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Research Article

The Influence of Surface Coatings of Silver Nanoparticles on the Bioavailability and Toxicity to *Elliptio complanata* Mussels

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Nanomaterials could be modified with various coatings which could modulate their behavior in the environment, bioavailability and toxicity. The purpose of this study was to determine if the selected coatings of silver nanoparticles (nAg) could influence the fate, bioavailability, and toxicity toward suspension feeding freshwater mussels, *Elliptio complanata*. Mussels were exposed for 96 h to 50 μg/L of nAg with the following surface coatings: citrate, silicate (Si), polyvinylpyrrolidone (PVP), and branched polyethylenimine (bPEI). After the exposure period, mussels were analyzed for total Ag, resistance to air emersion, oxidative stress, genotoxicity, and autophagosome protein uptake (protein ubiquitinylation) in gills and digestive glands. The data revealed that citrate- and PVP-coated nAg were 2 times more abundant in the digestive gland compared to bPEI- and Si-coated nAg with estimated bioaccumulation factors between 5 and 10. The data revealed that tissue Ag levels were closely associated with air survival time, weight loss during air exposure, DNA strand breaks, LPO, and protein-ubiquitin levels in the digestive gland. The data supports the hypothesis that the coatings could influence bioavailability and toxicity in freshwater mussels.

1. Introduction

Silver nanoparticles (nAg) are commercially produced for over a decade to fulfill many applications in our daily lives. Indeed, the main interest in nAg resides in their antimicrobial properties owing to the sustained release of ionic Ag^+, and they are incorporated in many consumer products such as clothes, medical devices, cosmetics, food packaging materials, electronics, and household appliances [1]. The contamination of the environment by the consumption of nAg raised the question about their potential impacts to the environment. For example, Ag was detected in treated municipal wastewaters at a concentration of 70 ng/L with colloidal Ag (nAg) representing circa 5-10% of total Ag [2]. Moreover, most of the nAg were below 100 nm and a secondary treatment process (biological and physicochemical treatments) removed about 75% of the colloidal Ag indicating that a significant amount is still being released in the environment. Hence, the occurrence of nAg in the aquatic environment could raise some concerns through bioaccumulation and toxicity to aquatic organisms. In this respect, mussels and clams represent species at risk to released nanoparticles in the environment given that they have sessile lifestyle, feed on suspended matter, and live for many years depending on the species [3, 4]. Surface modifications of nAg and other nanoparticles are made in the attempt to provide additional properties such optimized persistence and interaction with biological targets such as microorganisms. Hence, the coatings could also influence the bioavailability and toxicity of nAg in addition to the size and form. Indeed, it was found that surface coatings, surface charge, and particle size all contributed to toxicity in *Allium cepa* roots [5]. While ionic Ag was more toxic than nAg of different coatings (citrate, polyvinylpyrrolidone, and cetyltrimethylammonium bromide or CTAB), the highest toxicity was obtained with the positively charged CTAB-coated nAg which was associated with increased bioaccumulation of total Ag in the roots.

The toxicity of Ag and nAg was examined in many previous studies which revealed that nAg cause oxidative stress and protein oxidation, induce metallothioneins, and produce

Table 1: Characterization of silver nanoparticles.

Nanosilver (Nanocomposix)	Coating	Reported[1] size (nm) TEM	DLS	Ag commercial suspension (mg/mL)	Measured[2] hydrodynamic diameter (nm)	Zeta potential (mV)
nAg (AGCN50)	Citrate	51 ± 6	51	0.021	42 ± 3 (Milli-Q) 56 ± 1 (aquarium)	NA −10.4 ± 1.9
nAg (AGSH50)	Silica	Core: 49 ± 6 Shell: 18	101	1.07	87 ± 4 (Milli-Q) 87 ± 1 (aquarium)	NA −13.2 ± 3.0
nAg (AGPN50)	Polyvinylpyrrolidone (PVP)	51 ± 5	71	0.021	60 ± 2 (Milli-Q) 53 ± 3 (aquarium)	53 ± 3 (aquarium) −8.85 ± 1.8
nAg (AGBN50)	Branched polyethylenimine (bPEI)	47 ± 5	66	0.022	41 ± 0.5 (Milli-Q) 355 ± 16 (aquarium)	NA −23.3 ± 1.5

[1]According to the manufacturer's data sheet based on transmittance electron microscopy. [2]Measured by DLS as described in Materials and Methods at 50 μg/L concentration in either Milli-Q or aquarium water.

DNA damage [6–8]. In marine mussels exposed to 1 and 10 μg/L of nAg for 96 h, a significant increase in Ag in whole tissues with a concentration-dependent increase in micronucleus frequency (DNA damage) was observed [6]. Exposure to nAg also led to increased levels in lipid peroxidation (LPO) and metallothioneins suggesting that toxicity was mediated by the release of Ag^+ in part at least. Although the reported effects above could be observed by both ionic Ag and nAg, the nanoparticle produced changes in protein-ubiquitin levels which are involved in the autophagosome-mediated process for the removal of denatured proteins by tagging with ubiquitin [8]. Interestingly, ubiquitin (a 8 kD protein binding to lysine residues on denatured proteins) was also shown to form a corona on the nAg surface which suggests that alterations in these pathways were independent of Ag^+ release [9]. Hence, the toxicity of nAg involves oxidative stress (LPO and antioxidant enzymes such as peroxidases and glutathione S-transferase or GST), protein denaturation, and ubiquitin tagging for autophagosome processing and DNA damage. In another study with gold nanoparticles, the marine clam accumulated gold (Au) in a dose-dependent manner [10]. After 28 days, Au deposits were found in heterolysosomes, where autophagosomes fuse with lysosomes, in the digestive gland cells of clams suggesting again that metallic nanoparticles involve enhanced or altered protein signaling during autophagy. The influence of surface coatings on these pathways is not well understood at the present time as the interaction of ubiquitin at the surface of the various coatings might change. It is generally recognized that negative charges at the surface of the nanoparticle result generally in less toxicity compared to a positively charged surface [5]. In another study, the toxicity of positively coated and negatively coated cerium oxide nanoparticles was examined in *C. elegans* and revealed that mortality and reproductive toxicity were 2 orders of magnitude higher with a positively charge coating (diethylaminoethyl dextran) than with negatively charged (carboxymethyl dextran) and neutral (dextran) coatings [11].

The purpose of this study was therefore to examine the influence of coatings of nAg of the same size on bioavailability and toxicity in the freshwater mussel *Elliptio complanata*. Four different coatings were considered in this study: citrate, silicate (Si), PVP, and branched polyethylenimine (bPEI) coatings. A citrate coating confers a negative charge at the surface while the bPEI coating confers a positive charge at the surface of nAg. The null hypothesis is that the coatings have no bearing on bioavailability and toxicity responses. Toxicity was examined at the oxidative stress, protein denaturation tagging (ubiquitin), and DNA damage levels as described above. Impacts at the health status level were examined by their capacity to survive to air emersion in mussels exposed to the coated nAg.

2. Materials and Methods

2.1. Nanoparticle Handling and Characterization. Stock solutions of silver nanoparticles (nAg) of 4 different coatings were purchased from Nanocomposix Inc. (USA). The mean diameter of the nAg was constant at 50 nm (Table 1), and the following coats were used: citrate, silicate (Si), polyvinylpyrrolidone (PVP), and branched polyethylenimine (bPEI). The coated nAg were prediluted to 1 mg/L in Milli-Q water as this medium minimizes aggregation of particles with low zeta potential. For the exposure regime, *Elliptio complanata* mussels were exposed to a nominal concentration of 50 μg/L total Ag as citrate-, Si-, PVP-, and bPEI-coated nAg in aquarium water for 96 h at 15°C. This concentration was selected based on the information that heavy metal loads in municipal wastewaters are in the order of 10 μg-100 μg/L based on current literature (Gagnon et al., 2006). We selected this concentration as an upper limit given that nAg are increasingly used by our economy. The hydrodynamic diameter and zeta potential of nAg suspensions were analyzed at least three times using a light scattering instrument (Mobius Instrument, Wyatt Technologies, Santa Barbara, CA, USA) operating with a laser at a wavelength of 532 nm. Prior to measurements, the instrument was calibrated using standard suspensions of NIST Traceable Particle Size Standards of monodisperse polystyrene spheres from Bangs Laboratories Inc. (USA).

2.2. Mussel Exposure and Tissue Preparation. Elliptio complanata were collected in pristine lakes in the Laurentians under

a collection permit of the wildlife service of the province of Québec (Canada). Mussels were collected by hand and transported back to the laboratory in iceboxes at 4°C with water-saturated towels. They were placed in 60 L tanks in dechlorinated water and maintained at 15°C under constant aeration. They were fed three times weekly with commercial phytoplankton preparations (Phytoplex, Kent Marin, Franklyn, WI, USA) enriched with *Pseudokirchneriella subcapitata* algal preparations and maintained under these conditions for 4 weeks before initiating exposure. For the exposure experiments, 10 mussels (6-8 cm shell length) were placed in 20 L containers lined with polyethylene bags under constant aeration and exposed to 50 μg/L each of nAg coated with citrate, Si, PVP, and bPEI. Control tanks were also used containing the aquarium water only. The exposure experiment was repeated three times, and mussels were exposed to these conditions for 96 h at 15°C under 16 h light and 8 h dark cycles. The 96 h exposure period was used on the basis of allowing mussels time to accumulate silver nanoparticles and initiate defense mechanisms, and toxicity-given mussels are placed at 15°C. Water samples were taken (10 mL) for Ag analysis by ICP-mass spectrometry (XSERIES 2, ICP-MS, Thermo Fisher Scientific, USA) after overnight acidification with 1% v/v HNO_3 (Seastar Chemical, BC, Canada). The data were expressed as Ag in μg/g wet tissues with reproductivity being <5%.

At the end of the exposure period, mussels were placed in a 20 L clean aquarium overnight to allow depuration for the removal of loosely bound Ag on the fish surface and gut content. The mussels were then processed for biomarker analysis and total Ag in digestive gland and gills. Mussels were placed on ice, dissected for gills, gonads, and the digestive gland, and weighted. The condition index (CI) was determined by mussel weight/shell length (g/mm), the digestive gland index (DGSI) by digestive gland weight/soft tissue weight, and the gonadosomatic index (GSI) by gonad weight/soft tissues. For biomarker determinations, the tissues (half of the digestive gland and one set of gills) were homogenized in 100 mM NaCl, containing 25 mM HEPES-NaOH (pH 7.4), 1 μg/mL apoprotinin (protease inhibitor), 1 mM EDTA, and 1 mM dithiothreitol. The tissues were then homogenized using a Teflon pestle tissue grinder (5 passes) at 4°C, and a portion of the homogenate was decanted and centrifuged at 15000 x g for 20 min at 2°C for the supernatant (S15 fraction). The homogenates and S15 fractions were then stored at -85°C until analysis. Total proteins in the homogenate and S15 fraction were determined using the Coomassie Brilliant Blue dye methodology [12] using serum bovine albumin for calibration.

For total analysis by ICP-mass spectroscopy, the tissues were mixed with 8 mL of concentrated HNO_3, 1 mL of concentrated HCl (Seastar Chemical, BC, Canada), and 2 mL of 30% H_2O_2 (Seastar Chemical, BC, Canada) and adjusted to 12 mL with Milli-Q water. The tissues were then digested for 2 h using a gradient microwave digestion system [13]. Total Ag was then determined by ICP-mass spectrometry as described above. The data were expressed as Ag in μg/g wet tissues.

2.3. Stress on Stress Test. The stress on stress (SOS) test consists in finding the survival time when mussels are exposed to air following the 96 h exposure period to the coated nAg. The SOS test is a global indicator of mussel health and resilience toward air emersion. Air emersion could occur in the wild as the water levels could vary considerably during the warm summer months and low water levels in some rivers. Briefly, 10 mussels (*Elliptio complanata*) were exposed to air in a humidified (80%) incubator at 20°C for up to 60 days. Mussels were checked as regards weight loss and mortality as determined by shell opening twice each day. The weight loss at the time of death (% from initial time) and the number of days to shell opening were determined.

2.4. Biomarker Analyses. The levels of lipid peroxidation (LPO) were determined in the homogenate using the thiobarbituric acid reactant methodology (TBARS) for the fluorescence detection of malonaldehyde [14]. Fluorescence readings were performed at 540 nm and 590 nm for excitation and emission, respectively. Standard solutions of tetramethoxypropane (a stabilized form of malonaldehyde) were prepared for calibration. The data were expressed as μg TBARS/mg proteins. The levels of DNA strand breaks were also determined in the homogenate using the fluorescent alkaline DNA precipitation assay [15, 16]. Briefly, 25 μL of the homogenate from each tissue was mixed with 100 μL of 50 mM NaOH, 10 mM Tris base, 10 mM ethylenediamine tetraacetate, and 2% sodium dodecyl sulphate (SDS). After 5 min, one volume of 0.12 M KCl was added and heated at 60°C for 10 min. The mixture was cooled on ice for 10 min and centrifuged at 8000 x g for 10 min to precipitate SDS-associated protein and genomic DNA. A sample of this mixture was removed and mixed with SYTO Green dye in 3 mM sodium cholate, 0.4 M NaCl, and 100 mM Tris-acetate (pH 8) to control for the traces of SDS in the supernatant which could interfere with the fluorescence readings [17]. Fluorescence was measured at 485 nm excitation and 530 nm emission (Microplate, Synergy 4, BioTek, USA) using standard solutions of salmon sperm DNA for calibration. The data were expressed as μg supernatant DNA/mg proteins. The activity in arachidonate cyclooxygenase (COX) was determined by fluorescence detection of produced H_2O_2 [18]. A 50 μL volume of the S15 fraction was mixed with 150 μL of the assay mixture composed of 50 μM of arachidonate, 2 μM of dichlorofluorescein, and 0.1 μg/mL of horseradish peroxidase in 50 mM of Tris-HCl buffer (pH 8), containing 0.05% Tween 20. The reaction mixture was incubated for 30 min at 30°C, and fluorescence was measured at 485 nm for excitation and 520 nm for emission using a Synergy 4 microplate reader (BioTek Instruments Inc., USA). The data were expressed as the increase in relative fluorescence units/(min × mg proteins).

The activity of glutathione S-transferase (GST) was determined in the S15 fraction using a microplate spectrometric assay [19]. The activity was determined using reduced glutathione and 2,4-dichlorodinitrobenzene as the chromophore substrate at 340 nm. The data were expressed as the increase in absorbance at 340 nm/min/mg total proteins in the S15 fraction. The levels of polyubiquitinylated proteins were determined by the enzyme-linked immunosorbent assay

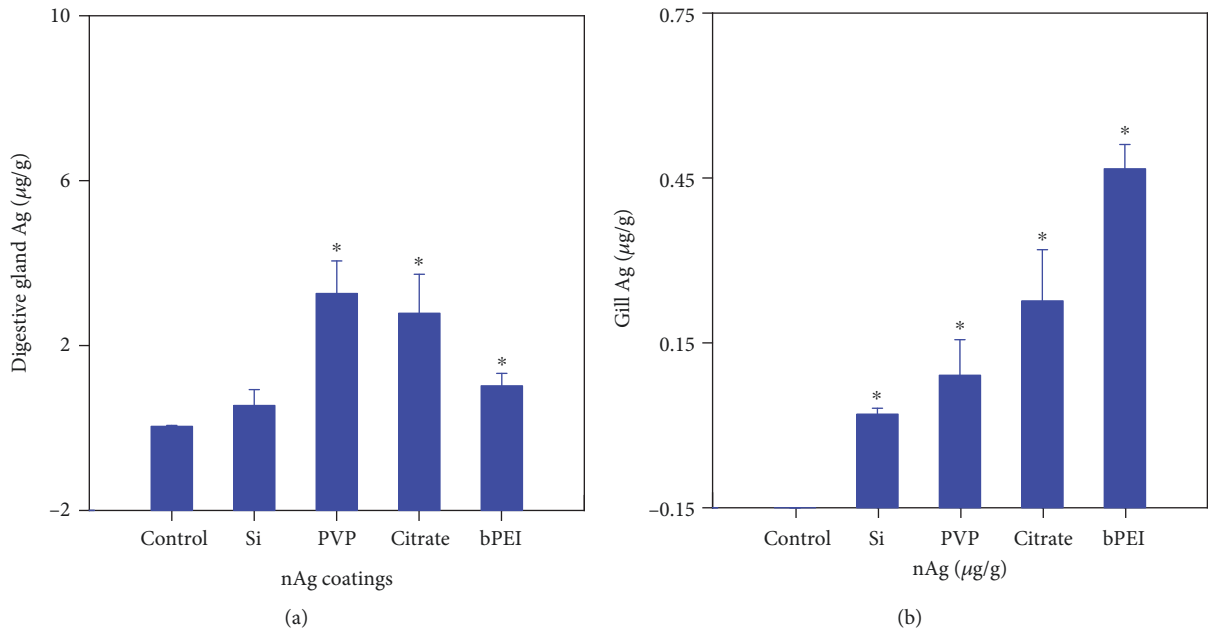

Figure 1: Bioavailability of nAg of different coatings in the digestive gland and gills of freshwater mussels. Mussels were exposed to 4 coatings of nAg for 96 h at 15°C. After the exposure period, mussels were placed overnight to allow depuration (elimination of nonadsorbed materials) from gills, digestive system, and shells. Total Ag was determined in the digestive gland (a) and gills (b). ∗ indicates significance from controls.

(ELISA) in the S15 fraction as described in a previous study [7, 8]. Standards of polyubiquitin (Ub2-7, K48-linked, Enzo Life Sciences, Farmingdale, NY) or the S15 fraction were used to coat the microplate wells (Immulon 4). The ubiquitin lys48-specific rabbit monoclonal antibody (clone Apu2; EMD Millipore, Billerica, USA) was diluted to 1/2000 in phosphate-buffered saline (140 mM NaCl, 5 mM KH_2PO_4, and 1 mM $NaHCO_3$ (pH 7.4)) containing 0.5% albumin and was added to each well. After incubation for 60 min, the wells were washed with 0.5% albumin and the secondary antibody (anti-rabbit Igg linked with peroxidase; ADI-SAB-300, Enzo, USA) diluted to 1/5000 and incubated for another hour. After well washing, the activity of peroxidase was detected using a highly sensitive chemiluminescence assay kit (Roche Diagnostics, QC, Canada). Data were expressed as ng of polyubiquitin/mg proteins.

2.5. Data Analysis. The data were obtained from $N = 8$ mussels per exposure condition, and each tank was run in triplicate. The normality and variance homogeneity were confirmed by Shapiro-Wilk's and Levene's tests, respectively. The data were subjected to an analysis of variance using the Fisher least square difference test to confirm changes in respect to controls (mussels in aquarium water only). Correlation analysis was determined by the Pearson-moment procedure. Cluster analysis of the data was determined by principal component and by discriminant function analyses to determine whether the coatings could be discriminated with each other and the controls. Significance was set at $\alpha = 0.05$ in all cases, and marginal difference was at $0.1 < \alpha < 0.05$. Graphical statistical tests were performed using the SYSTAT software package (version 13.2, USA).

3. Results

In this study, the effects of surface coatings of the nAg were examined on freshwater mussels. The surface coatings consisted of citrate, Si, PVP, and bPEI. Nanoparticles were prepared in both Milli-Q and aquarium waters (Table 1). After dilution in Milli-Q water, the measured sizes of the spherical nanoparticles obtained by dynamic light scattering (DLS) were in the range of the reported sizes of 50 nm. Specifically, for 50 μg/L of nAg, the measured hydrodynamic diameter was as expected for the citrate, PVP, and bPEI coatings. On the other hand, the measured size of the Si-coated nAg was 87 nm in both Milli-Q and aquarium water. For the bPEI-coated nAg, large aggregates formed when the particle was diluted in aquarium water, with an observed average hydrodynamic diameter of 355 nm as compared to 41 nm when measured in Milli-Q water. Particle stabilities were also measured with time (48 h) by measuring both changes in size and zeta potential. For PVP-nAg, citrate-nAg, and Si-nAg, they were no significant changes in either the mean size or zeta potential after 48 h. On the other hand, the mean size of the 50 nm bPEI-nAg increased from 353 ± 16 (1 h) to 411 ± 29 nm (48 h) in the aquarium water. The zeta potential for bPEI-nAg decreased from -12.4 ± 3.9 (1 h) to −23.4 ± 1.5 mV (48 h) during that time.

The bioavailability of nAg was examined in mussel digestive gland and gill tissues (Figures 1(a) and 1(b)). The data revealed that mussels readily accumulated nAg in the digestive gland but marginally so in gills. Indeed, the digestive gland contained circa 25 times Ag than gills on a dry weight basis. For bPEI, the digestive gland contained 4.4 times Ag than gills which indicates higher retention in gill tissues. There was no correlation between the Ag levels in gills and

Table 2: Morphometric data of mussels exposed to coatings of nAg.

nAg coatings	Condition factor[1]	STW ratio[1]	GSI[1]	DGSI[1]
Control	1.26 ± 0.06	0.289 ± 0.01	0.165 ± 0.01	0.085 ± 0.007
Si	1.21 ± 0.05	0.262 ± 0.01	0.161 ± 0.01	0.085 ± 0.002
PVP	1.23 ± 0.09	0.283 ± 0.01	0.169 ± 0.008	0.073 ± 0.004*
Citrate	1.29 ± 007	0.262 ± 0.01	0.183 ± 0.01	0.069 ± 0.005*
bPEI	1.29 ± 0.03	0.289 ± 0.01	0.179 ± 0.009	0.056 ± 0.005*

[1]Condition factor (mussel weight/shell length), soft tissue weight ratio (STW), gonadosomatic index (GSI), and digestive gland somatic index (DGI). *Significant from control ($p < 0.05$).

the digestive gland. Interestingly, the charged coatings bPEI and citrate were more retained in gills in respect to neutral coatings Si and PVP. The influence of coatings based on the levels of Ag in the digestive gland was as follows: citrate > PVP > Si~bPEI. The surface coatings readily influenced the estimated bioaccumulation factor ((concentration in the digestive gland + gills/added concentration in water) × hepatic somatic index) for nAg which was 0.8, 1.5, 5, and 8 for Si, bPEI, PVP, and citrate coatings, respectively.

Changes in morphological parameters (Table 2) and air-time survival (Figure 2) were examined in mussels exposed to coated nAg. There were no significant changes in the condition factor (mussel weight/shell length), soft tissue weight (g tissues/g mussel weight), and gonadosomatic index (GSI; g gonad/g tissues). The digestive gland somatic index (DGSI; g digestive gland/g tissues) was significantly reduced in mussels exposed to citrate- and bPEI-coated nAg after 96 h. There was a marginal decrease in soft tissue weights in mussels exposed to citrate-coated ($p = 0.09$) and Si-coated ($p = 0.1$) nAg. No changes were found for the GSI and the condition factor for the mussels exposed to the different nAg coatings. The effects on air-time survival and weight loss also revealed that the coatings for nAg had no influence on the mussel's sensitivity to emersion to air (Figure 2). The air survival time was significantly correlated with weight loss ($r = -0.75$). The weight loss was significantly correlated with the GSI ($r = 0.40$). The DGSI was significantly correlated with the condition factor ($r = 0.32$) and GST activity in the digestive gland ($r = -0.31$).

The effect of coatings of nAg on inflammation, oxidative stress, and damage was examined in freshwater mussels (Figures 3(a)–3(c)). COX activity was significantly decreased in the gills of mussels exposed to citrate-coated nAg compared to controls (Figure 3(a)). COX activity in the digestive gland was significantly correlated with the condition factor ($r = 0.35$) and COX activity in gills ($r = 0.62$). GST activity, a marker of oxidative stress and conjugation of organics, was significantly increased in the digestive gland of mussels exposed to bPEI-coated nAg and decreased in the digestive gland of mussels exposed to citrate-coated nAg compared to controls (Figure 3(b)). GST activity was significantly correlated with DGSI ($r = 0.31$) and COX activity ($r = 0.51$) in the digestive gland. There were no changes in LPO levels in the gills, but there was a decrease in LPO in the digestive gland of mussels exposed to Si-coated nAg (Figure 3(c)).

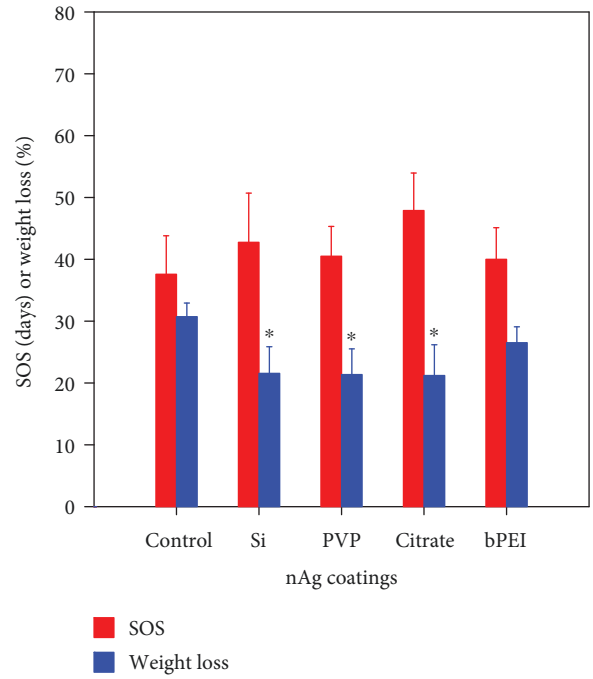

Figure 2: Health effects in mussels exposed to nAg with different coatings. Mussels were exposed to 50 μg/L nAg with 4 coatings (bPEI, citrate, PVP, and citrate) for 96 h. Air-time survival was analyzed by the time of days (SOS) and weight loss. $*$ denotes significant difference in respect to controls.

LPO levels in gills were significantly correlated with DNA damage in gills ($r = 0.53$).

The levels of protein ubiquitinylation and DNA damage were examined in mussels exposed to the various coated nAg (Figures 4(a) and 4(b)). In gills, protein-ubiquitin levels were significantly decreased in mussels exposed to bPEI-, citrate-, and PVP-coated nAg compared to control mussels. In the digestive gland, the levels were significantly decreased in mussels exposed to PVP- and Si-coated nAg compared to control (Figure 4(a)). This suggests that the mechanisms for denatured protein tagging for turnover are decreased in mussels. Protein-ubiquitin levels in gills were correlated with those in the digestive gland ($r = 0.36$). Protein-ubiquitin levels in gills were significantly correlated with DGSI ($r = 0.31$) and COX activity in gills ($r = 0.44$). Protein-ubiquitin levels and COX activity were also significantly correlated in the digestive gland ($r = 0.38$). DNA strand breaks were significantly increased in the digestive gland of mussels exposed to citrate-, PVP-, and Si-coated nAg compared to controls (Figure 4(b)). DNA strand breaks were significantly correlated with the GSI ($r = 0.39$).

In the attempt to gain a global view of the various responses, principal component analysis was performed to explore the relationships between the biomarkers (Figures 5(a) and 5(b)). The analysis revealed that the biomarkers explained 80% of the variance by 3 factors. Tissue Ag levels, which were highly influenced by the surface coatings, were closely associated with air survival time, weight loss, protein-ubiquitin, DNA strand breaks, GST activity, and LPO in the gills. Discriminant function analysis revealed

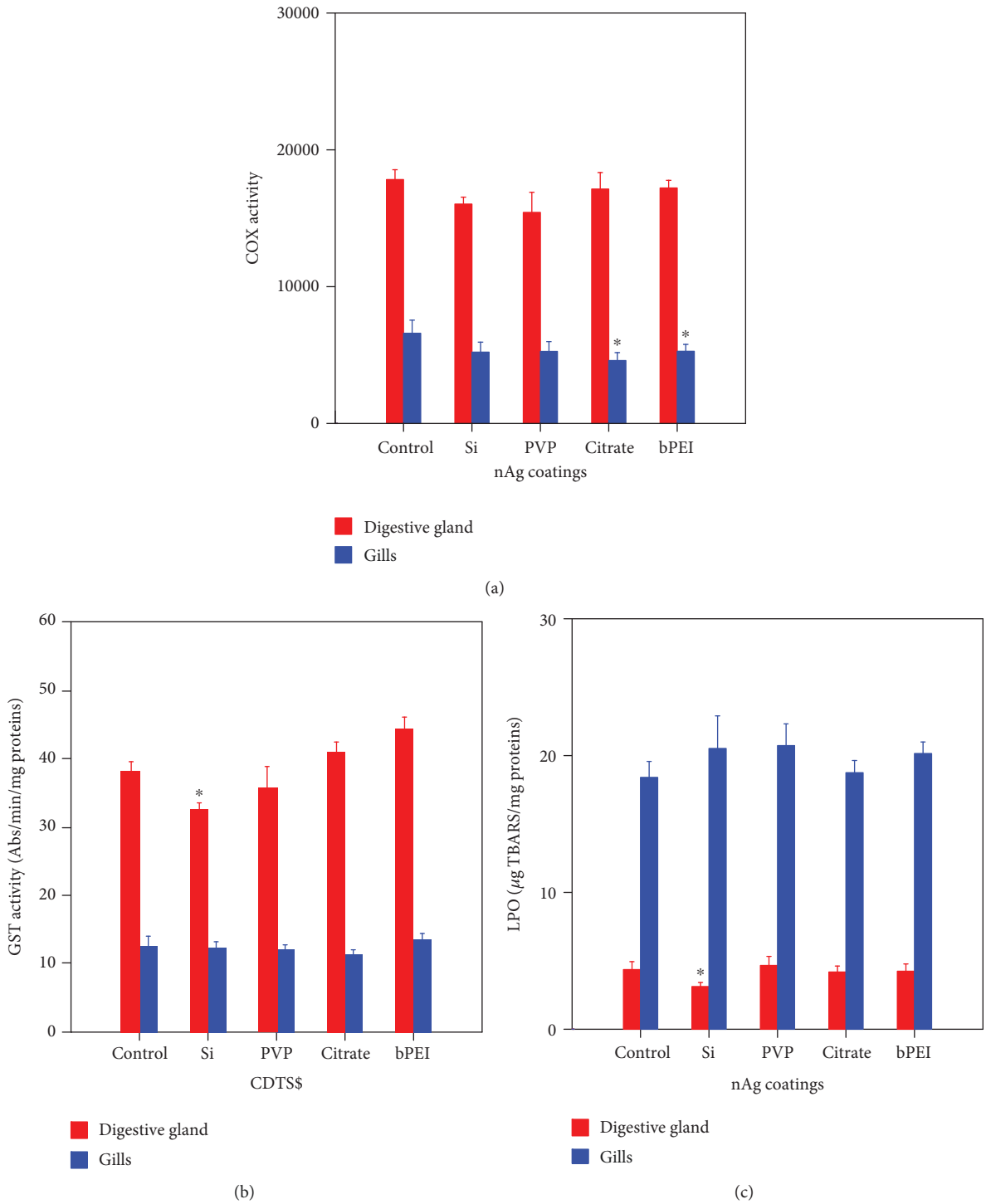

Figure 3: Oxidative stress and damage of coated nAg in mussels. Mussels were exposed to 50 μg/L nAg with different coatings for 96 h. The activities in COX (a) and GST (b) and LPO levels (c) were determined in the digestive gland (DG) and gills. $*$ indicates a significant effect between controls in the digestive gland and gills.

that the control group formed a distinct group from the coated nAg (Figure 5(b)). The mean classification efficiency was at 83%. Citrate- and PVP-coated nAg were closely related and less separated suggesting that they produced similar effects. Citrate- and bPEI-coated nAg formed two well-separated clusters based on the first component (x-axis) which were best explained by protein-ubiquitin levels of the gills and digestive gland and GST activity and DNA damage of the digestive gland.

4. Discussion

Silver nanoparticles were readily bioavailable in the digestive gland of mussels in this study. This is consistent with the

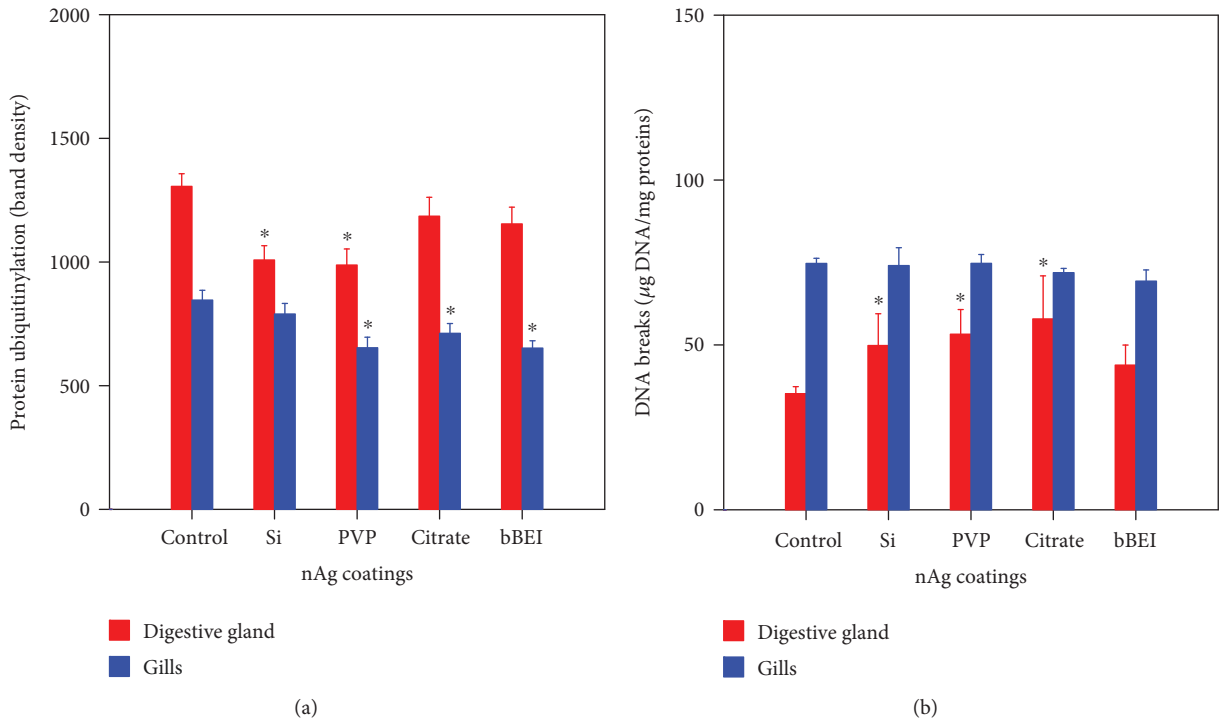

Figure 4: Protein turnover and DNA stand breaks in mussels exposed to nAg with different coatings. Mussels were exposed to 50 μg/L nAg with different coatings for 96 h. The levels of ubiquitin protein tagging (a) and DNA stand breaks (b) were determined in the digestive gland (DG) and gills. $*$ indicates significance from the controls.

particular biological characteristics of this invertebrate such as feeding on suspended particles in the water column, sessility, and longevity that can span decades. The gills are equipped with microvilli acting as a primordial mouth directing suspended matter in the digestive system. Although the nanoparticles would come in contact in gills, they would be quickly directed into the digestive system. It appears that the neutral and negatively charged coatings of nAg do not adhere strongly to gills in respect to the positively charged bPEI coating. Nevertheless, the above function of the gills and digestive system would explain the lack of correlation between gill and digestive gland Ag and the greater accumulation of Ag in the digestive gland. Hence, mussels were deemed to be ideal species to study bioavailability and toxicity of nanoparticles [3, 4]. In this respect, the digestive gland and gills would act as the primary target tissues for nanoparticles where particles trapped in gills are transported to the digestive gland. Based on the estimated bioaccumulation factor, the highest bioaccumulation factor was 8 for citrate-coated nAg of 50 nm diameter. In a previous study with *Elliptio complanata* mussels exposed to citrate-coated nAg, the reported bioaccumulation factors for 20 and 80 nm were 9 and 7, respectively, compared to 20 for dissolved Ag which were all in the same range in the present study [7, 8]. The Si and bPEI coatings were the least bioavailable in the digestive gland of mussels. The Si (Si-OH) coating is neutral while the bPEI coating introduces a positive charge from the imine groups. Although these coatings were less bioavailable, bPEI-coated nAg was reported as more toxic than citrate-coated nAg in *Xenopus laevis* frog embryos [20]. The sublethal effects of the coated nAg were discriminated by denatured protein tagging (ubiquitin), GST activity, and DNA damage. However, bPEI decreased DNA strand breaks in respect to the other coatings which suggests decreased repair activity. In zebra mussels *Dreissena polymorpha* exposed to PVP-coated nAg (50 and 80 nm diameter) and dissolved Ag, the nanoparticles tended to form aggregates but were still found in mussel tissues as with dissolved Ag but between 1.2 and 1.6 times less than the latter [21]. Dietary exposure to PVP/bPEI-(77 : 23) coated nAg (5 nm) was toxic to marine mussel embryos (*Mytilus galloprovincialis*) when fed with contaminated algae of 1 and 10 μg/L nAg [22]. The digestive gland accumulation factor was higher than that in gills and other tissues and ranged between 0.17 and 0.26 which was similar to values shown in the present study.

Exposure of mussels to coated nAg also leads to increased DNA strand breaks in the digestive gland. This suggests that the nanoparticles and their aggregates could proceed in the digestive system. The genotoxicity of nAg was also observed in hemocytes of the marine mussel *Mytilus galloprovincialis* exposed to 10 μg/L nAg for 15 days [23]. This study suggested that genotoxicity was mediated by oxidative stress, but this was not corroborated here since no significant correlations were observed between DNA damage with the biomarkers related to oxidative stress: LPO levels and COX or GST activities. In another study with freshwater mussels, citrate-coated nAg was able to induce DNA strand breaks and protein-ubiquitin levels in the digestive gland in a different manner than ionic Ag which is an oxidant metal [7, 8]. They also found that DNA damage was not entirely related to Ag^{+}-induced oxidative stress because although DNA breaks were correlated with LPO levels, the responses were

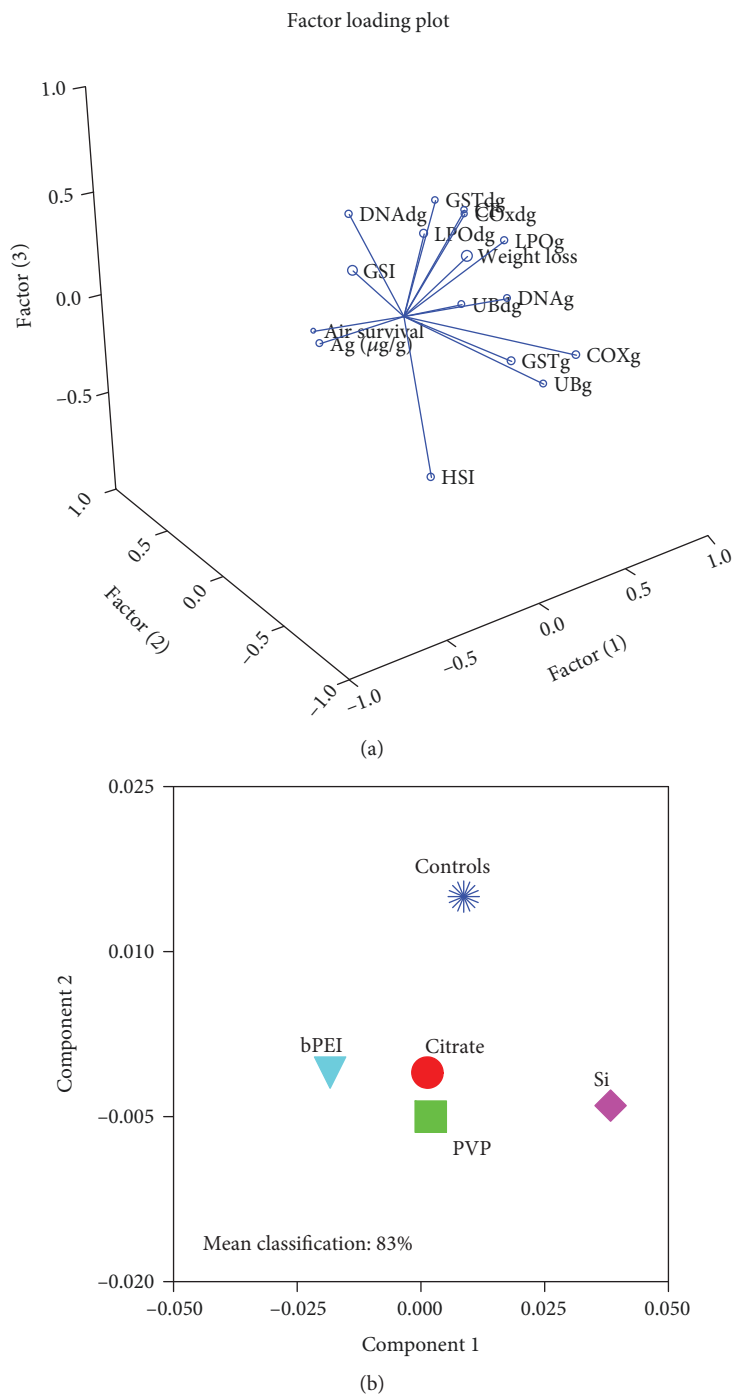

Figure 5: Discriminant function analysis of the biomarker responses. Principal component (a) and discriminant function (b) analyses were performed with biomarker data from mussels exposed to 50 μg/L nAg with 4 different coatings: citrate, PVP, silicate, and bPEI. For principal component analysis, the total variance was explained at 80%. dg: digestive gland; g: gills; GSI: gonadosomatic index; HIS: hepatosomatic index; CF: condition factor. For discriminant function analysis, the mean classification was at 83% and the total explained variance was 80%.

not influenced by induction of metallothionein, a detoxification mechanism for ionic Ag and reactive oxygen radicals. However, protein-ubiquitin levels in the digestive gland were closely associated with the inflammation markers COX and GST activity involved in thiol-mediated radical scavenging activity. This suggests that the various coatings of nAg decreased protein tagging for turnover which suggests that denatured proteins are maintained for longer times in the digestive gland and gills. This effect was seemingly impervious to the various coatings although PVP- and Si-coated nAg were more potent in decreasing protein-ubiquitin levels in the digestive gland. Ubiquitin-proteins and COX and GST activities were also closely related to mussels' condition factor (mussel weight/shell length) and the lower capacity of mussels to survive to air emersion. Furthermore, the response profiles of mussels exposed to citrate- and PVP-coated nAg

were very similar while bPEI- and Si-coated nAg were the least similar based on discriminant function analysis. The difference between the latter involved the DNA damage and GST activity. Given that citrate-coated nAg would release ionic Ag and produce oxidative stress, the different effects observed with bPEI- and Si-coated nAg could be related to mechanisms other than ionic Ag. The effects of citrate-coated nAg on protein carbonylation (oxidation) and ROS activity were observed in mussels and involved the release of ionic Ag, in part at least, and oxidative stress [7, 8, 24]. However, endocytosis pathways and autophagy could also modulate the effects of nAg in cells [25]. It seems that the toxicity of nAg is similar to the toxicity of soluble Ag^+ in *Scrobicularia plana* clams (Buffet et al., 2013). However, the effects on protein ubiquitinylation were related to the nanoparticulate form of Ag in *Elliptio complanata* mussels [7, 8]. Indeed, 20 and 80 nm diameter citrate-coated nAg and ionic Ag were able to increase the levels of metallothionein and LPO which suggests that ionic Ag was able to explain in part the toxicity of nAg. However, the nanoparticles were able to specifically produce changes in protein-ubiquitin levels and to a lesser extent actinomyosin-ATPase activity. Effects other than ionic Ag for nAg were also observed in the digestive gland of marine mussels [26]. Oxidative stress was not observed in the digestive gland of mussels exposed to nAg, but it was present in gills. In the present study, although LPO levels in gills were somewhat higher, the difference was not significant. This suggests that nAg and ionic Ag have distinct but with some overlapping properties [27]. Interestingly, ubiquitin has been shown to form a corona at the surface of nAg which interferes with ubiquitin protein modifications [9]. Ubiquitination is also responsible for the selectivity of autophagy for the clearance of damaged/denatured proteins. Thus, it appears that nAg toxicity is mediated by ubiquitin signaling associated with autophagy. Moreover, cation trapping by vacuole formation was also proposed as a means to the removal of cationic compounds in cells by lysosomes [28]. Cations are pumped into the autophagosome by V-ATPase where amine-coated (cationic) nanoparticles could partition in these vacuoles by a similar process. However, this will require experimental evidence to support this hypothesis.

In conclusion, the surface coatings of nAg could influence bioavailability where citrate- and PVP-coated nAg were more bioavailable in mussels than Si- and bPEI-coated nAg in the digestive gland of mussels. Gills contained about 25 times less Ag although bPEI was more strongly retained in gills compared to the others. Tissue Ag levels were significantly associated with air-survival time, weight loss, protein-ubiquitin, and DNA strand breaks. With the more bioavailable form of nAg (PVP- and citrate-nAg), the DGSI and weight loss during air emersion were reduced and increased, respectively, COX activity was reduced (citrate-coated nAg), protein-ubiquitins were reduced in the digestive gland in mussels, and DNA strand breaks were higher in citrate- and PVP-coated nAg. The coatings on nAg could therefore influence the nanoparticle's availability and the chronic toxicity in freshwater mussels but at a concentration that has yet to be found in municipal wastewaters.

Data Availability

The experimental data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflict of interest regarding the publication of this paper.

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