs, of the target cell, and of the composition of biological fluids, that appears to be species-specific.

Available ~~results obtained data~~ on the *in vitro* effects of NPs in marine invertebrate cells were carried out in sea water media, or in common media adjusted for ionic strength and pH (reviewed in

Canesi and Corsi, 2016), ~~since no specific medium has been developed so far for long term culture of these cells. In~~. However, it has been shown that in *Mytilus* hemocytes, responses to cytokines and bacterial challenge were significantly affected by the presence of hemolymph serum, this resulting in activation of distinct signalling components that induce or prevent lysosomal damage and apoptotic processes (Betti et al. 2006; Balbi et al., 2013; Pezzati et al., 2015). The ~~results of this work~~present data demonstrate the additional role of hemolymph serum in the interactions with PS-NH₂.

Overall, the results underline the importance of the physiological exposure medium and the determinant role of the recognizable biological identity during *in vitro* testing of NPs with invertebrate immune cells. ~~Although the formation of a protein corona may be particle specific, these interactions may contribute to explain the effects of different NPs observed in the hemocytes of mussels exposed *in vivo* at concentrations much lower than those utilized *in vitro* (Canesi et al., 2012; Canesi and Corsi, 2016).~~ However, the protein composition of extracellular fluids of invertebrates is largely unknown, given the large diversity of phyla and species, and different proteins may be involved in the formation of a stable corona around different NPs in different invertebrate groups (marine, freshwater, terrestrial). In ~~earthworm~~earthworm coelomocytes, lysenin was identified as the major corona protein for AgNP *in vitro* (Hayashi et al., 2013), and different secreted proteins related to cell-to-cell signalling were identified in *Daphnia magna* (Nasser and Lynch, 2015). In mussels, MgC1q6 may play a key role in the interactions occurring *in vivo* between the hemolymph and not only PS-NH₂, but also other types of NPs. As in earthworms, different NPs may induce secretion of specific proteins by mussel hemocytes. ~~Research~~However, it must be underlined that the results obtained *in vitro* do not entirely reflect a realistic exposure scenario and the more complex formation of a bio-corona that is ~~in progress~~likely to occur *in vivo* in invertebrate species. Further studies are needed to investigate the formation of NP protein corona in mussels exposed *in vivo* to different types of NPs at concentrations closer to those predicted in the marine environment. Both *in vitro* and *in vivo* studies will contribute to the understanding of NP

uptake and potential toxicity in marine invertebrates invertebrate species.

Finally, the results obtained with PS-NH₂ provide a further insight on the potential impact of nanoplastics in marine organisms. Occurrence of nanoplastics in the sea and their possible impact on marine biota is obviously part of the growing concern for the continuous increase of plastic wastes and plastic debris in the aquatic compartment, including estuarine and coastal areas (Moore, 2008; Wegner et al., 2012; Mattsson et al., 2015). Polystyrene (PS) is one of the most largely used plastics worldwide, accounting for 24% of the macroplastics in the estuarine habitat, and it can be found in the oceans and in marine biota as micro- and nano-debris (Browne et al., 2008; Moore, 2008; Andrady 2011; Plastic Europe, 2013). Recent data reported the formation over time (from 14 days) of nanoplastics of about 220 nm by degradation of polystyrene in controlled conditions (Lambert and Wagner, 2016). In the sea urchin embryo (*Paracentrotus lividus*), exposure to PS NPs, in particular PS-NH₂, caused severe developmental defects and induced changes in gene expression suggesting the involvement of apoptotic pathways (Della Torre et al., 2014). A recent study in brine shrimp larvae (*Artemia franciscana*) showed that PS NPs might affect food uptake (feeding), behavior (motility) and physiology (multiple molting) (Bergami et al., 2016). PS-NH₂ cause developmental defects and changes in gene expression also in *Mytilus* embryos (Canesi et al., 2015b, and ms. in preparation). Taken together, the results underline the potential impact of nanoplastics from the molecular to the organism and population level in marine invertebrates. In this light, knowledge of their interactions with cells within the physiological environment of model species represents the basis for understanding their fate and impact on marine biota.

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Author Contributions

L.C. and I.C. designed the research and supervised the project; C.C., R.F., T.B., E.B. performed the research and analysed the data; L.C. analysed the data and wrote the paper; L.C., I.C., K.D. and M.M. supervised the paper.

Additional Information

Competing financial interests: The authors declare no competing financial interests.

Figure legends

Figure 1 – Effects of PS-NH₂ suspensions in different media on hemocyte functional parameters.

Cells were incubated as described in methods with PS-NH₂ suspensions (1, 5, 50 µg/~~m~~mL) in either ASW or in HS. a) lysosomal membrane destabilization, evaluated as NR retention time; b) extracellular oxyradical production, evaluated as cytochrome *c* reduction. c) phagocytic activity, evaluated as uptake of Neutral-Red conjugated zymosan particles. Data, expressed as percent of control values (unrated hemocytes) and representing the mean±SD of four experiments in triplicate, were analysed by ANOVA followed by Tukey's post hoc test (p≤0.05).

* = all treatments vs controls; # = serum vs ASW.

Figure 2 – Electron microscopy of *Mytilus* hemocytes. Representative TEM images of control and PS-NH₂ exposed cells (15 min, 5 µg/mL) in the presence of ASW or HS. Scale bar 5 µm. a) control granular hemocyte with intact filopodia, several intracellular granules and well visible mitochondria; b) hemocyte exposed to PS-NH₂ in ASW, showing loss of filopodia and presence of plasma membrane blebs (arrows), and a large intracellular vacuole (arrowhead). c, d) hemocytes exposed to PS-NH₂ in HS, showing short blebbed pseudopodia / enlarged cytoplasmic extensions (arrows), irregular plasma membrane surfaces, a large vacuole (d, arrowhead). Fragments of broken filopodia can be also observed around hemocytes (c).

Figure 3 – Effects of incubation of mussel hemocytes with PS-NH₂ (5 µg/mL) for different periods of time (10-60 min) on p38 MAPK and PKC phosphorylation in the presence of ASW or hemolymph serum (HS). C = control. Protein extracts from control and PS-NH₂-treated hemocytes were subjected to 12% SDS-PAGE followed by Western blotting using polyclonal phosphospecific antibodies to p38 MAPK and PKC pan. Bands were detected using enhanced chemiluminescence reagents (see Methods). Results are representative of three independent experiments.

a, b) representative blot of phosphorylated p38 MAPK and PKC (75 kDa and 70 kDa p-PKC); anti-actin blots are shown as loading controls.

c, d) densitometric analysis of blots from three independent experiments (mean±SD). Relative increases in band optical densities (arbitrary units) were normalised for the control band in each series. * = P≤0.05. p < 0.05 Mann–Whitney U test.

Figure 4 – Separation of PS-NH₂ protein complexes from HS of *M. galloprovincialis* proteins by SDS-PAGE and staining with Coomassie Brilliant Blue. PS-NH₂ were incubated in HS at the nominal concentration of 25 µg NP/mg serum protein/mL, and samples were subjected to repeated centrifugation and washing steps as described in Methods. A whole serum sample (WS) in the absence of NPs was included (Lane 1). Lane 2: supernatant after the first centrifugation of the

PS-NH₂ suspension in HS (SN); Lanes 3-5: samples corresponding to the three washing steps (W1, W2, W3); Lane 6: long-lived, hard corona proteins (HC). A representative gel of three independent experiments is shown. The arrow indicates the position of band excised for tandem mass spectrometry analysis.

Fig. 5 – Representative images obtained by Field Emission Scanning Electron Microscopy (FESEM) on PS-NH₂ suspensions in ASW (a,c) and in samples of the hard corona proteins (HC) (b,d).

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2 **Interactions of cationic polystyrene nanoparticles with marine bivalve hemocytes in a**
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4 **physiological environment: role of soluble hemolymph proteins**
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1 **Key words:** *nanoplastics, marine invertebrates, immunity, hemocytes, hemolymph serum, protein*
2 *corona.*
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4 **Abstract**
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8 The bivalve *Mytilus galloprovincialis* has proven as a suitable model invertebrate for evaluating
9 the potential impact of nanoparticles (NPs) in the marine environment. In particular, in mussels, the
10 immune system represents a sensitive target for different types of NPs. In environmental conditions,
11 both NP intrinsic properties and those of the receiving medium will affect particle behaviour and
12 consequent bioavailability/uptake/toxicity. However, the evaluation of the biological effects of NPs
13 requires additional understanding of how, once within the organism, NPs interact at the molecular
14 level with cells in a physiological environment. In mammalian systems, different NPs associate
15 with serum soluble components, organized into a “protein corona”, which affects particle
16 interactions with target cells. However, no information is available so far on the interactions of NPs
17 with biological fluids of aquatic organisms.
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32 In this work, the influence of hemolymph serum (HS) on the *in vitro* effects of amino modified
33 polystyrene NPs (PS-NH₂) on *Mytilus* hemocytes was investigated. Hemocytes were incubated with
34 PS-NH₂ suspensions in HS (1, 5 and 50 µg/mL) and the results were compared with those obtained
35 in ASW medium. Cell functional parameters (lysosomal membrane stability, oxyradical production,
36 phagocytosis) were evaluated, and morphological changes were investigated by TEM. The
37 activation state of the signalling components involved in *Mytilus* immune response (p38 MAPK and
38 PKC) was determined. The results show that in the presence of HS, PS-NH₂ increased cellular
39 damage and ROS production with respect to ASW medium. The effects were apparently mediated
40 by dysregulation of p38 MAPK signalling. The formation of a PS-NH₂-protein corona in HS was
41 investigated by centrifugation, and 1D- gel electrophoresis and nano-HPLC-ESI-MS/MS. The
42 results identified the Putative C1q domain containing protein (MgC1q6) as the only component of
43 the PS-NH₂ hard protein corona in *Mytilus* hemolymph. These data represent the first evidence for
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1 the formation of a NP bio-corona in aquatic organisms and underline the importance of the
2 recognizable biological identity of NPs in physiological exposure medium when testing their
3 potential impact environmental model organisms. Although the results obtained *in vitro* do not
4 entirely reflect a realistic exposure scenario and the more complex formation of a bio-corona that is
5 likely to occur *in vivo*, these data will contribute to a better understanding of the effects of NPs in
6 marine invertebrates.
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15 Marine ecotoxicology of nanomaterials: toxicity and bioaccumulation of nanotitanium dioxide in
16 edible species in the presence of metals and dioxin.
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28 **1. Introduction**

29 The development of nanotechnology will inevitably lead to the release of consistent amounts of
30 nanoparticles (NPs) into aquatic environments, in particular in marine ecosystems, with potential
31 adverse effects for aquatic organisms (Baker et al., 2014; Corsi et al., 2014). Invertebrates are
32 emerging as suitable models for evaluating the impact of NPs in marine organisms (Matranga and
33 Corsi, 2012; Corsi et al., 2014; Canesi and Corsi, 2016). The bivalve mollusc *Mytilus spp.*
34 represents so far the most utilized invertebrate model (Canesi et al., 2012; Rocha et al., 2015,
35 Canesi and Corsi, 2016). The application of a battery of functional tests on *Mytilus* immune cells,
36 the hemocytes, has been proven as a powerful tool for the rapid screening of the
37 immunomodulatory effects of different types of NPs in cell models of marine organisms. They also
38 represent robust alternative methods for testing the toxicity of NPs and a possible basis for
39 designing *ecosafe* NP for marine ecosystem sustainability (reviewed in Canesi et al., 2012; Canesi
40 and Procházková, 2013; Corsi et al., 2014).
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60 The effects of NPs on mussel hemocytes were observed at concentrations ranging from 1 to 50
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$\mu\text{g/mL}$ in standard conditions utilizing artificial sea water (ASW) as exposure medium (Canesi et al., 2012; Canesi and Procházková, 2013; Canesi and Corsi, 2016). The immunomodulatory effects of NPs were confirmed *in vivo*, in mussels exposed to different types of NPs, in particular using TiO_2 as a model NP type, although at much lower concentrations ($\mu\text{g/L}$). 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 and Frimmel, 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., 2015a; Schaumann et al., 2015; Canesi and Corsi, 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) (Cederval et al., 2007; Nel et al. 2009; Fubini et al., 2010; Monopoli et al. 2012; Wang et al., 2013; Fleischer and Payne, 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

1 AgNPs (Hayashi et al., 2013). Recent data obtained in *Mytilus* hemocytes exposed to cationic
2 polystyrene NPs (PS-NH₂) in the presence of hemolymph serum, suggested that also in marine
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4 invertebrates components of biological fluids may affect NP interactions with immune cells (Canesi
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6 et al., 2015a). Mussels have an open circulatory system, where the blood (hemolymph) is in direct
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8 contact with cells and tissues; therefore, no distinction exists between plasma serum and
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10 extracellular medium. The protein composition of *Mytilus* hemolymph serum has been recently
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12 characterized (Oliveri et al., 2014; Campos et al., 2015). In this work, the influence of hemolymph
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14 serum (HS) on the *in vitro* effects of PS-NH₂ on *Mytilus* hemocytes and the possible formation of
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16 NP-protein complexes in HS were investigated.
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24 **2. Materials and methods**

25 *2.1. Particle characterization*

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28 Primary 50 nm amino polystyrene NPs (PS-NH₂), purchased from Bangs Laboratories at 50
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30 µg/mL were previously characterized (Della Torre et al., 2014 and Canesi et al., 2015) in MilliQ
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32 water and artificial sea water (ASW). Transmission Electron Microscope-TEM analysis confirmed
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34 primary particle nominal size of 50 nm. Dynamic Light Scattering-DLS analysis indicated no
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36 agglomeration, and a ζ-potential of +43 ± 1 mV in MilliQ water suspensions. In contrast, in ASW
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38 small aggregates were observed (Z-average=200.3 nm, PDI=0.302) and a lower ζ-potential (+14.2
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40 mV). For experiments carried out in mussel HS, PS-NH₂ suspensions (50 µg/mL) were freshly
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42 prepared in filter sterilized HS and vortexed prior to use. Particle size (Z-average and polydispersity
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44 index, PDI) was determined at different times (T0, T 1h, T 2h) by DLS (Malvern instruments LTD),
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46 using a Zetasizer Nano Series software, version 7.11 (Particular Sciences, UK). Measurements were
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48 performed in triplicate samples, each containing 10-14 runs of 10 seconds for determining Z-
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50 average. Samples were also observed by TEM.
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58 *2.2 Animals, hemolymph collection, preparation of hemocyte monolayers and hemocyte treatment*

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1 Cattolica (RN) were obtained from SEA (Gabicce Mare, PU) and kept for 1–3 days in static tanks
2 containing artificial sea water (ASW) (1 L/mussel) at 16°C. Sea water was changed daily.
3
4 Hemolymph was extracted from the posterior adductor muscle of 8–20 mussels, filtered and pooled
5
6 in 50 mL Falcon tubes at 4°C and hemocyte monolayers were prepared as previously described
7
8 (Canesi et al., 2008). Hemocytes were incubated at 16°C with different concentrations of PS-NH₂ in
9
10 ASW or filter sterilized hemolymph serum (HS), for different periods of time, depending on the
11
12 endpoint measured. PS-NH₂ were used at concentrations of 1, 5 and 50 µg/mL (corresponding to
13
14 1.46 x 10¹⁰, 7.31 x 10¹⁰, and 7.31 x 10¹¹ particles/mL, respectively), as previously described
15
16 (Canesi et al., 2015) and in analogy with studies carried out with functionalized PS NPs in human
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18 cells (Lunov et al., 2011; Wang et al., 2013). Untreated hemocyte samples (control in ASW or HS)
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20 were run in parallel.
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26 2.3. Hemocyte functional assays

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28 Lysosomal membrane stability-LMS, extracellular oxyradical production and phagocytosis were
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30 evaluated as previously described (Canesi et al., 2015). LMS in control hemocytes and hemocytes
31
32 pre-incubated with different concentrations of PS-NH₂ for 30 min was evaluated by the Neutral Red
33
34 (NR) Retention time assay. The endpoint of the assay was defined as the time at which 50% of the
35
36 cells showed sign of lysosomal leaking (the cytosol becoming red and the cells rounded).
37
38 Phagocytosis of neutral red-stained zymosan in 0.05 M Tris-HCl buffer (TBS), pH 7.8, containing
39
40 2% NaCl was added to each monolayer at a concentration of about 1:30 hemocytes:zymosan in the
41
42 presence or absence of PS-NH₂, and allowed to incubate for 1 h. Monolayers were then washed
43
44 three times with TBS, fixed with Baker's formol calcium (4%, v/v, formaldehyde, 2% NaCl, 1%
45
46 calcium acetate) for 30 min and mounted in Kaiser's medium for microscopical examination with a
47
48 Vanox optical microscope. Extracellular oxyradical production was measured by cytochrome *c*
49
50 reduction. Hemolymph was extracted into an equal volume of TBS (0.05M Tris-HCl buffer, pH
51
52 7.6, containing 2% NaCl). Aliquots (500 µl) of hemocyte suspensions were incubated with 500 µl
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54 of cytochrome *c* solution (75 µM ferricytochrome *c* in TBS), with or without PS-NH₂. Cytochrome
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c in TBS was used as a blank. Samples were read at 550 nm at different times (from 0 to 30 min) and the results expressed as changes in OD per mg protein. For each experiment, control hemocyte samples were run in parallel. Triplicate preparations were made for each sample. All incubations were carried out at 16°C.

2.4 Transmission electron microscopy

Hemocytes were incubated with PS-NH₂ suspensions (5 µg/mL) in ASW or HS in glass chamber slides (Lab-Tek, Nunc, 177380) for 15 min at 16 °C. Untreated controls were run in parallel. Samples were treated as previously described (Ciacci et al., 2012; Balbi et al., 2013). Cells were washed out in 0.1 M cacodylate buffer in ASW and fixed in the same buffer containing 2.5% glutaraldehyde, for 1 hour at room temperature. Cells were postfixed in 1% osmium tetroxide for 10 min and 1% uranyl acetate for 1 h, dehydrated through a graded ethanol series and embedded in epoxy resin (Poly-Bed; Polysciences, Inc., Warrington, PA) overnight at 60 °C. Ultrathin sections (50 nm) were observed with a Tecnai G2 Spirit BioTWIN electron microscope (Philips, Eindhoven, The Netherlands) without additional staining. Digital images were taken with Megaview 3 CCD camera and iTEM software and processed with Adobe Photoshop CS2.

2.5 Electrophoresis and Western blotting

Hemocyte monolayers were incubated with PS-NH₂ suspensions (1.5 mL, final concentration 5 µg/mL) in either ASW or HS at 16 °C for different periods of time. Control hemocytes were run in parallel. Levels of phosphorylated p38 Mitogen Activated Protein Kinase (MAPK) and Proteinkinase C (PKC) in hemocyte protein extracts were determined by SDS-PAGE and Western blotting using phosphospecific antibodies (Canesi et al., 2009; Ciacci et al., 2010). Samples (normalized to 30 µg of protein) were resolved by 12% (for p38 MAPK) or by 10% (for PKC) SDS-polyacrylamide gel electrophoresis (Laemli, 1970). Pre-stained molecular mass markers (Bio-Rad) were run on adjacent lanes. The gels were electroblotted and stained with Coomassie blue (Towbin et al., 1979). Blots were probed with human recombinant-specific, anti-phospho-p38 MAPK (Thr180/Tyr182, New England Biolabs Inc) and anti-phospho-PKC(pan) (ser660, New

1 England Biolabs Inc.) (1:1000) as primary antibodies, and horseradish peroxidase peroxidase-
2 conjugated goat anti-rabbit IgG (Sigma) (1:3000) as secondary antibody. Nitrocellulose membranes
3
4 were stripped and re-probed with rabbit polyclonal anti-actin antibody (Sigma) (1:1000) as loading
5
6 controls. Immune complexes were visualized using an enhanced chemiluminescence Western
7
8 blotting analysis system (Amersham–Pharmacia). Western blot films were digitized (Chemidoc-
9
10 Biorad) and band optical densities quantified using a computerized imaging system (QuantityOne).
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12 Relative optical densities (arbitrary units) at each time point were normalized against those of each
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14 control group.
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18 19 *2.6 Isolation of PS-NH₂-protein complexes*

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21 Hemolymph was drawn from about 60 mussels and filter sterilized HS was obtained as described
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23 above. Due to the low protein content of native HS (about 2 mg/mL), sample concentration was
24
25 performed in order to ensure full particle coverage. Samples were dialysed overnight at 16°C
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27 against MilliQ water with 10 kDa cutoff tubes, subsequently lyophilized and kept at -80°C.
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29 Samples were resuspended in sterilized ASW and protein content, evaluated following Harthree
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31 (1972) was adjusted to 10 mg/mL.
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36 PS-NH₂ were incubated with HS at the nominal particle concentration of 25 µg/mg protein for
37
38 24 h at 16°C on a rocking platform. All experiments were carried out at least twice in duplicates.
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40 Parallel samples for field emission scanning microscopy-FESEM analyses were also prepared (see
41
42 below). After incubation, particle–protein complexes were recovered by centrifugal isolation
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44 (Monopoli et al., 2013, with some modifications). Samples were centrifuged at 17,000 x g for 75
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46 min at 4 °C. The supernatant was stored at -80 °C (SN) and the pellet was re-suspended in ASW,
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48 transferred to a new vial, and centrifuged again to pellet the particle-protein complexes. This
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50 washing procedure, used for removing unbound and loosely bound proteins from nanoparticles,
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52 was repeated three times, to obtain W1, W2 and W3 samples. The pellet, containing the hard corona
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54 (HC) proteins, was re-suspended in 0.1 mL ASW and protein content was evaluated, as well as in
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56 SN, W1, W2, W3 samples. W3 samples did not contain any detectable amount of proteins. Samples
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1 were added with SDS-sample buffer and boiled for 5 min. Proteins (25 µg) were separated by 10%
2 SDS/PAGE (Pezzati et al., 2015).
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4 5 2.7 Nano -HPLC-ESI-MS/MS 6

7 Bands of interests were cut from the gel and destained, reduced, alkylated and digested with
8 trypsin following Shevchenko *et al.* (1996) as previously described (Pezzati et al., 2015).
9
10 Trypsinized peptides were analysed by HPLC-MS/MS using an Ultimate 3000 nano-HPLC system
11
12 (managed by CHROMELEON software, version 6.70 SP2a, LC Packings, Amsterdam, NL)
13
14 connected to a Hybrid Quadrupole-Orbitrap mass spectrometer (Q Exactive, Thermo Scientific).
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16 Data were submitted to the SEQUEST search engine against Uniprot sequence database for
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18 Bivalvia (See Supplementary Information).
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23 24 2.8 Field Emission Scanning Electron Microscopy 25

26 Samples of PS-NH₂ suspensions in ASW (25 µg/mL) were pelleted by centrifugation and
27
28 resuspended in MilliQ water, in order to eliminate the excess NaCl. In parallel, samples of the
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30 pellet obtained by the centrifugation procedure applied to the PS-NH₂ suspension in HS (25 µg/mg
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32 protein/mL) to separate the NP corona proteins, containing PS-NH₂ –protein corona complexes
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34 (HC), were resuspended in 1 mL of MilliQ water. Both samples were vortexed and two drops of
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36 each suspension were placed on a lacey carbon holder and left to dry in air without coating.
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38 Samples were observed by field emission scanning electron microscopy (FESEM) on a Zeiss
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40 SUPRA 40 VP scanning electron microscope operating at 20 kV.
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46 2.9 Statistical analysis 47

48 Data are the mean ± SD (triplicates) of at least 4 independent experiments. Statistical analysis
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50 was performed using ANOVA followed by Tukey's post hoc test. Data from Western blot analyses
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52 were analyzed by the Mann-Whitney U test with significance at p< 0.05.
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58 3. Results 59

60 3.1 Characterization of PS-NH₂ suspensions in different media 61 62 63 64 65

1 DLS analyses were performed to follow agglomeration of PS-NH₂ suspensions (50 µg/mL) in
2 mussel hemolymph serum (HS) at 0, 1 and 2 hrs (Table 1) and compared with those previously
3 obtained in MilliQ and ASW (Della Torre et al., 2014; Canesi et al., 2015). The formation of small
4 agglomerates was detected in HS, with Z-average and PDI values in the range of 178-186 nm and
5 0.34-0.37, respectively, from 0 to 2 h (Table 1, Fig. S1a). A representative TEM image of PS-NH₂
6 suspensions in HS is reported in Fig. S1b. The slightly smaller size of these agglomerates with
7 respect to those formed in ASW (200 nm) (Table 1) may be due to the lower pH of HS (7.3) with
8 respect to that of ASW (8.0), as well as to the presence of organic serum components in HS. No
9 agglomeration was observed in MilliQ water.

10 3.2 *Effects of HS on responses of mussel hemocytes to PS-NH₂*

11 The effects of PS-NH₂ suspensions in HS on hemocyte functional parameters were evaluated in
12 comparison with ASW medium and the results are shown in Fig. 1. As previously reported (Canesi
13 et al., 2015), short term incubation (30 min) of hemocytes with PS-NH₂ suspensions in HS induced
14 a large and dose-dependent decrease in LMS (from -30% with respect to controls) at 1 µg/mL);
15 much smaller effects were observed in ASW medium (Fig. 1a). In particular, at 5 µg/mL the
16 decrease in LMS was 15% in ASW and about 50% in HS. In addition, stimulation of extracellular
17 ROS production was two-fold higher in HS than in ASW both at 5 and 50 µg/mL (Fig. 1b). In both
18 exposure media, PS-NH₂ induced a general decrease in phagocytic activity. However, only in HS a
19 significant effect could be observed also at the lowest concentration tested (-20% at 1 µg/mL) (Fig.
20 1c).

21 In Fig. 2 representative TEM images of control hemocytes and hemocytes incubated for 15 min
22 with 5 µg/mL PS-NH₂ in ASW or HS are reported. Control hemocytes with several intracellular
23 granules and intact elongated filopodia were observed, both in HS (Fig. 2a) and in ASW (not
24 shown). Exposure to PS-NH₂ induced rapid morphological alterations in hemocytes: in ASW, loss
25 of filopodia as well as appearance of plasma membrane blebs were observed, together with the
26 presence of large vacuoles (Fig. 2b). Similar, but more evident alterations were observed in
27

1 hemocytes incubated with PS-NH₂ in HS, characterized by the presence of swollen cytoplasmic
2 extensions (blebbed pseudopodia) (Fig. 2c and 2d). In both conditions, no cellular uptake of
3 individual PS-NH₂ or their agglomerates could be observed.
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7 The effects of PS-NH₂ suspensions in HS and ASW on signaling components involved in
8 hemocyte activation were evaluated, and the results are reported in Fig. 3. Hemocytes were
9 incubated for different periods of time (from 10 to 60 min) with PS-NH₂ (5 µg/mL) and the
10 phosphorylation state of the stress activated p38 MAPK and of PKC was evaluated in cell protein
11 extracts by electrophoresis and Western blotting with specific anti-phospho-MAPK or anti-
12 phospho-PKC(pan) antibodies. Fig. 3a and Fig. 3b show representative blots of p-p38 and p-PKC
13 obtained after incubation with PS-NH₂ in ASW or HS. Densitometry analysis of phosphorylated
14 protein bands showed a progressive decrease in p38 MAPK phosphorylation in ASW (-55% with
15 respect to controls at 60 min). On the other hand, in the presence of HS, PS-NH₂ induced a time
16 dependent increase in p-p38 levels, that was maximal (up to 2,5 folds with respect to controls) at 60
17 min (Fig. 3c). PKC phosphorylation was evaluated utilizing anti-pan-phospho-PKC antibodies, that
18 recognize two phosphorylated protein bands of ~70 and 75 kDa, respectively, in mussel hemocytes
19 as already reported in mammalian cells (Canesi et al., 2006; Ciacci et al., 2010). In ASW, PS-NH₂
20 induced a transient increase in phosphorylation of both PKC bands at 30 min (+75% with respect to
21 control). On the contrary, in the presence of HS, significant decreases in the levels of p-PKC were
22 observed at 30 and 60 min (-25% and -20% of controls, respectively) (Fig. 3d).
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46 *3.3. Isolation of PS-NH₂ protein complexes and protein identification by nano-HPLC-ESI-MS/MS*

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48 PS-NH₂ suspensions in HS were subjected to a basic protocol utilized to isolate the NP-corona
49 proteins in mammalian serum (centrifugation, 1D gel electrophoresis, MS) (Monopoli et al. 2013)
50 with slight modifications. A representative gel is reported in Fig. 4. In lane 2 the classical protein
51 separation profile of whole *Mytilus* serum (WS) is reported, characterized by the absence of
52 proteins of MW higher than 100 kDa and by a major protein band around 35-30 kDa (Oliveri et al.,
53 2014; Pezzati et al., 2015). A similar pattern was observed in the supernatant obtained from the first
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1 centrifugation of the PS-NH₂ suspension in HS (SN). The procedure resulted in the strong
2 presence of 30-32 kDa protein band in wash 1 (W1) that became attenuated in W2, and was not
3 present in W3, confirming the successful application of the protocol. After the third washing step
4 the supernatant did not contain any detectable amount of proteins. In the final pellet, containing the
5 hard protein corona (HC), a single protein band of the apparent MW of a 32 kDa was observed.
6
7 Densitometric analysis of this band in all samples indicated that the protein content in the HC
8 sample was about 40% of that present in whole hemolymph serum (Fig. S2).
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11 The 32 kDa band was cut from the gel, trypsin digested and analyzed by nano-HPLC-ESI-
12 MS/MS. The results allowed to specifically identify the PN-NH₂ corona protein as the Putative C1q
13 domain containing protein MgC1q6 of *M. galloprovincialis* (F0V443), with high confidence
14 peptides corresponding to a sequence coverage of 75.76% and high sequest score of 423. Details
15 on all the identified peptides (seventeen in total) related with the MgC1q6 protein are reported in
16 Table S1.
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18 19 3.4 Field Emission Scanning Electron Microscopy (FESEM)

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21 FESEM observations were carried out on PS-NH₂ suspensions in ASW (0.25 mg/mL) and in
22 the NP pellet obtained by the centrifugation procedure applied to obtain the PS-NH₂-protein corona
23 complexes. As shown in a representative image in Fig. 5 (a and c), in ASW, agglomerates made up
24 of small clusters or chains of nanosized particles were observed. In samples containing PS-NH₂-
25 protein corona complexes, individual particles were embedded in an amorphous material forming
26 large agglomerates (about 1 μm in size) (Fig 5 b and 5d).
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31 32 4. Discussion

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34 In *Mytilus* hemocytes PS-NH₂ suspensions in ASW medium (1, 5, 50 μg/mL) have been recently
35 shown to induce a dose-dependent decrease in phagocytic activity and increase in lysozyme
36 activity; moreover, stimulation of oxyradical and nitric oxide production, was observed, with
37 maximal effects at lower concentrations. Clear signs of cytotoxicity (lysosomal damage and
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1 apoptotic processes) were evident only at the highest concentration tested (Canesi et al., 2015a).
2 The effects of PS-NH₂ in *Mytilus* hemocytes were comparable with those observed in mammalian
3 cells (Wang et al., 2013), although the responses were particularly rapid, in line with the
4 physiological role of invertebrate immune cells as first line of defence against non-self material
5 (Canesi and Procházková, 2013).
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11 The results of the present work demonstrate that the presence of biological fluids, i.e.,
12 hemolymph serum, significantly affects the responses of *Mytilus* hemocytes to PS-NH₂. In order to
13 evaluate particle behavior in exposure medium, DLS analysis of PS-NH₂ suspensions in HS was
14 performed: the results showed the formation of agglomerates of about 180 nm in size that were
15 stable throughout the time frame of hemocyte exposure. This size was slightly smaller than that
16 previously measured in PS-NH₂ suspensions in ASW (Canesi et al., 2015a). This slight difference
17 may be due to differences in pH between ASW and HS, as well as to the presence of organic
18 components in HS.
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31 Exposure to PS-NH₂ in HS significantly increased lysosomal membrane destabilization and
32 oxyradical production with respect to ASW exposure medium. TEM analysis indicated that PS-
33 NH₂ in ASW induced rapid cellular damage, in particular at the plasma membrane level (membrane
34 blebbing and loss of filopodia). More extensive damage was observed in HS, with formation of
35 short, blebbed pseudopodia. Large intracellular vacuoles were often seen in both conditions. No
36 uptake of PS-NH₂ agglomerates could be observed in either ASW or HS within the short exposure
37 conditions of the experiment (15 min). These effects were in agreement with the large decrease in
38 phagocytic activity of zymosan particles induced in both conditions at longer times of incubation,
39 indicating an overall disruption of phagocytic/endocytic pathways.
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53 The mechanisms involved in mediating the effects of PS-NH₂ in different exposure media were
54 investigated by evaluating the phosphorylation (activation) state of the stress activated p38 MAPK
55 and of PKC, the main cytosolic kinases involved in the immune function of mussel hemocytes
56 (Canesi et al., 2006a; Ciacci et al., 2010). In ASW, PS-NH₂ induced a decrease in p-p38 MAPK
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1 levels, and a transient phosphorylation of PKC (both the 75 and 70 kDa isoforms). In contrast,
2 different types of NPs (carbon black and metal oxides) have been previously shown to induce a
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4 transient phosphorylation of p38 MAPK (Canesi et al., 2008; Ciacci et al., 2012). In this light, the
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6 results show a distinct effect of cationic NPs on p38 MAPK and give a further insight on the
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8 specificity of the responses of mussel hemocytes to different types of NPs. Moreover, these data
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10 indicate that PKC, in addition to stress-activated MAPK, represents a potential target for NPs.
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14 Distinct effects were observed in the presence of HS, where a persistent increase in
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16 phosphorylation of p38-MAPK was observed, whereas the levels of p-PKC were slightly decreased.
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18 The extent and time course of activation of p38 MAPK and PKC are crucial in determining the
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20 outcome of the response to immune stimuli: in particular, in *Mytilus* hemocytes, transient
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22 phosphorylation is associated with efficient activation of the immune response, whereas persistent
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24 phosphorylation is generally related to lysosomal damage and immunotoxic effects (Betti et al.,
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26 2006; Canesi et al., 2008). The results indicate that the stronger effects of PS-NH₂ on functional
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28 parameters and cellular damage observed in the presence of physiological medium are partially
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30 mediated by dysregulation of p38 MAPK signaling. Overall, these data suggest that the presence of
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32 HS components may affect surface interactions between PS-NH₂ and the hemocytes, and
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34 consequent activation state of membrane receptors and related signaling pathways, leading to
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36 cellular responses distinct from those observed in ASW medium.
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43 In mammalian biological fluids, attachment of proteins and lipids results in the formation of
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45 hard and soft coronas around NPs, with long and short typical exchange times, respectively
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47 (Rahman et al., 2013; Docter et al., 2015). The lifetime of hard corona has been shown to be of
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49 several hours, long enough for many biological and physiological phenomena to occur: therefore,
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51 the hard corona defines the biological identity of the particle. The competition between different
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53 proteins in mammalian serum for adsorption on the NP surface changes the composition of the
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55 corona over time. In most cases, proteins with high abundance in the plasma are first adsorbed on
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57 the surface, and over the time, they are replaced by proteins with lower concentration but higher
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affinity (Rahman et al., 2013). In a first attempt to characterize the possible formation of a hard
protein corona in mussel serum, PS-NH₂ were incubated for 24 h in HS, and the suspension was
subjected to the standard procedure utilized for mammalian serum (Monopoli et al 2013), adapted
for mussel HS, for separation and identification of NP-protein corona complexes by centrifugation,
1D PAGE and MS. Although further studies are needed to better characterize and identify the corona
proteins using more adequate proteomic tools, this procedure allowed for the identification of the
only protein associated with PS-NH₂ as the Putative C1q domain containing protein MgC1q6 of *M.*
galloprovincialis (F0V443).

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A recent proteomics study demonstrated that in *M. galloprovincialis* MgC1q6 (or Extrapallial
protein-EP precursor) is the most abundant serum protein, with different bands detected by both 1-
and 2-D gel electrophoresis and MS analysis (Oliveri et al., 2014). Shotgun analysis of *Mytilus*
hemolymph proteome showed that MgC1q6 has a relative abundance about three times higher in
serum than in hemocytes (Campos et al., 2015). MgC1q6 or EP is the same protein previously
identified by different methods and named as serum protein band 1-SPB1, histidine-rich
glycoprotein-HRG, heavy metal-binding protein-HIP, keystonein (reviewed in Oliveri et al., 2014). It
is an acidic glycoprotein with a high histidine content that can bind Ca²⁺ and heavy metals.
Recently, by a MS-based approach, a complex and anomalous N-glycan structure was determined
in *M. edulis* EP (Zhou et al., 2013). Such unique structure and calcium and heavy metal-binding
properties indicate that this protein plays a key role in multiple biological functions including shell
formation, metal ion transportation and detoxification (Zhou et al., 2013). Moreover, we have
recently demonstrated that MgC1q6 has a key role in immunity, acting as a specific serum opsonin
that mediates adhesion and killing of invading bacteria carrying D-mannose-sensitive ligands
(Pezzati et al., 2015). These data supported the role of this protein in innate immune response, due
to the presence of conserved complement C1q domains (Gerdol et al., 2011).

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The results of the present study identify MgC1q6 as the only protein component of the hard
corona formed in *M. galloprovincialis* HS around PS-NH₂. Binding of MgC1q6 to PS-NH₂ could

1 be partly due to the positive charges retained by PS-NH₂ in high ionic strength media, as shown by
2 the values of ζ potential recorded in ASW (Canesi et al., 2015a). Protein glycosylation may also
3 contribute to interactions with PS-NH₂; glycosylation of the protein corona has been shown to play
4 an important role in maintaining the colloidal stability of NPs in human plasma (Wan et al. 2015).
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9 Overall, the results obtained so far underline the need of understanding how the formation of a
10 NP protein corona may affect the biological outcome of NP exposure, in marine organisms as in
11 mammalian systems. In general, the presence of proteins reduces NP surface energy by nonspecific
12 adsorption, leading to lowered membrane adhesion and uptake efficiency (Lesniak et al., 2013).
13 Therefore, the formation of the protein corona in mammalian serum is considered as a general
14 protective effect from the potential cytotoxicity of NPs. Similarly, in earthworms, incubation of
15 AgNPs with coelomic proteins lead to formation over time of AgNP-EfCP corona complexes that
16 induced significantly greater NP accumulation in coelomocytes. Therefore, it was hypothesized
17 that NP hard corona proteins function as recognizable molecular patterns, making the NP-protein
18 complexes “visible” for clearance by phagocytic cells (Hayashi et al., 2013). The results obtained in
19 this work indicate that in the marine mussel *Mytilus* the formation of a serum protein corona
20 increases, rather than decrease, the short term *in vitro* toxicity of PS-NH₂ towards immunocytes.
21 Similarly, a recent study reported that a single histidine rich glycoprotein-HRG, present in human
22 (and mouse) plasma, but not in foetal calf serum, forms a stable hard corona around SiO₂-NPs that
23 confers to these particles the ability to evade their capture by macrophages, thus potentially
24 changing their biological fate (Fedeli et al., 2015). In different model organisms, the outcome of
25 the response may depend on the type of NPs, of the target cell, and of the composition of biological
26 fluids, that appears to be species-specific.
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53 Available data on the *in vitro* effects of NPs in marine invertebrate cells were carried out in sea
54 water media, or in common media adjusted for ionic strength and pH (reviewed in Canesi and
55 Corsi, 2016). However, it has been shown that in *Mytilus* hemocytes, responses to cytokines and
56 bacterial challenge were significantly affected by the presence of hemolymph serum, this resulting
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in activation of distinct signalling components that induce or prevent lysosomal damage and apoptotic processes (Betti et al. 2006; Balbi et al., 2013; Pezzati et al., 2015). The present data demonstrate the additional role of hemolymph serum in the interactions with PS-NH₂.

Overall, the results underline the importance of the physiological exposure medium and the determinant role of the recognizable biological identity during *in vitro* testing of NPs with invertebrate immune cells. However, the protein composition of extracellular fluids of invertebrates is largely unknown, given the large diversity of phyla and species, and different proteins may be involved in the formation of a stable corona around different NPs in different invertebrate groups (marine, freshwater, terrestrial). In earthworm coelomocytes, lysenin was identified as the major corona protein for AgNP *in vitro* (Hayashi et al., 2013), and different secreted proteins related to cell-to-cell signalling were identified in *Daphnia magna* (Nasser and Lynch, 2015). In mussels, MgC1q6 may play a key role in the interactions occurring *in vivo* between the hemolymph and not only PS-NH₂, but also other types of NPs. As in earthworms, different NPs may induce secretion of specific proteins by mussel hemocytes. However, it must be underlined that the results obtained *in vitro* do not entirely reflect a realistic exposure scenario and the more complex formation of a bio-corona that is likely to occur *in vivo* in invertebrate species. Further studies are needed to investigate the formation of NP protein corona in mussels exposed *in vivo* to different types of NPs at concentrations closer to those predicted in the marine environment. Both *in vitro* and *in vivo* studies will contribute to the understanding of NP uptake and potential toxicity in invertebrate species.

Finally, the results obtained with PS-NH₂ provide a further insight on the potential impact of nanoplastics in marine organisms. Occurrence of nanoplastics in the sea and their possible impact on marine biota is obviously part of the growing concern for the continuous increase of plastic wastes and debris in the aquatic compartment, including estuarine and coastal areas (Moore, 2008; Wegner et al., 2012; Mattsson et al., 2015). Polystyrene (PS) is one of the most largely used plastics worldwide, accounting for 24% of the macroplastics in the estuarine habitat, and it can be found in

1 the oceans and in marine biota as micro- and nano-debris (Browne et al., 2008; Moore, 2008;
2 Andradý 2011; Plastic Europe, 2013). Recent data reported the formation over time (from 14 days)
3 of nanoplastics of about 220 nm by degradation of polystyrene in controlled conditions (Lambert
4 and Wagner, 2016). In the sea urchin embryo (*Paracentrotus lividus*), exposure to PS NPs, in
5 particular PS-NH₂, caused severe developmental defects and induced changes in gene expression
6 suggesting the involvement of apoptotic pathways (Della Torre et al., 2014). A recent study in brine
7 shrimp larvae (*Artemia franciscana*) showed that PS NPs might affect food uptake (feeding),
8 behavior (motility) and physiology (multiple molting) (Bergami et al., 2016). PS-NH₂ cause
9 developmental defects and changes in gene expression also in *Mytilus* embryos (Canesi et al.,
10 2015b, and ms. in preparation). Taken together, the results underline the potential impact of
11 nanoplastics from the molecular to the organism and population level in marine invertebrates. In
12 this light, knowledge of their interactions with cells within the physiological environment of model
13 species represents the basis for understanding their fate and impact on marine biota.
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45 46 47 48 49 **Acknowledgements**

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53 54 55 **Author Contributions**

56
57 L.C. and I.C. designed the research and supervised the project; C.C., R.F., T.B., E.B. performed the
58 research and analysed the data; L.C. analysed the data and wrote the paper; L.C., I.C., K.D. and
59 M.M. supervised the paper.

Additional Information

Competing financial interests: The authors declare no competing financial interests.

Figure legends

Figure 1 – Effects of PS-NH₂ suspensions in different media on hemocyte functional parameters. Cells were incubated as described in methods with PS-NH₂ suspensions (1, 5, 50 µg/mL) in either ASW or in HS. a) lysosomal membrane destabilization, evaluated as NR retention time; b) extracellular oxyradical production, evaluated as cytochrome *c* reduction. c) phagocytic activity, evaluated as uptake of Neutral-Red conjugated zymosan particles. Data, expressed as percent of control values (untreated hemocytes) and representing the mean±SD of four experiments in triplicate, were analysed by ANOVA followed by Tukey's post hoc test (p≤0.05).

* = all treatments vs controls; # = serum vs ASW.

Figure 2 – Electron microscopy of *Mytilus* hemocytes. Representative TEM images of control and PS-NH₂ exposed cells (15 min, 5 µg/mL) in the presence of ASW or HS. Scale bar 5 µm. a) control granular hemocyte with intact filopodia, several intracellular granules and well visible mitochondria; b) hemocyte exposed to PS-NH₂ in ASW, showing loss of filopodia and presence of plasma membrane blebs (arrows), and a large intracellular vacuole (arrowhead). c, d) hemocytes exposed to PS-NH₂ in HS, showing short blebbed pseudopodia / enlarged cytoplasmic extensions (arrows), irregular plasma membrane surfaces, a large vacuole (d, arrowhead). Fragments of broken filopodia can be also observed around hemocytes (c).

Figure 3 – Effects of incubation of mussel hemocytes with PS-NH₂ (5 µg/mL) for different periods of time (10-60 min) on p38 MAPK and PKC phosphorylation in the presence of ASW or hemolymph serum (HS). C = control. Protein extracts from control and PS-NH₂-treated hemocytes

1 were subjected to 12% SDS-PAGE followed by Western blotting using polyclonal phosphospecific
2 antibodies to p38 MAPK and PKC pan. Bands were detected using enhanced chemiluminescence
3 reagents (see Methods). Results are representative of three independent experiments.
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7 a, b) representative blot of phosphorylated p38 MAPK and PKC (75 kDa and 70 kDa p-PKC); anti-
8 actin blots are shown as loading controls.
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12 c, d) densitometric analysis of blots from three independent experiments (mean±SD). Relative
13 increases in band optical densities (arbitrary units) were normalised for the control band in each
14 series. * = $P \leq 0.05$. $p < 0.05$ Mann–Whitney U test.
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22 Figure 4 – Separation of PS-NH₂ protein complexes from HS of *M. galloprovincialis* proteins by
23 SDS-PAGE and staining with Coomassie Brilliant Blue. PS-NH₂ were incubated in HS at the
24 nominal concentration of 25 µg NP/mg serum protein/mL, and samples were subjected to repeated
25 centrifugation and washing steps as described in Methods. A whole serum sample (WS) in the
26 absence of NPs was included (Lane 1). Lane 2: supernatant after the first centrifugation of the PS-
27 NH₂ suspension in HS (SN); Lanes 3-5: samples corresponding to the three washing steps (W1,
28 W2, W3); Lane 6: long-lived, hard corona proteins (HC). A representative gel of three
29 independent experiments is shown. The arrow indicates the position of band excised for tandem
30 mass spectrometry analysis.
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46 Fig. 5 – Representative images obtained by Field Emission Scanning Electron Microscopy
47 (FESEM) on PS-NH₂ suspensions in ASW (a,c) and in samples of the hard corona proteins (HC)
48 (b,d).
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Table 1. DLS analysis of PS-NH₂ suspensions (50 µg/ml) in different media, showing Z-average (nm) and polydispersity index (PDI). HS : *Mytilus* hemolymph serum (0.22 µm filtered). Data on HS are reported at different times of incubation (0, 1 and 2 h). Data are reported as mean ± SD. MQ: Milli-Q water; ASW: artificial sea water.

	Z-Average (nm)	PDI
HS T ₀	178± 2	0.37 ± 0.01
HS T ₁	179 ± 1	0.35 ± 0.01
HS T ₂	186 ± 3	0.34 ± 0.05
MQ*	57 ± 2	0.07 ± 0.02
ASW*	200 ± 6	0.30 ± 0.02

* Data from Canesi et al., 2015.

Figure1

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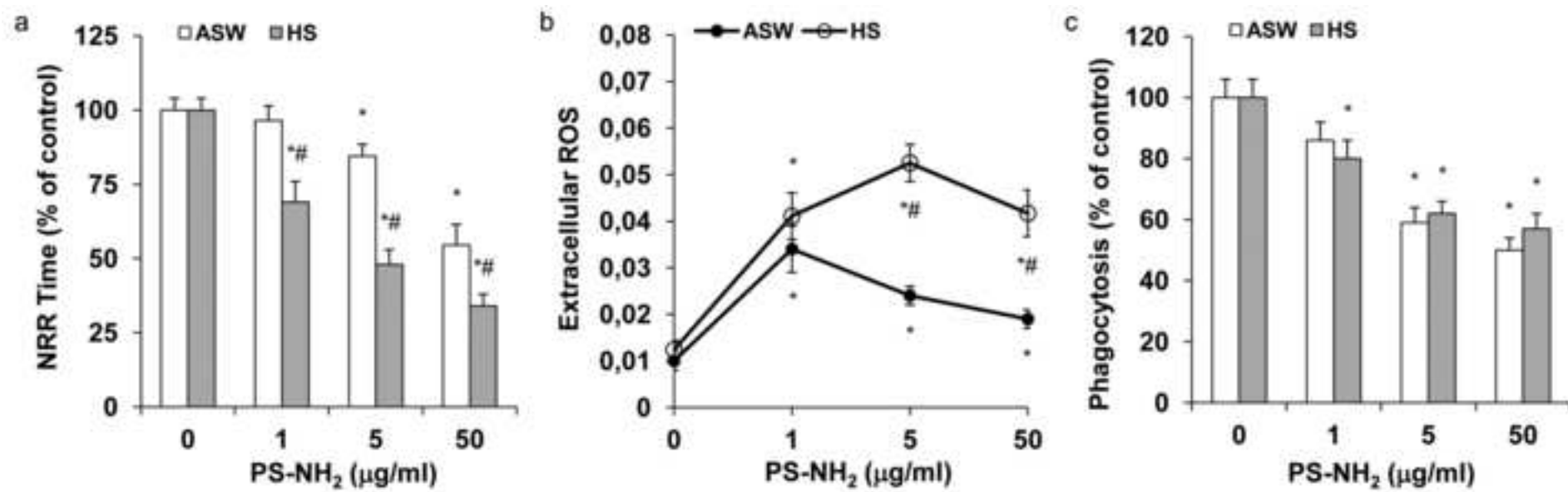


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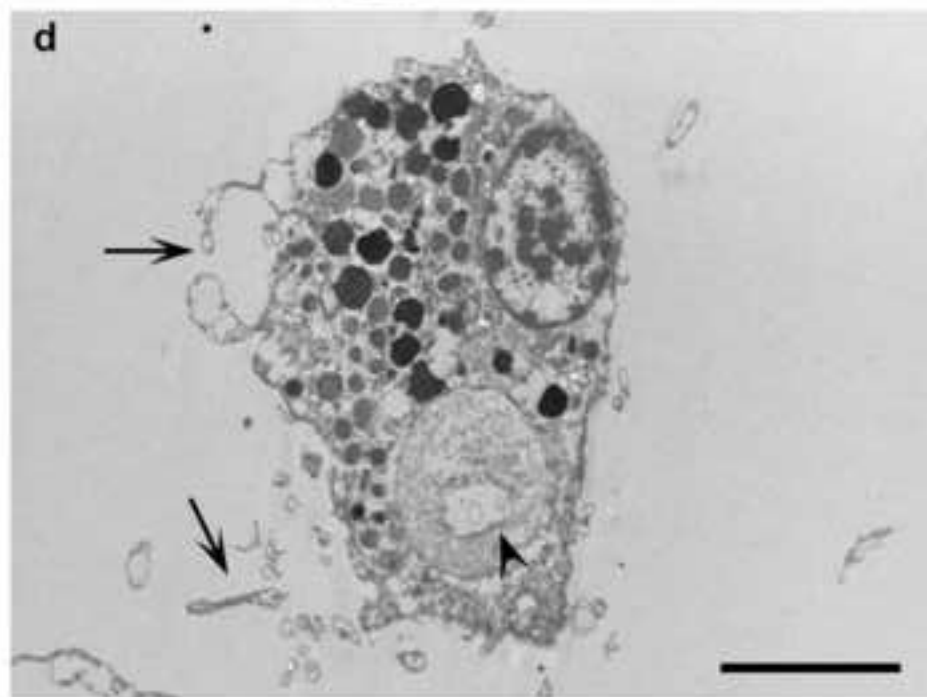
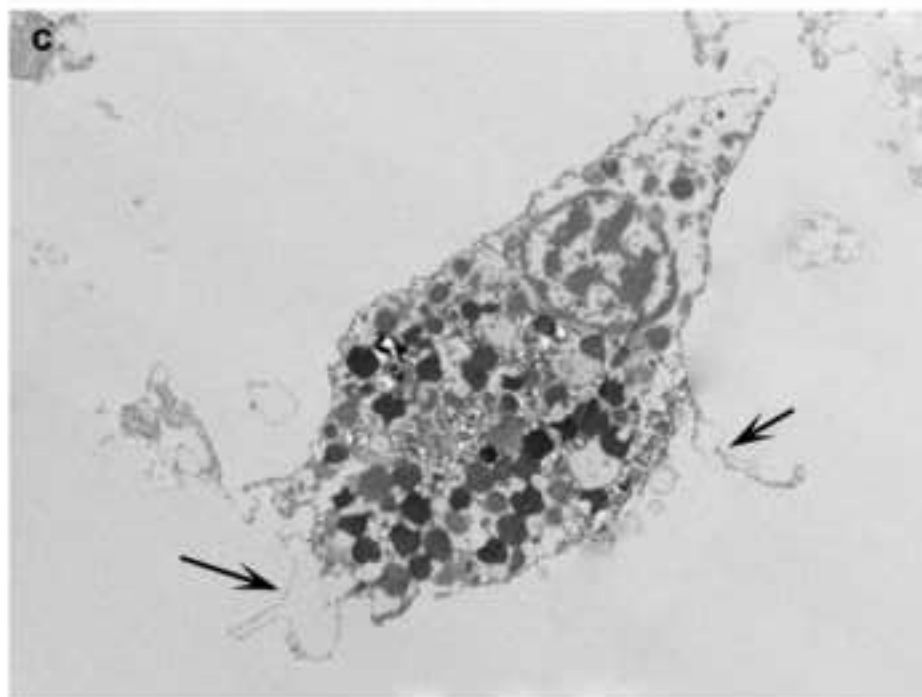
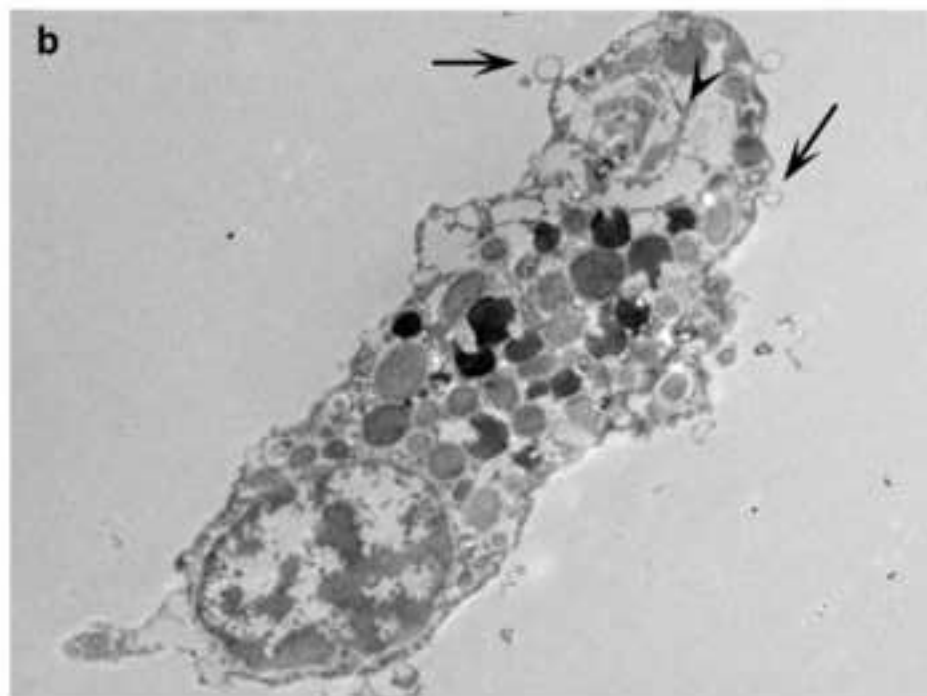
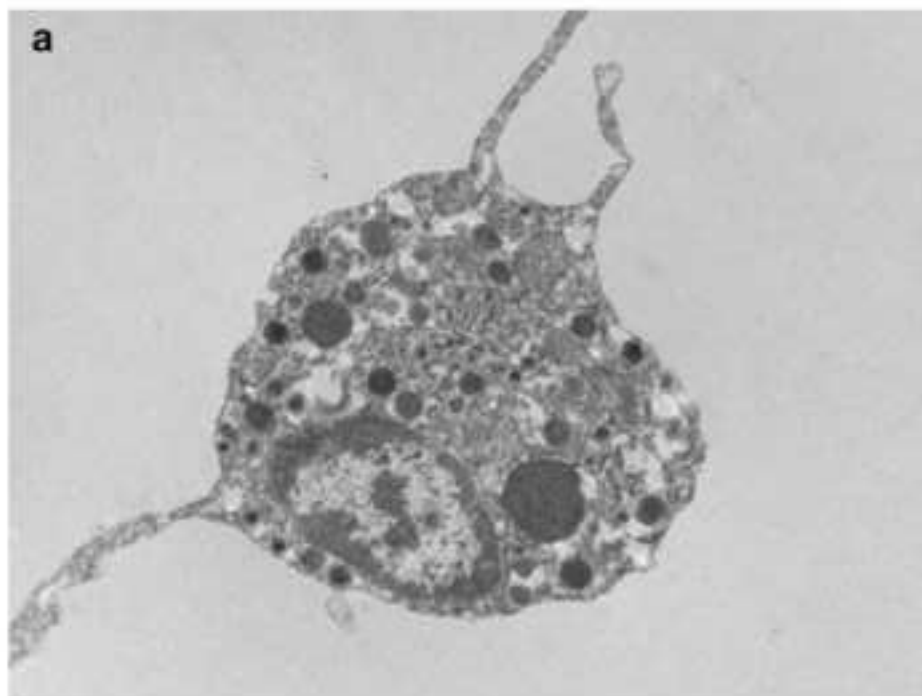


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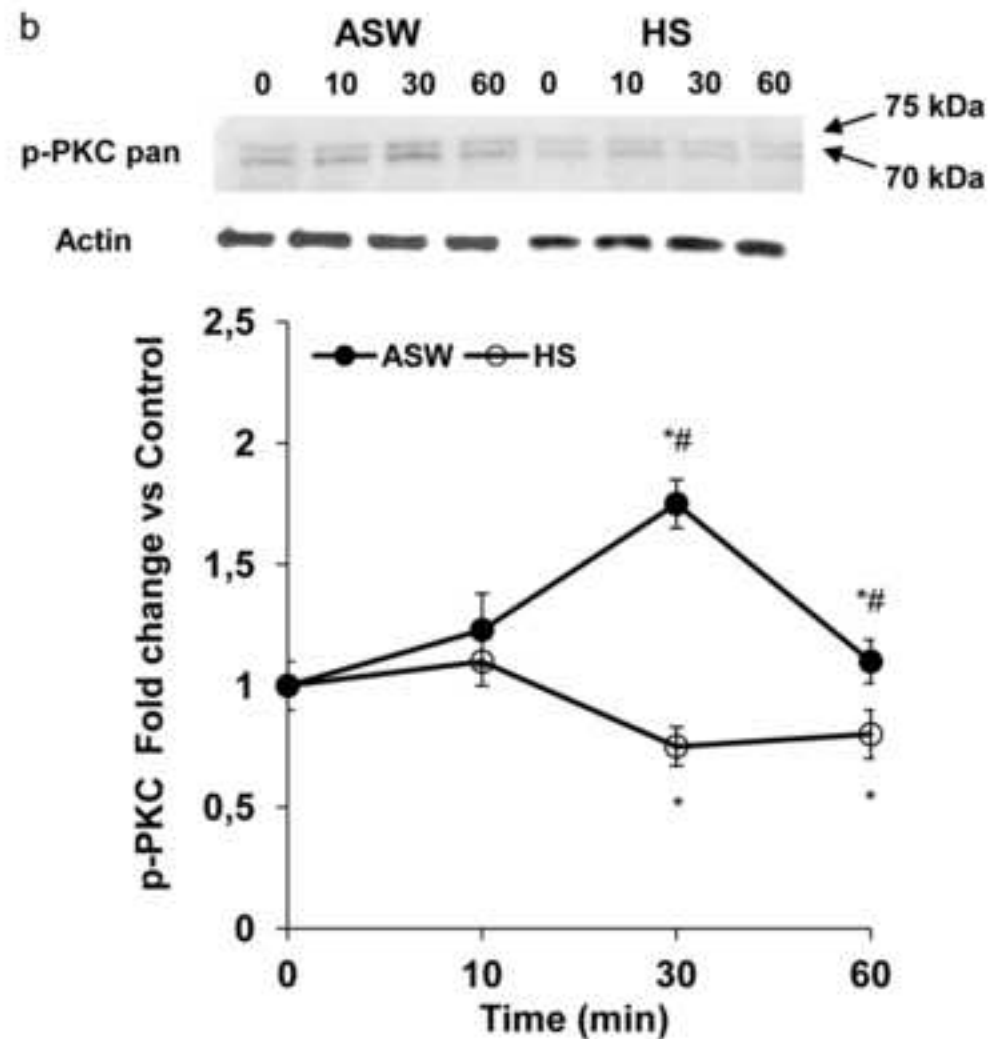
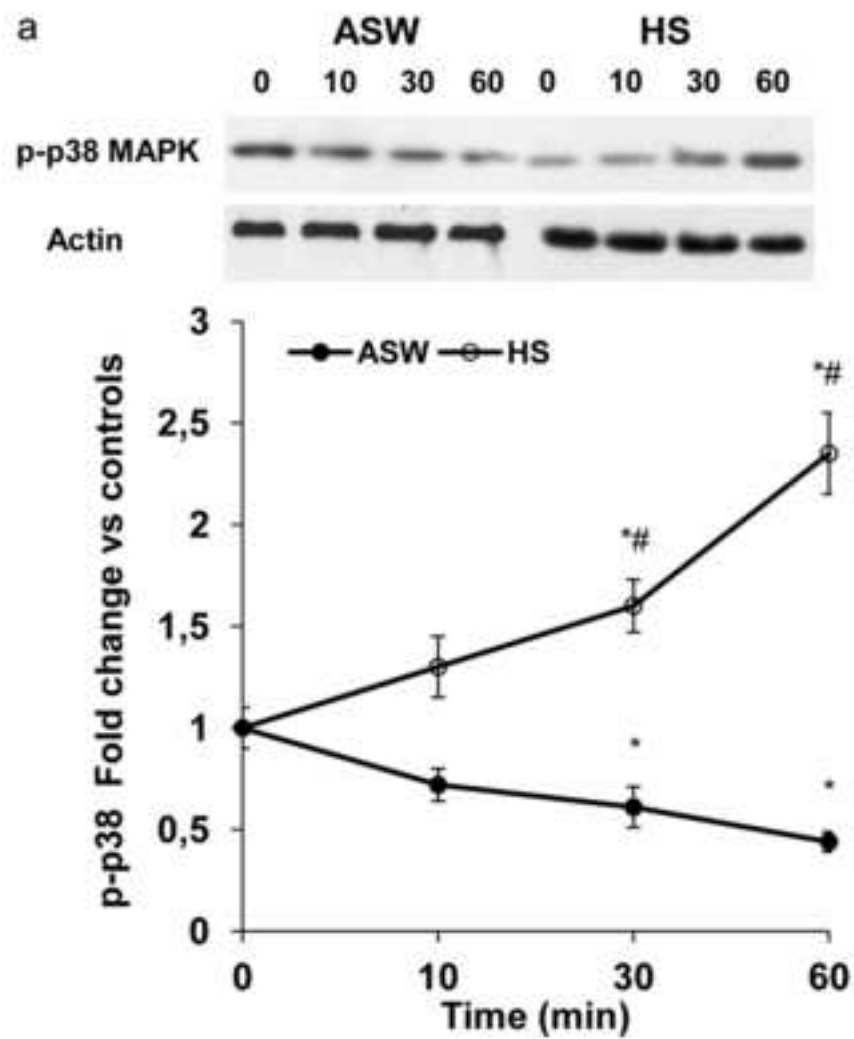


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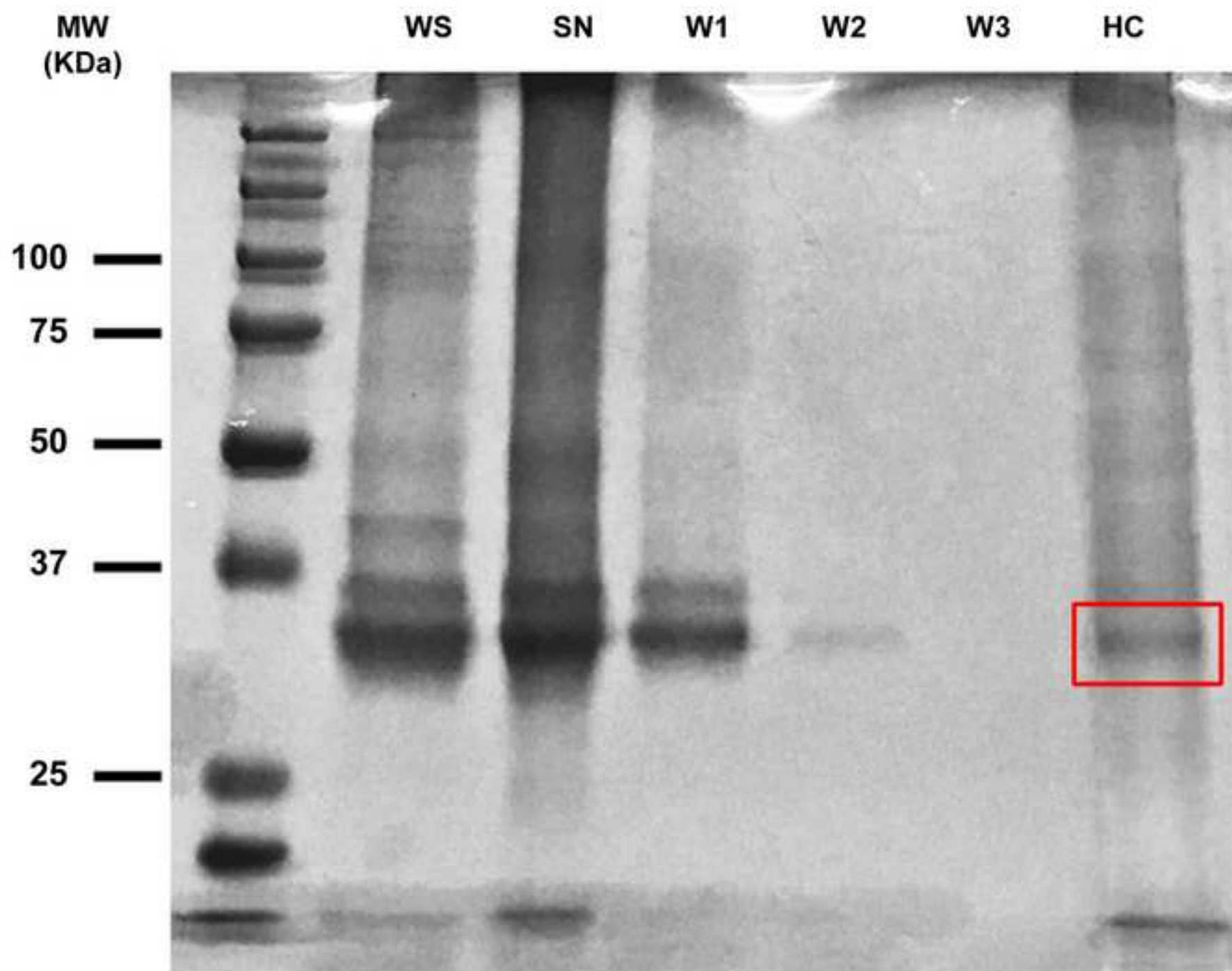
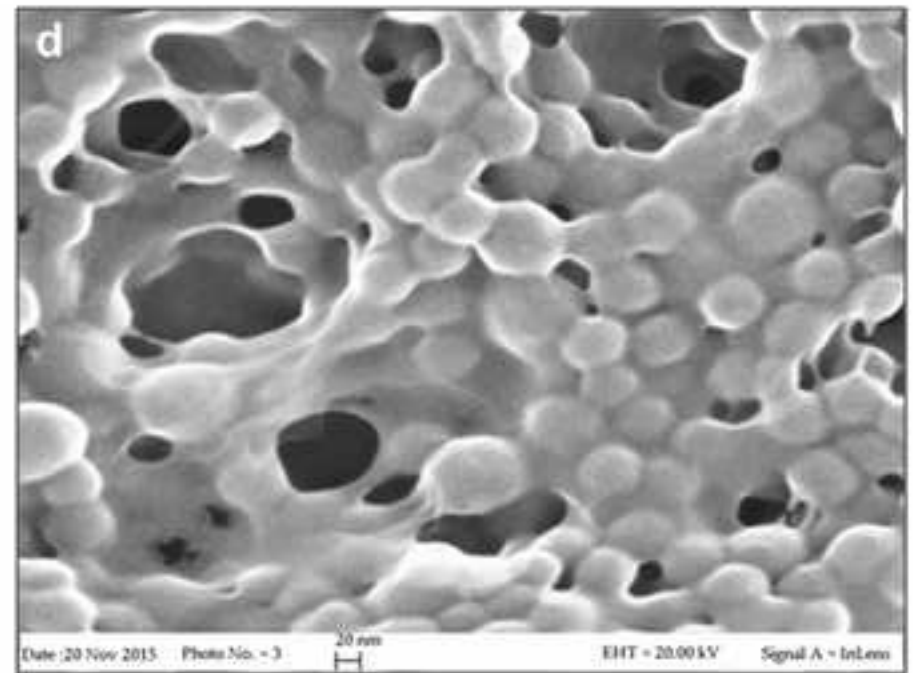
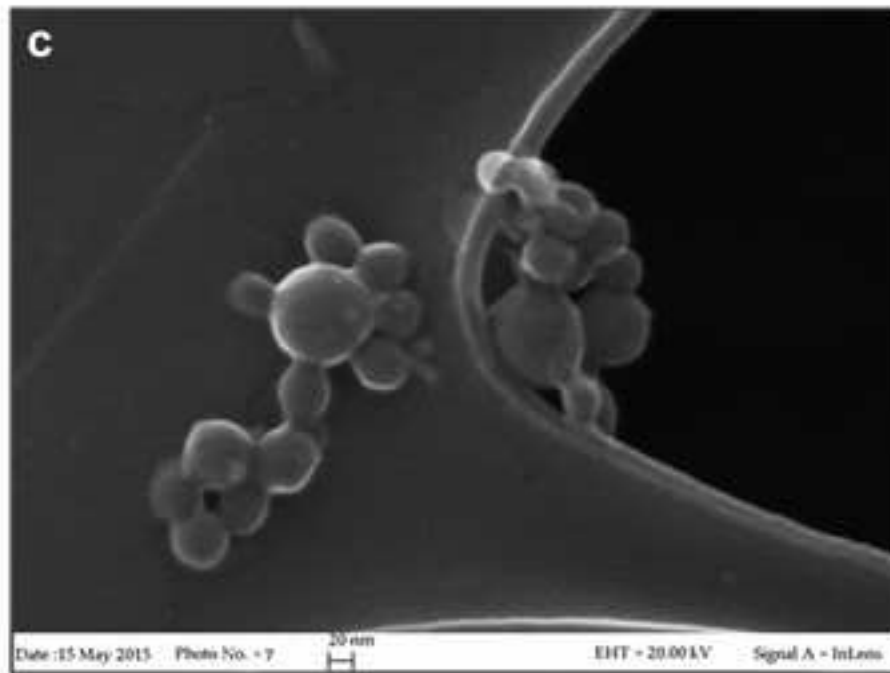
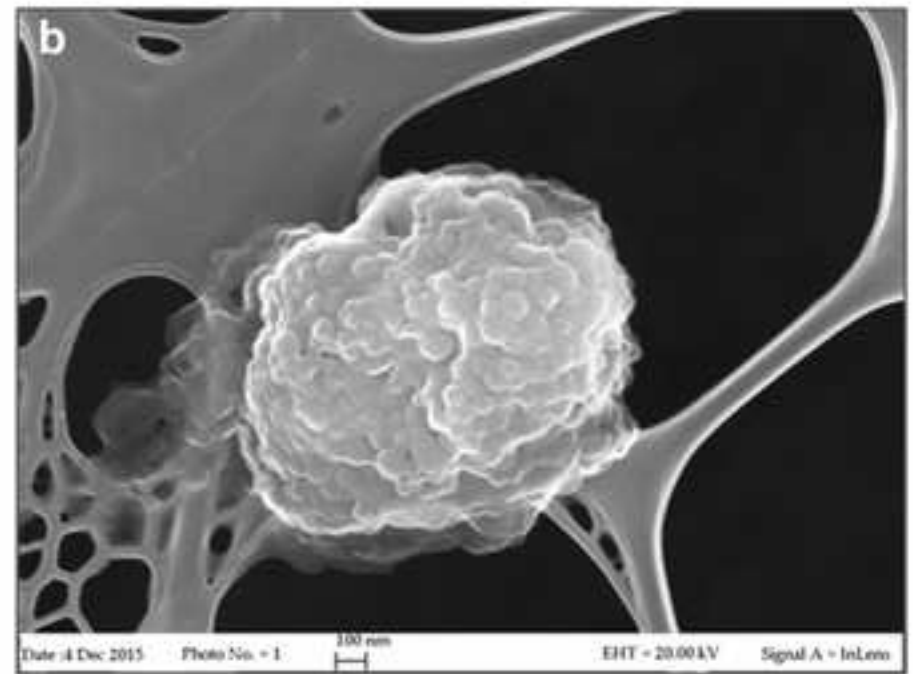
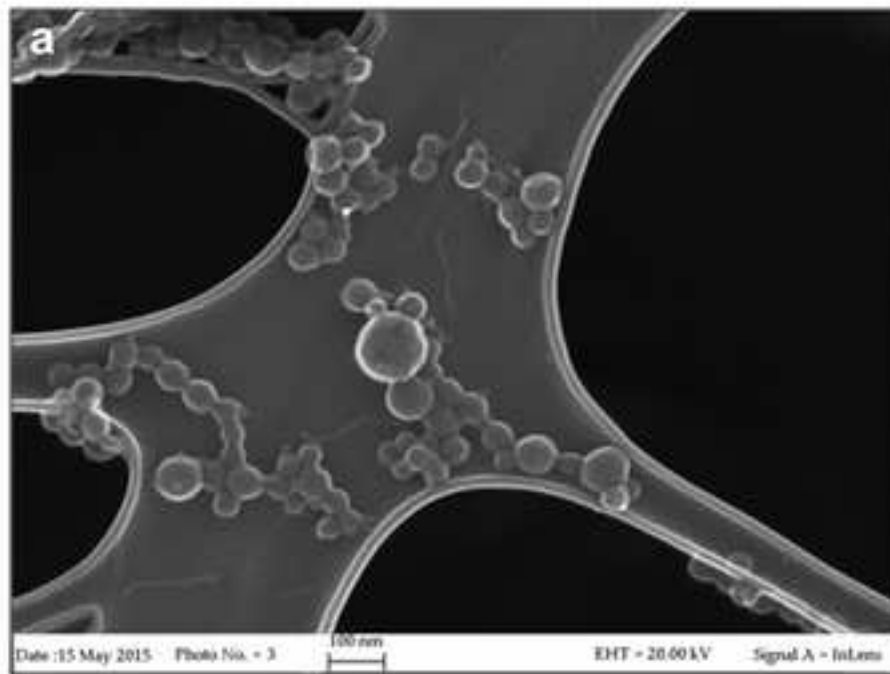


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