Impact of exposure time, particle size and uptake pathway on silver nanoparticle effects on circulating immune cells in *Mytilus galloprovincialis*

Younes Bouallegui, Ridha Ben Younes, Faten Turki and Ridha Oueslati

Research Unit for Immuno-Microbiology Environmental and Cancerogenesis, Sciences Faculty of Bizerte, University of Carthage, Bizerte, Tunisia

**ABSTRACT**
Nanomaterials have increasingly emerged as potential pollutants to aquatic organisms. Nanomaterials are known to be taken up by hemocytes of marine invertebrates including *Mytilus galloprovincialis*. Indeed, assessments of hemocyte-related parameters are a valuable tool in the determination of potentials for nanoparticle (NP) toxicity. The present study assessed the effects from two size types of silver nanoparticles (AgNP: <50 nm and <100 nm) on the frequency of hemocytes subpopulations as immunomodulation biomarkers exposed in a mussels host. Studies were performed using exposures prior to and after inhibition of potential NP uptake pathways (i.e. clathrin- and caveola-mediated endocytosis) and over different durations of exposure (3, 6 and 12 h). Differential hemocyte counts (DHC) revealed significant variations in frequency of different immune cells in mussels exposed for 3 hr to either AgNP size. However, as exposure duration progressed cell levels were subsequently differentially altered depending on particle size (i.e. no significant effects after 3 h with larger AgNP). AgNP effects were also delayed/varied after blockade of either clathrin- or caveola-mediated endocytosis. The results also noted significant negative correlations between changes in levels hyalinocytes and acidophils or in levels basophils and acidophils as a result of AgNP exposure. From these results, we concluded AgNP effects on mussels were size and duration of exposure dependent. This study highlighted how not only was NP size important, but that differing internalization mechanisms could be key factors impacting on the potential for NP in the environment to induce immunomodulation in a model/test sentinel host like *M. galloprovincialis*.

**Introduction**

Nanoparticles (NP) are defined as materials with all dimensions in nanoscale [1–100 nm] (Luoma 2008). Silver nanoparticles (AgNP) have become the fastest growing product category in nanotechnology due to their thermo-electrical conductivity, catalytic activity and nonlinear optical behavior and have great value in the formulation of inks, microelectronic products and biomedical facilities (i.e. imaging devices) (Tiede et al. 2009; Katsumiti et al. 2015). Their exceptional broad-spectrum bactericidal properties and biocompatibility (i.e. as drug delivery agent) have also made AgNP extremely useful in a diverse range of consumer goods (Luoma 2008; Rainville et al. 2014; Cozzari et al. 2015; Katsumiti et al. 2015; Marisa et al. 2016).

Worldwide AgNP production is estimated at ~55 tonne/yr (Piccinno et al. 2012). However, release of AgNP into aquatic environs can happen through wastewaters generated during AgNP synthesis and/or incorporation into goods and consumer products (Canesi et al. 2012; Matranga & Corsi 2012; Katsumiti et al. 2015; Marisa et al. 2016). As such, AgNP have emerged as potential stressors that might enter marine environment (Luoma 2008). A lack of appropriate tools to evaluate effective NP (of AgNP in particular) levels in aquatic environments make selection of appropriate testing levels a major problem in risk assessment of engineered NP. As a result, predicted environmental concentrations for AgNP are often set at a level of ~0.01 μg/L (Tiede et al. 2009; Katsumiti et al. 2015). Even so, levels much lower than that have commonly been used in aquatic species ecotoxicity tests (1–100 μg/L) (Tiede et al. 2009; Canesi & Corsi 2016), including those with mollusk models.

In the mussel *Mytilus galloprovincialis* (filter-feeding organism), hemocytes are hemolymph cells responsible for immune defence and serve as a first line of defence against foreign substances (Gosling 2003; Parisi et al. 2008; Giron-Perez 2010; Matozzo & Balo 2015). Immune defences carried out by hemocytes constitute important targets for potential NP toxicity (Canesi et al. 2012; Canesi & Prochazkova 2013; Katsumiti et al. 2015).

Several studies have shown that different NP types, that is, carbon black, C₆₀ fullerenes, TiO₂, SiO₂, ZnO, CeO₂, Cd-based, Au-based and Ag-based, are rapidly taken up by hemocytes. Internalization of these NP subsequently impacted on morphologic/functional characteristics including immune responses (Canesi et al. 2008, 2010a, b, 2012; Katsumiti et al. 2015; Marisa et al. 2016). Various mussel hemocyte parameters, including total hemocyte count (THC), differential hemocyte count (DHC), hemocyte viability, phagocytic activity and lysosomal membrane stability, have been used as a tool for screening of immunomodulatory effects of differing NP (Matozzo et al. 2007; Parisi et al. 2008; Hoher et al. 2013; Matozzo & Balo 2015; Canesi & Corsi, 2016; Marisa et al. 2016). Specifically, hyalinocytes and granulocytes have been assessed for morphological changes among hemocytes in *Mytilus galloprovincialis* (Pipe et al. 1997; Chang et al. 2005; Garcia-Garcia et al. 2008).
While granulocytes are large ovoid-shaped cells with a small eccentric nucleus and granulated cytoplasm (low nucleus/cytoplasm [N/C] ratio) that are able to spread out and produce pseudopodia, haemocytes are small round cells with an agranular (zero)-few granules) small cytoplasm surrounding a large nucleus (high N/C ratio) (Carballal et al. 1997; Parisi et al. 2008; Cima 2010; Matozzo & Balio 2015). Overall, haemocytes can be classified into two types, granulocytes and haemolymphocytes (so-called agranulocytes), based on morphological characteristics (the presence/absence of granules in cytoplasm). Staining of the cytoplasm by certain dyes allows for sub-distinguishing of acidophils from basophils among the granulocytes. Ultimately, the basophils of M. edulis appear as granulocytes with small granules, while acidophilic granulocytes contain large granules. In comparison to the granulocytes, haemolymphocytes in bivalve have only basophilic properties. Thus, in earlier studies that described haemocyte subpopulations, the authors indicated that basophilic cells (haemolymphocytes + basophils) made up about 40% of the total haemocyte pool in bivalves/mussels while eosinophils accounted for the remaining approximately 60% of all haemocytes (Chang et al. 2005; Garcia-Garcia et al. 2008).

Cellular uptake by endocytosis (clathrin- or caveolae-mediated routes) are crucial for a variety of cellular and physiological activities (i.e. nutrient uptake, immune defence) (Hauke 2006; Sandvig et al. 2011); each has also been identified as potential means for NP entry into cells (Moore 2006; dos Santos et al. 2011; Khan et al. 2015). Clathrin-dependent endocytosis involves formation of a clathrin (protein)-coated pit used in enzymatic destruction of internalized contents. Caveolae-dependent endocytosis occurs via cell-surface flask-shaped invaginations enriched with caveolin (cholesterol-binding proteins) (Nichols & Lippincott-Shwartz 2001; Razani & Lisanti 2002) that permit sub-cellular movements of ingested materials through a series of endosomal compartments of increasing acidity allowing for hydrolytic breakdown (Moore 2006; Putheveedu & von Zastrow 2006; Doherty & McMahon 2009). Each route can be modified with inhibitors. For inhibitor-treated groups, mussels were incubated for 3 h with 100 μM nystatin (NYS), mussels were exposed with 50 μg/ml Sigma) was prepared in dimethyl sulfoxide (DMSO) vehicle (Sigma); the final concentration of DMSO in all Nystatin exposures was 0.05% (v/v). Exposures to vehicle alone or in the presence of AgNP of differing sizes were conducted to assure effects were not caused by any carrier modulation of NP behavior or by the carrier itself. Effective concentration ranges used were chosen based on previous study by Khan et al. (2015).

Endocytic internalization blockers

A stock solution of amantadine (3 mg/mL; Sigma, Steinheim, Germany) was prepared in ultrapure water. Nystatin (Sigma) stock solution (5 mg/mL; Sigma) was prepared in dimethyl sulfoxide (DMSO) vehicle (Sigma); the final concentration of DMSO in all Nystatin exposures was 0.05% (v/v). Exposures to vehicle alone or in the presence of AgNP of differing sizes were conducted to assure effects were not caused by any carrier modulation of NP behavior or by the carrier itself. Effective concentration ranges used were chosen based on previous study by Khan et al. (2015).

Sampling and experimental design

Mature mussels (M. galloprovincialis) of average shell length 75 [±5] mm were collected from Bizerte lagoon (Tunisia) and maintained in oxygenated ASW (35% salinity, pH 8.0; as for local natural seawater) in static tanks under standard conditions (aeration, 12/12 h photoperiod, 16°C). Animals used for exposure experiments were acclimated for 1–3 days (Canesi et al. 2010b) and were not fed during either acclimation or exposure. Exposure in each tank was 1 mussel/0.5 L ASW in all studies. As only predicted environmental concentrations (PEC) were available in literature, the chosen dose of 100 μg AgNP/L was selected as the test concentration; this dose is usually used in ecotoxicity tests on aquatic species and would be effective in producing adverse effects that could be correlated with outcomes of previous in vitro studies (Katsumi et al. 2015; Canesi & Corsi 2016).

Mussels (n = 10/group) were separately exposed to AgNP <50 nm (AgNP50) or AgNP <100 nm (AgNP100) for 3, 6 and 12 h with/without initial treatment with the pharmaceutical inhibitors. For inhibitor-treated groups, mussels were incubated for 3 h with 100 μM amantadine (AMA), then placed in AgNP250 nm exposure solutions (without AMA) for the required times. For nystatin (NYST), mussels were exposed with 50 μg M NYS for 1 h before and then continuing over into the AgNP exposure time frames (Ivanov 2008; Angel et al. 2013; Khan et al. 2015). Control groups (n = 10) of mussels were maintained in oxygenated tanks of only ASW and/or ASW with the inhibitors exactly as above with the AgNP treatments. All exposures were done in triplicate.

Material and methods

Silver nanoparticles (AgNP) source and characterization

Poly-vinyl-pyrrolidone (PVP)-coated AgNP of <100 nm (99.5% pure) were purchased from Sigma (Steinheim, Germany). PVP-coated AgNP <50 nm were produced by a modified process wherein AgNO3 (Sigma) was dissolved in ethylene glycol (EG) solvent (ACROS Organics, 98%, Geel, Belgium) in the presence of PVP (K30, Sigma) as a capping agent (Mezni et al. 2014a,b).

A stock solution of each AgNP size was suspended in artificial seawater (ASW; 58.5% NaCl; 26.5% MgCl2; 9.8% Na2SO4; 2.8% CaCl2; 1.65% KCl; 0.5% NaHCO3; 0.24% KBr; 0.07% H3BO3; 0.0095% SrCl2; 0.007% NaF (Pinnino et al. 2015)). Prior to use, each AgNP stock was mixed several times and an aliquot removed as a working solution that was sonicated 15 min in alternating cycles (2 × 30 s) in an ultrasonic bath (VWR, Strasbourg, France). Primary physicochemical properties of each AgNP was confirmed by transmission electron microscopy (TEM) coupled with a microanalysis characterization (TECNAI G20, Ultra-Twin, FSB, Bizerte, Tunisia) and ultraviolet-visible (UV-Vis) spectroscopy (T60; PG-Instruments, Leicestershire, UK). X-ray diffraction (XRD) characterization was performed using a D8 Advance diffracto-meter (Bruker, Bizerte), with analyses performed in Bragg–Brentano configuration at 40 kV and 40 mA.

Pappenheim’s panoptical staining (MGG) and differential hemocyte counts (DHC)

At the completion of the given exposure period, hemolymph samples were quickly withdrawn (to minimize stress inflicted)
from the adductor muscles of each animal, using an 18-G needle fitted onto a 3-mL syringe. All samples were collected at 16 °C. For each sample, hemolymph of all 10 individuals/treatment regimen was pooled; the material was then filtered through 1-mm³ mesh sterile gauze into a 5-mL tube at 4 °C to avoid aggregation (Canesi et al. 2010a). After mixing, 40 μL aliquots were deposited onto glass slides; after 15 min, the attached cells were fixed with methanol and then the hemocytes were stained with May-Grünwald solution (Bio-optica, Milan, Italy). Slides were then counterstained with 5% Giemsa, air-dried, and then mounted using a mounting medium (Entellan Neu, Merck, Darmstadt, Germany) and cover slipped. Slides were then evaluated using a GX-10 light microscope (Olympus, Tokyo, Japan); differential hemocyte counts were made from counts of stained cells in 10 different fields/slide. A minimum of 350 cells/slide was counted. Ten slides/experimental condition were evaluated.

**Statistical analysis**

All results are expressed as percentages (±SD) of total hemocytes. Normal distribution and homogeneity of variance were tested using Shapiro–Wilk and Bartlett tests prior to statistical analysis. Statistical analysis of absolute percentages was performed using a one-way analysis of variance (ANOVA) with a Tukey’s HSD post hoc test. Modulation in the percentages of hemocyte subpopulations were compared to those of controls (untreated mussels). Correlation tests were used to determine relationships among modulated hemocyte subpopulations. Significance overall and within any correlation (confirmed by linear regression test) was accepted at p < 0.05.

**Results**

**Source and characterization of AgNP**

Purchased AgNP (<100 nm; AgNP₁₀₀) were characterized; characterizations met the manufacturer supplied valued (99.5% trace metal basis). Representative TEM showed homogeneous spherical characteristics with an approximate primary size of 90 nm (Figure 1(A)); size distribution histograms revealed a median size of 85.0 ±32.6 nm (Figure 1(C)). Representative TEM of synthesized AgNP (<50 nm; AgNP₅₀) demonstrated homogeneous spherical characteristics with an approximate size of 50 nm (Figure 1(B)); size distribution histograms revealed a median size of 41.6 ±18.8 nm (Figure 1(D)). Analyses of each sample indicated that the level of particles <50 nm within the AgNP₅₀ mixture was ≈ 1.38/each 100 particles from AgNP mixture (i.e. <1.5%).

The XRD pattern recorded from a representative batch of silver powder is shown in Figure 1(E). The crystalline nature of the AgNP was demonstrated by diffraction peaks that matched the face-centered cubic (fcc) phase of silver. The absorption maximum of the measured UV–vis spectrum of the colloidal solution provides information on the average particle size, whereas its full width at half-maximum (λₘₜₐₓ) can be used to estimate particle dispersion as demonstrated by Leopold and Lendl (2003). Agglomeration status analyses performed prior to exposure was confirmed by absorbance spectra measures at λₘₜₐₓ = 400 nm (Figure 1(F)) that clearly indicated the AgNP had a homogenous dispersion in aqueous solutions.

**Determination of hemocyte subpopulations**

Evaluations based on cytoplasmic granules (presence or absence) and stained granule color (Figure 2) showed that levels of circulating hemocytes from mussels exposed to AgNP suspensions at the same dose (100 μg/L) varied as a function of differing particle size. For example, when exposed to AgNP₅₀ for only 3 h, mussels evinced a significant increase in acidophilic granulocytes (acidophils) (78.93 ±6.29%L) compared to levels in controls (60.28 ±8.63%L); however, the AgNP₁₀₀ at this timepoint imparted no significant effect. Conversely, exposure to either size AgNP led to a significant decrease in basophilic granulocyte (basophils) levels in the same timeframes (i.e. 10.76 ±2.78%L for AgNP₅₀ and 13.43 ±0.90%L for AgNP₁₀₀) vs. control (19.77 ±2.89%L).

No significant variations were noted in levels of hyalinocytes (10.30 ±3.68%L for AgNP₅₀, 10.37 ±3.33%L for AgNP₁₀₀, 19.94 ±5.77%L control). Conversely, when exposed to AgNP₅₀ for 6h, mussel levels of hyalinocytes displayed a significant increase (16.21 ±3.69%L) versus control values (7.48 ±3.43%L). No other significant variations were recorded for basophils (16.24 ±2.49%L for AgNP₅₀, 14.27 ±1.97%L for AgNP₁₀₀, 15.32 ±1.82%L control) or acidophils (67.54 ±6.07%L for AgNP₅₀, 77.49 ±6.96%L for AgNP₁₀₀, 77.19 ±4.21%L control) in the same timeframe. For the 12-h exposure, no significant variations in hemocyte sub-populations were noted with either AgNP [hyalinocytes = 16.63 ±5.37%L AgNP₅₀, 18.02 ±3.52%L AgNP₁₀₀, 20.33 ±1.44%L control]; basophils = 24.11 ±7.03%L AgNP₅₀, 19.62 ±2.33%L AgNP₁₀₀, 17.58 ±0.96%L control; acidophils = 59.20 ±12.30%L AgNP₅₀, 62.35 ±2.23%L AgNP₁₀₀, 62.07 ±0.52%L control (Figure 3(A)).

**Effect of uptake pathway on circulating hemocytes**

**Caveolae-mediated endocytosis inhibition (amantadine [AMA])**

Significant increases in basophils were seen [16.02 ±1.62%L vs. AMA at 12.00 ±0.90%L in hosts exposed to AgNP₅₀ for 3 h but not to AgNP₅₀ [15.03 ±1.99%L]. No significant variations were recorded with any 6-h exposures (hyalinocytes: 15.49 ±0.93%L AMA, 13.8 ±2.09%L AMA + AgNP₅₀, 18.12 ±1.10%L AMA + AgNP₁₀₀; basophils: 15.12 ±0.95%L AMA, 16.52 ±2.09%L AMA + AgNP₅₀, 14.79 ±2.11%L AMA + AgNP₁₀₀; acidophils: 69.37 ±1.88%L AMA, 70.57 ±6.15%L AMA + AgNP₅₀, 67.07 ±3.21%L AMA + AgNP₁₀₀]. At 12 h, acidophilic levels were significantly increased in hosts exposed to either AgNP [74.23 ±2.81%L AgNP₅₀, 73.85 ±0.77%L AgNP₁₀₀, 68.28 ±0.63%L AMA. Conversely, basophilic levels were significantly decreased in mussels exposed for 12 h to AgNP with clathrin path blocking (14.51 ±0.15%L vs. AMA at 19.29 ±1.33%L) but not to AgNP₅₀ (19.29 ±1.53%L). Hyalinocyte levels were also significantly reduced in mussels exposed for 12 h to AgNP₅₀ with clathrin path blocking (8.76 ±0.12%L vs. AgNP₅₀ 11.79 ±3.01%L); AgNP₁₀₀ imparted no significant effect (11.63 ±0.76%L) (Figure 3(B)).

**Caveolae-mediated endocytosis inhibition**

**Effect of exposure to AgNP in presence of DMSO (Vehicle)**

Percentages of circulating hemocytes in mussels exposed to DMSO (0.05%) alone for 3, 6 or 12 h were not significantly changed from levels in untreated mussels (control) (Figure 4(A)). However, in the presence of AgNP₅₀ or AgNP₁₀₀ only a significant decrease in basophilic levels was noted at the 6-h timepoint (13.46 ±3.78%L and 12.07 ±2.65%L, respectively) as compared to in hosts exposed only to DMSO (18.25 ±9.06%L). No other significant changes due to either form of AgNP at all other timepoints was noted (Figure 4(B)).
Effect of exposure to AgNP in presence of nystatin (NYS; caveolae blocker)

No significant changes in circulating hemocytes sub-populations were evident for either size AgNP with 3 h of exposure in the presence of NYS (hyalinocytes: 13.91 ±3.64% AgNP\textsubscript{50}, 10.39 ±2.31% AgNP\textsubscript{100}, 10.13 ±3.37% NYS). In contrast, exposure to AgNP\textsubscript{50} for 6 h in the presence of NYS caused only a significant decrease in acidophils [79.15 ±1.02% vs. NYS at 84.51 ±2.14%] and a significant increase in basophils.
Figure 2. Representative light micrograph of *Mytilus galloprovincialis* hemocyte sub-populations. May–Grünwald–Giemsa (MGG) staining. AG: acidophilic granulocytes; Endo: endoplasm (dense stained granules); Ect: ectoplasm (hyaline with thin pseudopodia); Hy: hyalinocytes; BG: basophilic granulocytes. Magnification = 400×.

| Time (h) | Cont | Ag50 | Ag100 |
|----------|------|------|-------|
| 3h       |      |      |       |
| 6h       |      |      |       |
| 12h      |      |      |       |

Figure 3. Variations in circulating hemocyte sub-populations (%) as marker of immunomodulation from AgNP. (A) Exposure to only AgNP. (B) Exposure to AgNP and Amantadine. Data shown are percentages. Hyalinocytes (dark grey), basophilic granulocytes (light grey), acidophilic granulocytes (medium grey). Cont: untreated, Aman: amantadine, Ag50: AgNP < 50 nm, Ag100: AgNP < 100 nm for 3, 6 or 12 h. N = 10/group. Value significantly different from negative control [*p < 0.05.*]
[11.97 \pm 3.64\%] \text{ vs. NYS at 8.71 \pm 3.37\%}. Significant increases in acidophils were evident only after 12 h of exposure to AgNP_{100} in the presence of NYS [73.89 \pm 0.56\%] \text{ vs. NYS at 63.62 \pm 2.08\%}%; in contrast, a significant decrease in basophils was noted with exposures to either size AgNP in the presence of NYS in this same timeframe [20.09 \pm 0.49\% AgNP_{50}, 15.74 \pm 0.89\% AgNP_{100}, 23.23 \pm 1.08\% NYS]. For hyalinocytes, a significant increase was only evident with exposure to AgNP_{50} in the presence of NYS for 12 h [17.44 \pm 1.96\%] \text{ vs. NYS at 13.13 \pm 1.01\%}; no significant effects were induced with AgNP_{100} (10.35 \pm 0.51\%) (Figure 4(C)).

Correlation between variations in hemocyte sub-populations

The variations in hemocyte sub-population levels under the conditions tested here were seen to be intercorrelated. Mussels exposed under differing conditions for 3 h demonstrated significant negative correlations between changes in levels hyalinocytes and acidophils or in levels basophils and acidophils (\(r = -0.73\) and \(r = -0.90\), respectively). No significant correlation was found between levels of hyalinocytes and basophils (\(r = 0.46\)) (Table 1). With 6-h exposures, a significant [positive] correlation was seen between changes in levels of hyalinocytes and basophils...
changes in the levels of hyalinocytes and of basophils was noted

\[ r = 0.757, \text{respectively}. \] (Table 2). The 12-h exposure gave rise to significant negative correlations among the variations in levels of hyalinocytes and acidophils and of basophils and acidophils (\( r = 0.824 \) and \( r = -0.757, \) respectively). No significant correlations between changes in the levels of hyalinocytes and of basophils was noted (\( r = 0.255 \)) (Table 3).

### Discussion

The present in vivo study aimed to elucidate the ability of AgNP to enter into *Mytilus galloprovincialis* marine mussels and modulate the percentages of their immune system cell sub-populations. Previous studies noted the ability of environmental pollutants, such as mercury and cadmium, to significantly enhance variations with 6- and 12-h exposures) and a significant increase in levels of acidophils percentages in mussels, while no variations were recorded after 6 or 12 h. This short “toxicity timeframe” may indicate any putative cytotoxic effect caused by AgNP could potentially be neutralized by the increased presence of acidophils; this is plausible in that other studies have described a prominent role for acidophils in host internal defense (Chang et al. 2005; Garcia-Garcia et al. 2008; Parisi et al. 2008; Matozzo & Bailo 2015).

Apart from any increased presence of “NP-detoxifying acidophils,” the current results showing that the effect of the AgNP was duration of exposure-related effect could also be a result of changes in the bioavailability of these NP over time. As bioavailability of NP is a major factor in ultimate toxicity, surrounding environment effects on particle size stability, shape, surface charge, etc. are key variables that will determine effects on exposed hosts, including mollusks (Levard et al. 2012; Liu et al. 2012; Dobias & Bernier-Latmani 2013; Yu et al. 2014; Katsumita et al. 2015; Minetto et al. 2016). Canesi and Corsi (2016) hypothesize putative trans-formations of NP including how extracellular proteins could be adsorbed onto a NP surface, forming a protein corona of naturally occurring colloids, particles and macromolecules in the water column. The protein corona could then impact how specific cellular receptors, cellular internalization pathways, and ultimately in immune responses as well, see and respond to the now-modified NP.

The results also indicated significant decreases in basophil levels with host exposures for 3 h to either size AgNP (but no significant variations with 6- and 12-h exposures) and a significant increase in hyalinocytes levels only with AgNP50 for 6 h. Here, the variations showed again that AgNP effects were duration-of-exposure-dependent. In this same context, the recorded variations in the different sub-populations could be explained by an ability of other cell categories, apart from acidophils, to be activated as part of the immune response. This result was in agreement with outcomes of studies conducted with bacteria in mussels by Parisi et al. (2008) showed that dramatically varied proportions of the three cell categories clearly reflected how hyalinocytes participated in antibacterial responses despite being reported as “less active” than granulocytes. It was thus concluded that more than one cell type had been involved in immune defense. Such activation of different cell types as immune effectors corroborates the hypothesis of Ottaviani et al. (1998) that suggested that, in bivalve hemolymph (*M. galloprovincialis*), there is only one hemocyte type – with two or more different maturation (aging)-related stages, that is, hyalinocytes in a proliferative stage mature to become granulocytes (Ottaviani et al. 1998).

In the present study, the reasonable choice to have used AgNP with sizes of <50 and <100 nm was based on the literature on potential uptake pathways for each size particle. Typical clathrin-coated pins (vessels for clathrin-mediated endocytosis) have diameters in the range 120 nm; conversely, internalization via caveolae-mediated endocytosis is considered the predominant mechanism of entry for structures of 40–50 nm (and below)

### Table 1. Correlations of percentage variations in hemocyte sub-populations from mussels exposed for 3 h.

|          | Hyalinocytes | Basophils | Acidophils |
|----------|--------------|-----------|------------|
| Hyalinocytes | 1.0000       | –         | –          |
| Basophils  | 0.4661       | 1.0000    | –          |
| Acidophils | –0.7738***   | –0.9008** | 1.0000     |

**Value significantly correlated at \( p < 0.01. \)**

### Table 2. Correlations of percentage variations in hemocyte sub-populations from mussels exposed for 6 h.

|          | Hyalinocytes | Basophils | Acidophils |
|----------|--------------|-----------|------------|
| Hyalinocytes | 1.0000       | –         | –          |
| Basophils  | 0.7034*      | 1.0000    | –          |
| Acidophils | –0.9511**    | –0.8866** | 1.0000     |

Value significantly correlated at \( *p < 0.05 \) or \( **p < 0.01. \)

### Table 3. Correlations of percentage variations in hemocyte sub-populations from mussels exposed for 12 h.

|          | Hyalinocytes | Basophils | Acidophils |
|----------|--------------|-----------|------------|
| Hyalinocytes | 1.0000       | –         | –          |
| Basophils  | 0.2530       | 1.0000    | –          |
| Acidophils | –0.8243**    | –0.7577** | 1.0000     |

**Value significantly correlated at \( p < 0.01. \)**
in diameter. Thus, while effects on clathrin-mediated endocytosis would reflect how the cells interacted with both size AgNP here, any impact of exposure on caveolae-mediated endocytosis would then be more directly impactful upon the AgNP <50 nm only (Moore 2006; Doherty & McMahon 2009; Khan et al. 2015). This is an important distinction in that these studies did not segregate out the relatively few particles <50 nm from the AgNP100 parent sample so as to provide hypothetical data for AgNP50 versus AgNP51–100. While such analyses would be interesting and informative, the reality is that there is no way in the real world to face such segregated selections from a parent mixture of particles (any type) even if the original cutoff value was set at 100 nm. Further, as the AgNP100 samples only contained 1.4% particles <50 nm, their relative contribution to the observed outcomes for the AgNP100 would be expected to be nominal.

Apparently in keeping with this assumption, an AgNP size-dependent effect variation in the percentages of cell categories was in fact observed here. Other studies also reported size-dependent toxicity of AgNP, that is, with maltose-stabilized AgNP (Katsumiti et al. 2015). In that study, small NP (Ag30-Mal) were significantly more toxic than larger NP (Ag40-Mal and Ag100-Mal). Such outcomes were expected based on a concept proposed by Hine (1999) that posited differences in phagocytosis between granulocytes and hyalinocytes were related to characteristics of the involved particles (i.e. differences in size properties here) rather than differences in immune cell ability to phagocytize/process the particles.

The present study also sought to clarify the role of varying uptake mechanisms for NP (here AgNP) in influencing effects on the frequency of immune cell types. The variations in the percentages of different sub-populations seen here showed that when clathrin- or caveolae-mediated endocytosis was inhibited, effects caused by either size AgNP were delayed. Such results might be due to a potential ability of either uptake route to initially “mitigate” toxic effects of AgNP as each pathway enables any early-internalized particles to be broken-down/digested. While this might reduce initial levels of intracellular AgNP, it conversely increases the presence of the AgNP externally (such as in an actual water environment) to putatively serve as continuous source of Ag ions due to particle oxidation (involving dissolved O₂ and protons in aqueous system) (Dobias & Bernier-Latmani 2013; Gliga et al. 2014; Yu et al. 2014). Over time, the now increasingly present Ag⁺ ions could then impart their own forms of cytotoxicity as was demonstrated in studies by Park et al. (2013) and Katsumiti et al. (2015).

Conclusions

Overall, the results here showed how silver nanoparticles (AgNP) may influence the frequency of different hemocyte sub-populations as biomarker of the immunomodulation of mussel hemocytes by NP. It was clearly noted that nanotoxicity of AgNP was size and indirectly duration of exposure dependent. The internalization mechanism of NP most likely considered as major factor underlying NP effects in hemocytes of _M. galloprovincialis_. Lastly, it is highly recommended further research be undertaken to clarify how specific uptake routes could be involved in determining NP toxicity.

Acknowledgements

This study is funded by the immunomicrobiology, environmental and cancerogenesis IMEC Research Unit, Sciences Faculty of Bizerte, University of Carthage, Tunisia. The authors acknowledge Prof. David Sheehan at the Proteomic Research Group in the School of Biochemistry and Cell Biology at University College Cork (Ireland), for reviewing this paper.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This study is funded by the immunomicrobiology, environmental and cancerogenesis IMEC Research Unit, Sciences Faculty of Bizerte, University of Carthage, Tunisia.

References

Angel B, Batley G, Jarolimek C, Rogers N. 2013. The impact of size on the fate and toxicity of nanoparticulate silver in aquatic systems. Chemosphere. 93:359–365.

Canesi L, Corsi I. 2016. Effects of nanomaterials on marine invertebrates. Sci Total Environ. 565:933–940.

Canesi L, Prochážková P. 2013. Invertebrate immune system as a model for investigating the environmental impact of nanoparticles. In: Boraschi D, Duschl A, editors. Nanoparticles and the Immune System. Oxford: Academic Press; p. 91–112.

Canesi L, Ciacci C, Betti M, Fabbri R, Canonico B, Fantinati A, Marcomini A, Pojana G. 2008. Immunotoxicity of carbon black nanoparticles to blue mussel hemocytes. Environ Int. 34:1114–1119.

Canesi L, Ciacci C, Betti M, Valotto D, Gallo G, Marcomini A, Pojana G. 2010a. In vitro effects of suspensions of selected nanoparticles (C60 fullerene, TiO₂, SiO₂) on _Mytilus_ hemocytes. Aquat. Toxicol. 96:151–158.

Canesi L, Ciacci C, Fabbri R, Marcomini A, Pojana G, Gallo G. 2012. Bivalve mollusks as a unique target group for nanoparticle toxicity. Marine Environ Res. 76:16–21.

Canesi L, Fabbri R, Gallo G, Valotto D, Marcomini A, Pojana G. 2010b. Biomarkers in _Mytilus galloprovincialis_ exposed to suspensions of selected nanoparticles (Nano-carbon black, C60 fullerene, Nano-TiO₂, Nano-SiO₂). Aquat Toxicol. 100:168–177.

Carballal M, Lopez M, Azevedo C, Villalba A. 1997. Hemolymph cell types of the mussel _Mytilus galloprovincialis_. Dis Aquat Org. 29:127–135.

Chang S, Tseng S, Chou H. 2005. Morphological characterization via light and electron microscopy of the hemocytes of two cultured bivalves: A comparison study between hard clam (_Mercenaria mercenaria_) and Pacific oyster (_Crassostrea gigas_). ZooL Studies. 44:144–153.

Cima F. 2010. Microscopy methods for morpho-functional characterization of marine invertebrate hemocytes. In: Méndez-Vilas A, & Díaz J, editors. Microscopy: Science, technology, applications and education. Mexico City: Formatec; p. 1100–1107.

Cozzari M, Ela A, Pacini N, Smith B, Boyle D, Rainbow P, Khan F. 2015. Bioaccumulation and oxidative stress responses measured in the estuarine ragworm (_Nereis diversicolor_) exposed to dissolved, nano- and bulk-sized silver. Environ Pollut. 189:32–40.

Dobias J, Bernier-Latmani R. 2013. Silver release from silver nanoparticles in natural waters. Environ Sci Technol. 47:4140–4146.

Doherty G, McMahon H. 2009. Mechanisms of endocytosis. Annu Rev Biochem. 78:857–902.

dos Santos T, Varella J, Lynch I, Salvati A, Dawson K. 2011. Effects of transport inhibitors on the cellular uptake of carboxylated polystyrene nanoparticles in different cell lines. PLoS One. 6:e24438.

García-García E, Prado-Alvarez M, Novoa B, Figueras A, Rosales C. 2008. Immune responses of mussel hemocyte sub-populations are differentially regulated by enzymes of the PI-3-K, PKC, and ERK kinase families. Develop. Comp Immunol 32:637–653.

Giron-Perez M. 2010. Relationships between innate immunity in bivalve mollusks and environmental pollution. Invert Survival J. 7:149–156.

Gliga A, Skoglund S, Wallinder I, Fadeel B, Karlsson H. 2014. Size-dependent cytotoxicity of silver nanoparticles in human lung cells: The role of cellular uptake, agglomeration and Ag release. Part Fibre Toxicol 11:27.

Godling E, editor. 2003. Bivalve mollusks: Biology, ecology and culture. Oxford: Blackwell Publishing.

Haucke V. 2006. Cargo takes control of endocytosis. Cell. 127:35–37.
