Article

Eco-Corona vs Protein Corona: Effects of Humic Substances on Corona Formation and Nanoplastic Particle Toxicity in *Daphnia magna*

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ABSTRACT: Despite many studies on the toxicity of nanoplastic particles (NPPs) to aquatic invertebrates, the effects of ecological constituents such as humic substances (HSs) are often neglected. In our study, *Daphnia magna* was used to evaluate the effects of three HSs, natural organic matter (NOM), fulvic acid (FA), and humic acid (HA), on NPP toxicity and corona formation. Acute toxicities of NPPs were reduced by all HSs at environmentally relevant concentrations. NPPs elicited the upregulation of all genes related to detoxification, oxidative stress, and endocrine activity after 7 days of exposure. The presence of NOM or HA resulted in the mitigation of gene expression, whereas significantly higher upregulation of all of the genes was observed with FA. The presence of FA led to increased protein adsorption on NPPs in *D. magna* culture medium (eco-corona, EC) and homogenates (protein corona, PC), while there was less adsorption in the presence of HA. The highly abundant proteins identified in EC are involved in immune defense, cell maintenance, and antipredator response, while those in PC are responsible for lipid transport, antioxidant effects, and estrogen mediation. Our findings revealed the key influence of HSs on the toxicity of NPPs and provide an analytical and conceptual foundation for future study.
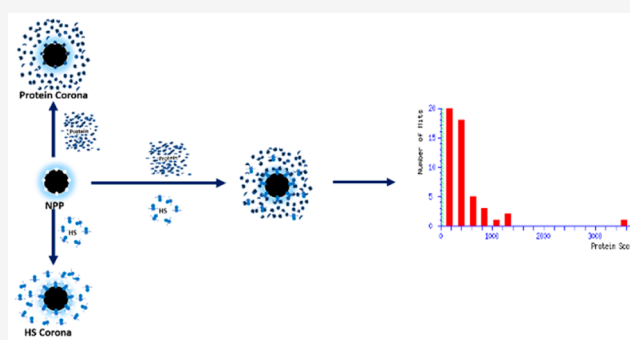
INTRODUCTION

Micro-/nanoplastics (M/NPs) are formed from the degradation of mismanaged plastics or from intentionally produced plastic particles released into the environment. The presence and accumulation of M/NPs in aquatic systems have raised serious concerns due to their deleterious effects on aquatic animals.[1] A number of recent studies have isolated microplastic particles from both aquatic and terrestrial environments, and M/NPs were shown to induce behavioral[2] and physiological changes,[3,4] immunotoxicity,[5] reproductive defects,[6] transgenerational effects,[7] embryotoxicity, and apoptosis.[8,9] Nevertheless, a wide knowledge gap in M/NP interactions with aquatic invertebrate organisms still exists at the molecular level[10] with respect to their toxicity.

In addition to microplastics, another class of prevalent substances present in aquatic ecosystems consists of humic substances (HSs), naturally occurring assemblies of complex and heterogeneous organic compounds of varying molecular weights. HSs originate from a vast range of byproducts of bacterial metabolism and various oxidation processes in the environment.[11,12] Three types of compounds, humic acid (HA), fulvic acid (FA), and humin, have been identified in HSs, according to the International Humic Substances Society (IHSS, 2007).[13] HSs have major influences on not only various environmental processes and organisms[14,15] but also the behavior and properties of M/NPs, such as their colloidal stability, aggregation, reduction, and solubility[16−22] in the aquatic environment. HA and FA are found mainly in aqueous systems, while humin is found in terrestrial systems[13] due to its aqueous insolubility. Structurally, FA has a lower molecular weight, higher oxygen content, and more functional groups, such as carboxyl and hydroxyl groups, than HA, whereas HA contains a higher number of covalently linked phenolic rings. As a result, FA tends to possess a greater negative charge and be more water soluble after the deprotonation of its functional groups,[23] but HA can more easily bind to the hydrophobic surface of nanoparticles (NPs) due to its heterogeneous functional groups and complex-formation ability.[24,25]

When M/NPs encounter organic biomolecules, the latter compete for attachment to the M/NP hydrophobic surfaces,[26] forming what is generally accepted as an eco-corona (EC) or a protein corona (PC) depending on the nature or source of the bound biomolecules. An EC is normally formed when M/NPs

enter an aquatic medium and biomolecules such as proteins adsorb on the particle surface. These biomolecules are primarily the products of aquatic organisms' metabolic activities (e.g., extracellular polymeric substances (EPS)) or the exoproteome (i.e., proteins secreted for signaling and habitat adaptation).[27] Similarly, the PC is formed from adsorbed endogenous proteins. The driving forces of PC formation are hydrophobic and electrostatic interactions,[28−30] which dictate the new chemical nature, bioavailability, transport, degradation, and toxicity of the particles. For instance, corona formation has been reported to reduce the cytotoxic effects of NPs.[26] Additionally, NPs have been identified as protein/peptide aggregation inducers and inhibitors involved in protein conformational diseases such as Alzheimer's disease.[26,31] These biological effects (i.e., the conformational changes in protein) have been used to predict the cellular response of vertebrates to NPs, which has found applications in nanomedicine and human nanosafety.[12] Taken together, these findings indicate a significant relationship between corona formation and NP toxicity.

In a similar vein, we and others recently demonstrated the formation of an HA corona on polystyrene (PS) nanoplastic particles (NPPs)[32,33] that alleviates the toxicity of the NPPs to *D. magna*. Therefore, we aimed to determine and compare the influences of different HSs on the corona formation on NPPs and the toxicity of NPPs to *D. magna*. We hypothesized that HSs compete with secreted proteins during corona formation, altering the toxicity of NPPs to the animal. Additionally, we examined the influence of HSs on PC formation in *D. magna* homogenate, which may reflect the nature and extent of NPP toxicity. To achieve this, we incubated NPPs in *D. magna*-secreted protein or *D. magna* homogenate in both the presence and absence of different HSs. The NPPs were recovered for identification and quantification of the highly abundant proteins adsorbed on the particles. Remarkably, our results show a distinct compositional difference in the corona formed from the *D. magna* eco-medium and that from the *D. magna* homogenate, suggesting different modifications of the NPP behavior around and within the organism as well as a possible mechanism of interaction. Interestingly, most identified high-abundance proteins in both corona formations were found to be toxicologically relevant to the animal's protective mechanism.

MATERIALS AND METHODS

Chemicals and Materials. Natural organic matter (NOM; 2R101N), Suwannee River FA III (3S101F), and Suwannee River HA III (3S101H) standards were purchased from the IHSS (Atlanta, GA, US). Stock solutions (200 mg L^{-1}) of each HS (NOM, FA, and HA) were prepared by shaking on a mechanical shaker overnight and then filtering through a 0.45 μm membrane. Each solution was adjusted to pH 7.6−7.8 and stored at 4 °C in the dark. For corona formation interactions, a 10 mg L^{-1} concentration of each HS was used, which is within the range of humic substances in the environment.[34−38] All HSs were characterized using Fourier transform infrared (FTIR) spectrometer (FT/IR-6100, JASCO Corporation, Tokyo, Japan) (Figure S1 in the Supporting Information). PS NPPs (PS-NH$_2$, 1 g mL^{-1}, spherical surface, nonlabeled) were obtained from Sigma-Aldrich (St. Louis, MO, US) as 1% (w/v) aqueous suspensions. The hydrodynamic diameter and ζ potential of the NPPs in the presence of each HS were determined (Figure S2 in the Supporting Information). The

aqueous NPP suspensions were subjected to ultracentrifugation (Optima L-100 K, Beckman Coulter, Indianapolis, IL, US) for 1 h at 35000 rpm and 4 °C to precipitate the plastic particles. The resulting pellet was resuspended in Milli-Q water, and the process was repeated three times to remove the surfactant and preservatives. The particles were finally resuspended in the culture medium and placed in an ultrasonic bath for 30 min before use. The stock solutions of the particles were all adjusted to 1 g L^{-1} in culture medium and stored at room temperature. The physicochemical characteristics of the particles have been reported elsewhere.[32]

HES01 solution was obtained from Nanjing Health Biological Technology Co. Ltd. (Nanjing, China). Sodium dodecyl sulfate polyacrylamide (SDS) was purchased from Sigma-Aldrich (St. Louis, MO, US). The Coomassie stain InstantBlue was from Expedeon (Cambridgeshire, CB, U.K.). A BCA protein assay kit was obtained from CWBIO (China). Ammonium persulfate (APS) was purchased from MACKLIN (Shanghai, China). Glycerol was obtained from Biotech (China). N,N,N′,N′-Tetramethylethylenediamine (TEMED) was procured from Coolaber (China). GoTaq qPCR master mix was acquired from Promega (Madison, WI, US). TRIzol reagent was purchased from Ambion (Carlsbad, CA, US). Acrylamide/bis(acrylamide) (A/B 29/1), tris(methyl)-aminomethane 1 M Tris-HCl (pH 6.8 and 8.8), Page Ruler Plus prestained protein ladder, SDS sample buffer, lysis buffer, phosphate-buffered saline (PBS), and Tris-glycine running buffer 5× were obtained from Solarbio (Beijing, China). Ethanol, acetone, chloroform, and isopropyl alcohol were of analytical grade unless otherwise specified. All of the solutions were prepared in ultrapure water (resistivity of 18 MΩ cm at 25 °C) from a Millipore Milli-Q system (Merck Millipore, China).

Culture of *Daphnia magna*. A *D. magna* strain obtained from the University of Birmingham, United Kingdom (Bham2), has been cultured in our laboratory for two years (see the Supporting Information for details).

Acute Toxicity Assay (96 h). Fifteen neonates were placed in each beaker, and three beakers were set as a group for each treatment. NPP suspensions of varying concentrations from 1 to 400 mg L^{-1} were prepared and used for the assay. An acute toxicity test in line with OECD guidelines for *Daphnia* was adopted. No food was administered throughout the experimental period (0−96 h). Immobilization and mortality were recorded at 24 h intervals. To investigate the influence of HSs on the toxicity of PS NPPs, the response of *D. magna* to NPPs was recorded in the presence of the three HSs in parallel experiments with the following setup: each HS at 10 mg L^{-1} was introduced into solutions with different concentrations of NPPs (1, 10, 50, 100, 200, and 400 mg L^{-1}).

Preparation of *D. magna* Secreted Protein Extracts and Homogenate. Neonates were collected from multiple adults within <24 h of birth and were separated into a new medium, where they remained for 48 h to obtain sufficient metabolized biomolecules in the medium. During the experiment, no food was introduced to avoid protein or metabolic byproducts from the algae. Likewise, *D. magna* homogenate was obtained from neonates following an established procedure[39] with little modification (see the Supporting Information for details).

Plastic Particle−Protein Interactions. Using the protocol of Docter et al.,[40] NPPs (100 μg mL^{-1}) were incubated separately in crude protein (homogenate) or secreted protein
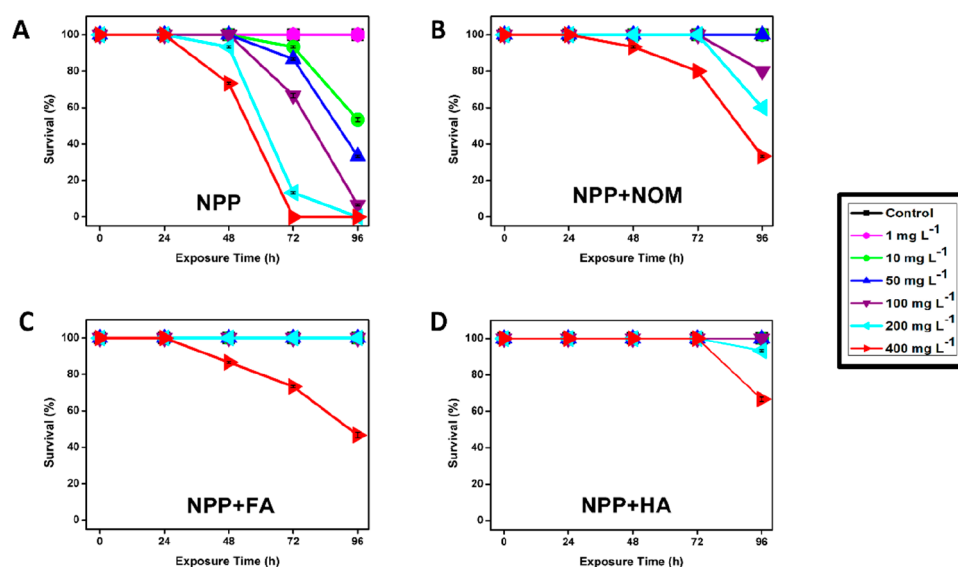
Figure 1. Acute toxicity of NPPs in *D. magna* in the presence or absence of three different types of HSs: NOM, FA, and HA. (A) Percentage of surviving *D. magna* exposed to 1−400 mg L^{-1} PS NPPs for 96 h. The control for (A) was constituted medium without NPPs. (B−D) Percentage of surviving *D. magna* exposed to 1−400 mg L^{-1} NPPs for 0−96 h in the presence of 10 mg L^{-1} of NOM, FA, and HA, respectively. The control group for each of the experimental setups was HS at 10 mg L^{-1}. Data represent the mean of three biological replicates (n = 3). Error bars represent the SD.

(200 μL) from the animals at the same concentration for 24 h in the dark at RT with mechanical shaking. Each HS (10 mg L^{-1} solution) was introduced in a parallel experiment. After incubation, the nanoparticle−protein complexes were recovered by centrifugation at 18000g and 4 °C for 30 min. The precipitated particles were washed with PBS (1 mL), and the recovery procedure was repeated twice. Then, 100 μL of SDS sample buffer was added to the NPP-PC and incubated for 5 min at 95 °C to remove the protein from the NPPs. The mixture was centrifuged to precipitate the NPPs, and the supernatant containing the protein was transferred to a new tube. The protein concentration was determined using the Bradford assay (Figure S3 in the Supporting Information).

Total RNA Isolation and Real-Time Reverse Transcription PCR. Approximately 2400 neonates from a pool of harvested offspring were randomly assigned to 48 groups for exposure assays (see the Supporting Information for details). The primer sequences used for qPCR have previously been reported elsewhere and are shown in Table S1 in the Supporting Information. β-Actin was used as the endogenous control, and gene expression fold changes over the control group were calculated using the $2^{-\Delta\Delta Ct}$ method. All reactions were carried out in three replicates.

SDS-PAGE and Gel Staining. The supernatants obtained from the NPP-PC were mixed with 4× SDS-PAGE sample loading buffer (0.125 M Tris buffer (pH 6.8), 50% (v/v) glycerol, 8% (w/v) SDS, 4% β-mercaptoethanol, and 0.04% (v/v) bromophenol blue). The individual sample mixtures (30 μL) were loaded and separated by SDS-PAGE over a 12−15% gradient using a Mini-Protean Tetra Cell (Bio-Rad, Hercules, CA). Electrophoresis was performed at 4 °C in 5× running buffer (25 mM Tris and 192 mM glycine); the voltage was initially held at 80 V for approximately 1 h and then increased to 100 V for 2.5 h. After electrophoresis, the gels were incubated in InstantBlue staining solution (Expedeon, Cambridgeshire, CB, U.K.) for 20 min or more on a mechanical shaker at 60 rpm for proper visualization, washed

three times with 10 mL of ultrapure Millipore water, and then scanned on a UMAX gel scanner (MagicScan V6.0). The whole process from protein harvesting to documentation of the stained gels was repeated multiple times to ensure reproducibility.

Quantification and Identification of High-Abundance Proteins. The intensity of each protein band was determined using free protein quantification software (GelQuant.NET V 1.8.2) provided by BiochemLabSolutions.com. Protein gel bands of interest were carefully extracted from the InstantBlue-stained gel into a clean tube using a surgical blade, destained, and digested with trypsin. The desalted peptide mixture was loaded onto an Acclaim PepMap C18 reversed-phase column (75 μm × 2 cm, 5 μm, 100 Å, Thermo Scientific) and separated with a reversed-phase C18 column (75 μm × 10 cm, 5 μm, 300 Å, Agela Technologies) mounted onto a Dionex Ultimate 3000 nano LC system. Peptides were eluted using a gradient of 5−80% (v/v) acetonitrile containing 0.1% formic acid over 48 min at a flow rate of 300 nL min^{-1}. The eluates were fed directly into a Q Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), with detection in positive ion mode, data-dependent full MS scanning from m/z 350 to 2000, a full scan resolution of 70000, and an MS/MS scan resolution of 17500. MS/MS scans were performed with a minimum signal threshold of 1 × 10^5 and an isolation width of 2 Da. To evaluate the performance of the two MS/MS acquisition modes, higher-energy collision-induced dissociation (HCD) was employed. To optimize the MS/MS acquisition efficiency of the HCD, the normalized collision energy (NCE) was systemically examined. The generated peak lists were searched against the UniProt database (see the Supporting Information for details) for protein identification.

Statistical Analysis. Statistical analysis was carried out using IBM SPSS 20.0 software. One-way analysis of variance (ANOVA) with an LSD post hoc test was used to evaluate the significant difference between the exposure group and control.

Experimental data are reported as the mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Acute Toxicity Assay. HSs are ubiquitous compounds in the environment that are formed as byproducts of the continuous decomposition of NOM in both aquatic and terrestrial environments.[13] HSs have been shown to interfere with the toxicity of nanomaterials, including NPPs.[41−45] Hence, we investigated and compared the influence of three types of HSs on the toxicity of NPPs to *D. magna* by carrying out a 96 h acute toxicity test with varying concentrations of NPPs (1−400 mg L^{-1}) in the presence and absence of three types of HSs (10 mg L^{-1}), and the percentage of surviving *D. magna* was determined. As shown in Figure 1A, up to 100% mortality of *D. magna* was observed after exposure to 200 and 400 mg L^{-1} NPPs for 96 and 72 h, respectively, whereas no toxicity was observed with 1 mg L^{-1} NPPs throughout the experimental period. The presence of HSs reduced the toxicity of NPPs. After 96 h of exposure, the percentage of surviving *D. magna* increased from 0 to 30% in the 400 mg L^{-1} treatment and 60% in the 200 mg L^{-1} treatment in the presence of NOM (Figure 1B). A similar trend was observed in the presence of HA and FA, but the toxicity was reduced more in the presence of HA and FA than in the presence of NOM (Figure 1C,D), with an overall order for the reduction in toxicity of HA > FA > NOM. HSs at 5 and 50 mg L^{-1} were found to be nontoxic to *D. magna* in a preliminary 21 day chronic toxicity assay (Figure S2 in the Supporting Information).

Similar reductions in the toxicity of NPs to aquatic animals have been previously reported for HSs.[33,41,44,45] HSs are an essential component of aquatic systems, and their effects on NPP toxicity have been attributed to different mechanisms of the reactions, such as adherence to the hydrophobic surface of the NPPs and charge exchange, which modify the surface chemistry of the NPPs.[17,46] This surface modification dictates the subsequent interactions of the NPPs with both the bodily surface and internal organs of animals,[33] thereby reducing the toxic effect of the NPPs and the mortality. The similarity in the toxicological behavior of the tested HSs is not surprising due to similarities in their chemical compositions (Table S2 in the Supporting Information); however, their chemical interactions[47] vary, which may be responsible for the observed variations in their effects on NPP toxicity.

Gene Expression. The genetic response of *D. magna* to NPP exposure at two different concentrations (50 and 100 mg L^{-1}) in the presence and absence of different types of HSs (10 mg L^{-1}) was compared by monitoring the change in the expression of well-known stress response and detoxification genes as well as the endocrine-related genes (*GST*, *CAT*, *P-GP*, *HSP70*, and *VTG*). With 50 mg L^{-1} NPPs (in the presence or absence of any of the HSs), no statistically significant ($p > 0.05$) induction of *GST* expression was observed (Figure 2). However, a significant expression change was observed at 100 mg L^{-1} NPP both with and without FA or NOM. The presence of HA significantly diminished the effect of NPPs. Specifically, 100 mg L^{-1} of NPP (NPP100), 100 mg L^{-1} of NPP plus NOM (NPP100 NOM), and 100 mg L^{-1} of NPP plus FA (NPP100 FA) led to 3.6-, 2.9-, and 17.2-fold changes in *GST* gene expression, respectively. The change in catalase (*CAT*) expression was significant ($p < 0.05$) in only the NPP100 FA group, and a similar trend was observed in the *P-GP* and *VTG* expression. In general, HSs exert an antagonistic
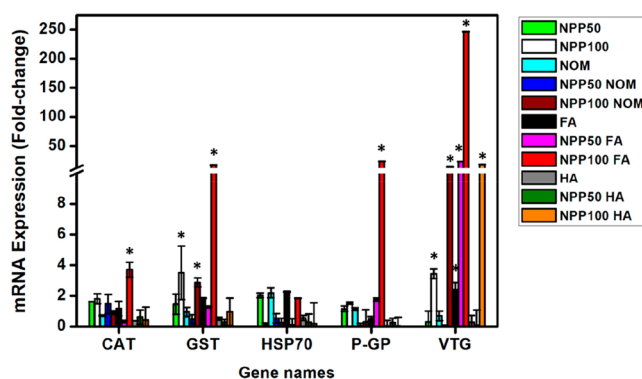

Figure 2. Relative mRNA expression of different *D. magna* genes in response to 50 and 100 mg L^{-1} NPP exposure in the absence and presence of HSs NOM, FA, and HA after 7 days of of exposure. Data are presented as the mean fold change ± SD relative to that in the control group ($n = 3$). *$p < 0.05$ indicates a significant difference in comparison with the control group.

influence on the effects of NPPs on *D. magna* gene expression, which is in accordance with the 96 h acute toxicity results. However, it is noteworthy that, although there was an observed increase in the percentage of surviving animals in the NPP 100 FA group (Figure 1C), all genes were significantly induced (Figure 2). This is in accordance with the observations that FA reduces oxidative damage by increasing the levels of antioxidant enzymes such as SOD, CAT, and GPx.[48] In another aspect, with a higher negative charge than NOM and HA, FA has a better chance of interacting with the positive charge of the animal's protein when it is in contact with the animal. Hence, FA can modulate the immune system,[25] by exerting both proinflammatory and anti-inflammatory effects in animal systems[25] and reducing the release of proinflammatory mediators in cells.[49−51] Therefore, the regulation of the genes observed showed that the alleviating effect of FA on NPP toxicity is minimal in comparison with other HSs, even though there was a reduction in the mortality of the animals.

Corona Isolation and Characterization. Aquatic organisms' continuous exchange of biological materials with their surroundings is key to their survival and evolution.[52] This exchange involves not only their feeding or metabolic waste but also the compounds released to help modify and influence their environment for habitation, such as toxins, antibiotics, and pheromones.[52] Most of these activities are carried out by proteins. Hence, information about the ecological survival tactics of an organism as they relate to the activities within its environment could be determined by evaluating its surrounding biomolecules, including how they interact with NPs. Lynch et al. noted the interactions between HSs/proteins and nanomaterials.[12] Biological molecules either in the environment or within an organism, upon coming into contact with NPs, compete for binding sites on these particles, thereby resulting in corona formation (EC or PC). This in return dictates the composition, uptake, toxicity, and cellular response to the NPs.[26] For instance, NPP interactions with *D. magna* secreted protein were reported by Nasser and Lynch, in which an EC resulted in higher uptake of NPPs and higher toxicity.[27] Hence, we purified the protein coronae formed on NPPs after incubating in the culture medium of *D. magna* (secreted biomolecules or exoproteome[52]), commonly referred to as EC, and within the physiological fluid of *D. magna* (homogenate), called the PC. Both coronae were subjected to a standard
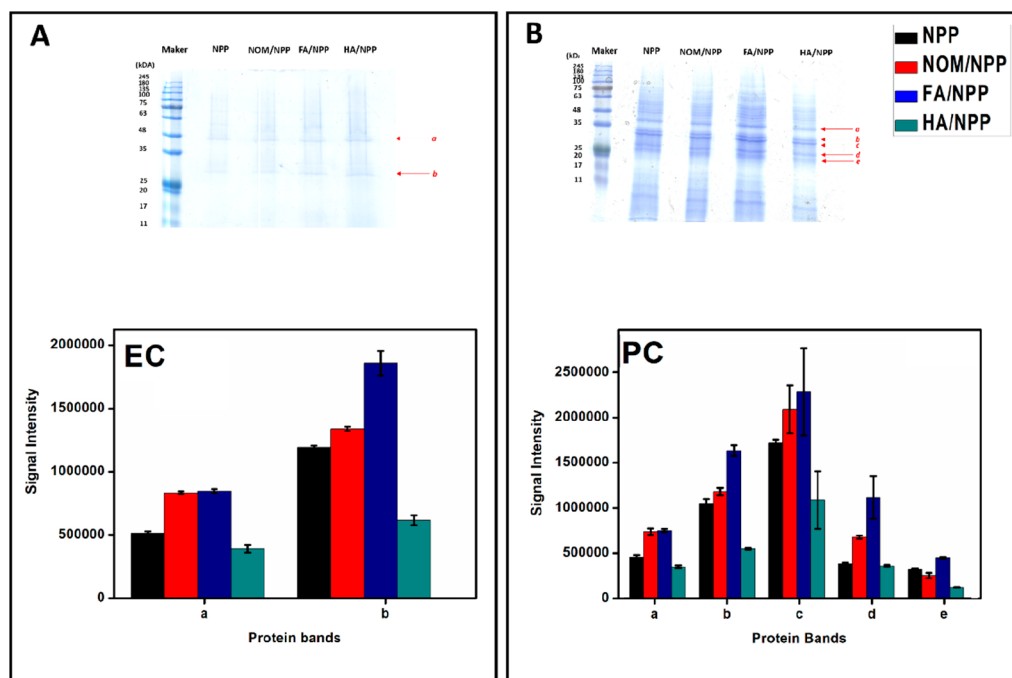
Figure 3. Corona formation on NPPs in the presence and absence of HSs (NOM, FA, and HA). Images of the electrophoresis gels of PC (A) and EC (B), showing the protein band patterns and the abundance (intensity) of selected bands measured with GelQuant.NET (lower panels).
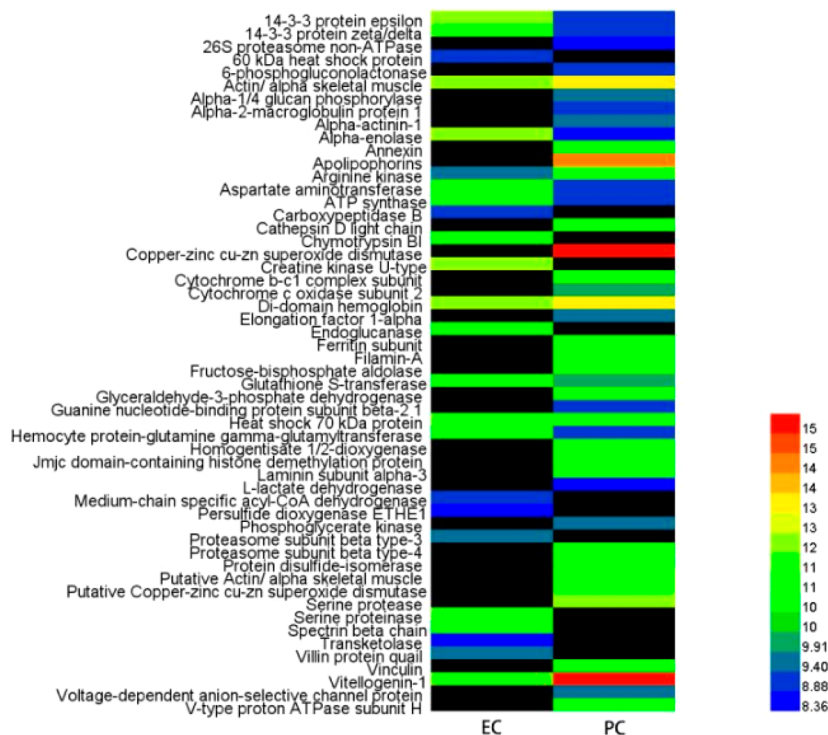

Figure 4. Profile of the most abundant proteins in the EC and PC of *D. magna* (<24 h neonates) medium and homogenates, respectively, on the basis of a score above 300 and at least 30% coverage. The scale represents the intensity of 2^x.

purification process to obtain the hard corona after incubation in the presence and absence of HSs.

SDS-PAGE was employed to reveal the protein composition of the EC and PC formed on the NPPs. After staining the gels, we observed a clear difference in the intensities of the protein bands from both coronae in the presence and absence of the three HSs. Additionally, the patterns of the protein profiles of

EC and PC were distinct. The most abundant protein bands for EC had molecular weights different from those for PC (Figure 3). In addition, the relative distribution of the high-abundance proteins in EC differed substantially from that in PC. This is not surprising, because the protein sources differed (i.e., proteins secreted by *D. magna* and its endogenous proteins). Moreover, protein–particle interactions are an

intrinsically selective mechanism;[53] hence, NPPs exhibited adsorption toward specific proteins in varied proportions in both the presence and absence of HSs. These proteins have been reported in the literature to ultimately trigger biological responses.[54,55] We then quantified selected high-abundance proteins in both coronae (Figure 3, lower panels). The results clearly showed that, for both coronae, the presence of HA reduced the protein binding, whereas the presence of FA or NOM had the opposite effect, indicating a dynamic influence of each HS on the corona formation of NPPs.

Proteomic analysis of both coronae formed in the presence and absence of the three HSs was carried out using LC-MS/MS. By using MASCOT software and a *p* value of 0.05, where *p* is the probability that an observed match is a random event, we identified a total of 1004 proteins, with 281 and 723 unique proteins (Figure 4 inset) for EC and PC, respectively, and 106 common proteins in both coronae. Proteins with an ion score allocation >300 and a percent coverage >30% were designated high-abundance proteins. The unique EC proteins of *D. magna* bound to NPPs are presented in Table S3 and the unique PC proteins in Table S4, along with their gene name, description, accession number, and biological function. The unique proteins accounted for 62.1% and 52.4% of the total proteins identified for EC and PC, respectively.

As shown in Figure 4A for the EC profile, the 14-3-3 protein (epsilon/zeta/delta), the actin alpha skeletal muscle protein, alpha-enolase, didomain hemoglobin, the 70 kDa heat shock protein (HSP70), and vitellogenin-1 were the significant proteins with scores above 1000. Other identified proteins in this category were aspartate aminotransferase and mitochondrial creatine kinase U-type protein. Interestingly, most of the proteins bound to NPPs in *D. magna* medium play protective roles in animals; for example, the 14-3-3 protein (epsilon/zeta/delta) acts as an antioxidant,[56,57] alpha-enolase confers tolerance to hypoxia and heavy metals,[58−60] and didomain hemoglobin enables oxygen binding and transport.[61] The response of *Daphnia* to habitat changes has been attributed to the regulation of its specialized phenotypes by developing an exaggerated morphological defense to evade predation.[62] These adaptive responses to environmental stressors include the diverse regulation and expression of its genes to produce specific sets of proteins for survival and maintenance of cellular activities[63] within the organism as well as its environment. For instance, hemoglobin levels in the hemolymph of daphnids can rise by a factor of 15−20 in response to environmental fluctuations.[62] The adsorption of these functional proteins onto the NPP surface in the *D. magna* culture medium may suggest that NPPs interfere with the normal adaptive mechanism of *D. magna* but are not directly toxic to the animals. For instance, M/NPs have been reported to disrupt quorum sensing in the aquatic luminescent bacterium *Aliivibrio fischeri*.[56] Furthermore, HSP70 is a chaperone involved in protein folding and is either constitutively expressed or induced by stress to preserve protein properties and functions.[57] HSP70 levels have been shown to increase under stress such as exposure to diclofenac,[57] cadmium,[58] and plastic particles.[59] HSP70 was also identified in high proportions on the NPPs in our study, indicating its tendency to detoxify NPPs in the aquatic medium.

Interestingly, Effertz et al. identified vitellogenin (VTG) as a protein involved in *Daphnia*'s response to fish kairomones.[60] Similarly, Trotter et al. demonstrated that the exposure of *Daphnia* to kairomones resulted in delayed primiparity in the animal.[64] To date, no information is available on the function of VTG in the ecological habitat of *D. magna*. We speculate that VTG plays a major role in the antipredation response or immune mechanism of *D. magna* in its environment. It will be interesting to determine how this organism balances its extracellular and intracellular VTG levels. The adsorption of VTG by NPPs in the medium may suggest its toxicological effect in the aquatic environment as a chemical signal disruptor. Hence, we propose that the level of VTG in the experimental medium should also be evaluated, as this can give a more convincing evidence of its role in the detoxification of NPPs in the aquatic system.

Actin alpha skeletal muscle is a cytoskeletal protein involved in the maintenance of cell shape, mobility, intracellular coordination, and transport. This protein has been reported to play a major role in defense against oxidative stress.[59] Since actin alpha protein is a constitutional protein that may be released to an organism's surroundings during molting, no specific ecological role is expected. However, the abundant adsorption of actin by NPPs suggests a high tendency of NPP interactions with the *D. magna* body surface, resulting in higher toxicity to *D. magna* in terms of immobility, than through NPP ingestion, as demonstrated in our previous work.[32] On the other hand, it expressed the possible detoxifying effects of actin on NPP toxicity by corona formation. Importantly, aspartate aminotransferase and creatine kinase U-type are important for the biological functions within an animal, but their environmental functions have not yet been identified. However, their abundance on the surface of NPPs deserves further investigation.

For NPPs to induce toxicity, there must be interactions between the NPPs and cellular components, which are mostly proteins. For aquatic invertebrates, information on PC formation on NPPs is still scant. Here, we employed proteomic techniques to identify proteins that can be modulated by NPPs in the presence or absence of HSs using animal homogenate to simulate the total protein content[39] of *D. magna*. From our results, three proteins (Figure 4) were highly modulated by NPPs in *D. magna* physiological fluids on the basis of their frequency in our proteomic data: apolipophorins, copper−zinc superoxide dismutase (Cu/Zn-SOD), and vitellogenin-1. Apolipophorins are lipoproteins that serve as lipid transporters in *D. magna*. In terrestrial invertebrates, apolipophorins carry out detoxification and the cellular immune response and influence hemocyte adhesion, phagocytosis, nodule formation, and gut immunity.[65] Previous studies on NPP PC with human serum identified lipophorins as one of the most abundant proteins, suggesting the tendency of this protein to act as a carrier of NPPs in organs and across physiological barriers.[53] The presence of lipophorins in NPP corona composition could denote the promotion of detoxification of NPPs. This may be further investigated using an *in vivo* approach. In addition, Cu/Zn-SOD is a representative antioxidant enzyme that is responsible for the conversion of the highly reactive superoxide anion ($O^{2−}$) to oxygen and hydrogen peroxide (H_2O_2) in aerobic organisms.[66] It has been detected in both the intracellular and extracellular spaces of crustaceans, serving a major role as a radical scavenger. The antioxidant defense system in aquatic organisms is crucial in neutralizing the reactive oxygen species (ROS) generated by environmental stressors,[67] including NPPs, which can damage lipids, proteins, and DNA. The interaction between this enzyme and NPPs indicates the capability of NPPs to disrupt *D. magna*'s

antioxidant mechanism and may support previous findings on the upregulation of SOD in *Daphnia* as a response to NPP toxicity.[68] Notably, in the presence of FA, NPPs induced higher expression of *GST* and *CAT* in *D. magna* (Figure 2), further confirming the potential of NPPs to generate oxidative stress in animals via quenching of viable enzymes.

VTG plays an essential role in oocyte and embryo development. The high abundance of VTG suggests the possible influence of NPPs as endocrine disruptors in *D. magna*, altering the molecular mechanism involved in vitellogenesis by binding to the estrogen receptor.[69] This is in line with our result (Figure 2) and some earlier reports that implicated NPPs in embryogenic and reproductive alterations in *D. magna*.[70−72] Moreover, VTG is a lipid transporter, and an increase in lipid droplets has been observed in *Daphnia* during the reproductive phase. Cui et al.[73] observed a significant change in lipid storage in embryos of pregnant *D. galeata* exposed to NPPs, which resulted in an altered hatching rate. These authors also noticed a decrease in the number of lipid droplets both in pregnant and nonpregnant *D. galeata* exposed to NPPs, suggesting a strong relationship between lipid transport and toxicity in *Daphnia*.

Taken together, the results of our study of the EC indicate that the most significant inferences are the secreted proteins adsorbed on the NPPs that were influenced by the HSs, which may disrupt ecological signals but do not directly cause mortality. This indicates a major role of eco-proteins in defense and survival strategies against environmental stressors, including predators and exposure to NPPs in the aquatic environment. On the other hand, PC analysis revealed proteins that may be highly modulated by NPPs in *D. magna*, leading to adverse effects on the animal's internal organs.

In summary, we present the *in vivo* study on the toxicity of NPPs to *D. magna* in the presence and absence of different types of HSs. The HSs ameliorated the toxicity of NPPs to varying degrees depending on their composition and corona formation. Using an *in vitro* approach, we gained insight into the possible mechanisms of NPP toxicity and alleviation in the presence of HSs through corona formation. Through the combination of SDS-PAGE and LC/MS/MS, we revealed the most abundant proteins that may influence the NPP toxicity to *D. magna*, thereby providing an analytical and conceptual framework for a further knowledge-based *in vivo* study. Our observations indicate the importance of considering the influence of other environmental factors, such as pH, salinity, and persistent organic pollutants, on the toxicity of NPPs. The empirical data from such findings, combined with the results of this study, will enhance our knowledge of the environmental risk associated with plastic particle proliferation in the environment.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.0c00615.

Detailed methods, FT-IR spectra of HSs, ζ potential and hydrodynamic diameter plots of NPPs, protein quantification, primer sequences, HS elemental composition, 21 day chronic toxicity study, and lists of EC and PC proteins and their functions (PDF)

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Notes
The authors declare no competing financial interest.

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