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Protein Corona Analysis of Silver Nanoparticles Exposed to Fish Plasma

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Abstract

Nanoparticles (NPs) in contact with biological fluids experience changes in surface chemistry that can impact their biodistribution and downstream physiological impact. One such change involves the formation of a protein corona (PC) on the surface of NPs. Here we present a foundational study on PC formation following the incubation of polyvinylpyrrolidone-coated AgNPs (PVP-AgNPs, 50 nm) in the plasma of smallmouth bass (*Micropterus dolomieu*). PC formation increases with exposure time and is also affected by gender, with AgNPs incubated in male plasma having slightly thinner PCs and less negative zeta potentials than those incubated in female plasma. Proteomic analysis also revealed gender-specific differences in PC composition: in particular, egg-specific proteins (vitellogenin (VTG) and zona pellucida (ZP) were identified only in PCs derived from female plasma, raising the possibility of their roles in AgNP-related reproductive toxicity by promoting their accumulation in developing oocytes.

Introduction

Silver NPs (AgNPs) have been studied extensively as agents of concern in ecotoxicology. In fish populations, numerous studies have shown that AgNPs can cause hatching delays, abnormal larval development, and early mortality in juveniles.^{1–3} AgNPs are known to induce the expression of genes related to metal detoxification and radical scavenging action, and can also activate pro-inflammatory responses to oxidative stress that can result in cellular and DNA damage.^{4, 5} AgNPs are widely distributed throughout the fish body: in addition to the vascular system and alimentary canal, they have been found in the brain, heart, yolk, retina, gill arches, and ovaries.^{1, 6} The toxicological impact of AgNPs on humans and other organisms has not yet been adequately defined by scientific data, due in part to limitations in mechanistic insights. However, it is known that NPs in mammalian serum or plasma nucleate the rapid and dynamic formation of a protein corona (PC), whose “biological identity” can influence their biodistribution and uptake with subsequent effects on cell and organ function.^{7–11} For example, PC composition can promote or deter the

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Supporting Information

Western blot analysis of vitellogenin, UV-vis absorption and NTA data of PVP-AgNPs and PC-AgNPs, additional proteomics analysis including tables of specific proteins identified under specific conditions.

interaction of NPs with outer membrane receptors for specific cell uptake,^{12, 13} or impact their blood circulation lifetime.^{14, 15}

PC formation is governed both by protein–NP and protein–protein interactions. Factors that influence PC composition can be intrinsic (size and topology, surface chemistry, charge density) or extrinsic (protein activities, pH, ionic strength).^{16–18} Exposure time is also important, as the dynamics of surface adsorption and exchange can cause PC size and composition to evolve substantially over relatively short periods. For example, PCs formed on silica and polystyrene NPs of variable size and surface chemistry have been observed to increase in thickness over time, with PC composition remaining roughly constant.¹⁸

Nearly all PC studies are based on NPs exposed to mammalian serum or plasma.^{19–24} Similar phenomena should occur in other vertebrate species such as fish; however to our knowledge no such studies have been reported using fish plasma. Here we provide a foundational study on PCs formed upon exposure of NPs to fish plasma, one that offers valuable insights on how NPs might accumulate in specific organs. Using plasma extracted from male and female smallmouth bass (*Micropterus dolomieu*), we observed PCs on polyvinylpyrrolidone (PVP)-coated AgNPs to undergo time-dependent changes in size and composition. Notably, compositional differences are gender-dependent, with PCs derived from female bass containing significant levels of vitellogenin (VTG) and zona pellucida (ZP), proteins known to be critical for egg development. These novel findings suggest a mechanism for the accumulation of NPs in ovaries and developing eggs, via targeted delivery to follicular cells expressing cognate receptors.

Materials and Methods

Smallmouth Bass Plasma Collection.

Ten adult fish (6 females and 4 males) were collected from the St. Joseph River (Elkhart, IN, USA) during the peak of their spawning season (mid- to late March). Fish were captured uninjured using electrofishing methods. Blood samples (~1 mL) were collected and kept on ice prior to centrifugation (1,000 *g*, 20 min); the resulting plasma was frozen and stored at –80 °C until further processing. Fish were dissected after bleeding for confirmation of gender.²⁵

VTG Analysis.

The presence or absence of VTG in plasma was confirmed by Western blotting (Fig. S1, Supporting Information). The primary antibody used was a polyclonal anti-VTG from Biosense (Bergen, Norway), imaged by a secondary antibody labeled with IRDye 700 (Li-Cor, Lincoln, NE, USA). Vascular endothelial growth factor (VEGF) was used as a reference protein, as it is expressed at steady levels in fish plasma, and detected using a polyclonal antibody from Anaspec (Fremont, CA, USA) and a secondary antibody labeled with IRDye 800 (Li-Cor). Plasma samples were pooled by gender, as VTG was found only in female plasma.

Characterization of PVP-AgNPs and Their Protein Coronae.

50-nm PVP-AgNPs (Nanocomposix, San Diego, CA, USA) were used as provided, and incubated with either female or male bass plasma (NP:protein weight ratio = 1:500) for 1 h or 24 h. Untreated PVP-AgNPs and fish plasma without PVP-AgNPs were included as controls. Dispersion stability was characterized by ultraviolet–visible (UV-Vis) spectroscopy using a Cary-50 spectrophotometer (Varian, Palo Alto, CA, USA). Nanoparticle tracking analysis (NTA) was performed at 25 °C using a Nanosight LM-10 (Malvern Instruments, Marlborough, MA, USA) to quantify particle size distribution. Hydrodynamic size and zeta potentials were measured at 25 °C using a Zetasizer NanoZS (Malvern).

Isolation and SDS-PAGE Analysis of PC-coated NPs.

PVP-AgNPs (1 µg/mL) coated with fish plasma proteins (1 µg/mL) were incubated at 30 °C for 1 h or 24 h, then collected by centrifugation (15,000 *g*, 20 min at 4 °C). Solid pellets were separated from free plasma, then redispersed in fresh solution and digested following a published protocol.²⁶ Aliquots were eluted by 12% SDS-PAGE and visualized with a silver stain kit (Thermo Fisher Scientific, Rockford, IL, USA).

Liquid Chromatography–Mass Spectrometry Analysis.

Protein corona pellets were digested for LC-MS/MS analysis,²⁷ using a Dionex UltiMate 3000 RSLC Nano System coupled to a Q Exactive™ HF Hybrid Quadrupole-Orbitrap MS (Thermo Scientific, Waltham, MA, USA). A database search of non-redundant proteins from chordates (NCBI) was performed using the Mascot MS/MS Ion Server (Matrix Science, Boston, MA, USA). Relative mass fractions of proteins were estimated with the exponentially modified protein abundance index;²⁸ additional details are provided in Supporting Information.

Statistical Analysis.

All statistical analyses were conducted using SPSS 22.0. Oneway analysis of variance (ANOVA) followed by post-hoc Tukey's multiple comparison tests were used to compare means across treatments.

Results and Discussion

UV-vis analysis of PVP-AgNPs indicates an absorption peak at 430 nm, with a 10-nm redshift upon PC formation (Figure S2, Supporting Information). A 5-nm increase in hydrodynamic size is observed after the first hour of incubation with fish plasma, with further increase (2–3 nm) after a 24-hour period (Figure 1). In addition, the zeta potentials for PC-coated AgNPs are less negative after the first hour of incubation, and remain essentially the same after 24 hours. The changes in size and zeta potential are both expected: with regard to the latter, protein adsorption onto negatively charged NPs has been observed previously to reduce zeta potentials.^{18, 29, 30} It should be mentioned that incubation of PVP-AgNPs in fish plasma resulted in some agglomeration and colloidal instability, especially for AgNPs incubated in male plasma for 24 h. This is again not surprising, as the kinetic destabilization of imperfectly passivated metal colloids is well known. Nevertheless, NTA

indicates the great majority of AgNPs to be stable, with an overall size increase due to PC formation (Figure S3, Supporting Information).

We also observe a significant, gender-based difference in PC formation. Specifically, PVP-AgNPs added to male plasma exhibit a larger change in zeta potential relative to those added to female plasma ($p < 0.05$), whereas differences in hydrodynamic size are less significant. This observation is in contrast to the only other study on gender-related effects in PC formation using 20-nm AgNPs exposed to human plasma, which reported minimal physical or biochemical differences.³⁰ We attribute the gender-related differential in zeta potentials to variations in PC composition, to be discussed in the sections below.

SDS-PAGE analysis provides additional evidence that PC composition is both gender-specific and time-dependent (Figure 2). PCs derived from male fish plasma (Lanes 6 and 7) contain significant quantities of smaller proteins (< 25 kDa), whereas those from female plasma (Lanes 3 and 4) are at or below the limit of detection. The profiles of corona proteins from either gender are also quite different than those in the parent fish plasma (Lanes 2 and 5), indicating that PC formation is an innately selective process.

LC-MS/MS data indicate PCs derived from fish plasma to contain a higher fraction of low molecular-weight proteins (< 25 kDa; $> 50\%$ by mass) and a lower fraction of high molecular-weight proteins (> 100 kDa; $< 10\%$ by mass) relative to bulk plasma (Figure S4, Supporting Information). In particular, PCs derived from female fish plasma carry an especially large fraction of proteins below 20 kDa, which increased to over 50% by mass after 24 hours of incubation. The protein sizes and distributions in these PCs are within a similar range as those reported in studies involving citrate- and PVP-coated AgNPs (20 nm) in human plasma, in which the majority of the proteins were under 60 kDa.³⁰ It is worth noting that PC composition is also influenced by the chemistry of the core NP: for example, PCs formed on polystyrene NPs contain mostly proteins in the 60–70 kDa range, whereas PCs formed on silica NPs exposed to the same plasma source contain much larger proteins (150–200 kDa).¹⁸

A total of 337 proteins were identified in PC-coated AgNPs by LC/MS-MS proteomic analysis (Figure S5, Supporting Information). For PCs derived from female fish plasma, 135 and 147 proteins were identified from AgNPs incubated for 1 h and 24 h respectively; for those derived from male plasma, 194 and 193 proteins were identified. Less than 18% (60 proteins) were common to all PCs, and less than 40% (128 proteins) were shared between genders. These values are much lower compared to PCs formed on 20-nm AgNPs in human plasma, which shared 70% of all proteins between genders.³⁰ In PCs derived from female fish plasma, roughly two-thirds of the proteins (89/135) are common to both 1-h and 24-h incubation samples, while the number of proteins unique to either condition is relatively low ($n = 19$ or 26, respectively). On the other hand, while more than half of the proteins in male-derived PCs (109/193) are found in both 1-h and 24-h incubation conditions, a surprisingly large number of proteins are unique to a given sample ($n = 67$ and 64, respectively), an interesting finding that warrants further investigation.

The relative distribution of proteins in PCs derived from female and male fish plasma can be broken down according to their primary functions (Figure 3; for a complete list, see Tables S1 and S2). The most abundant proteins are those associated with the immune system (immunoglobulins, complement proteins), followed by those for vascular and oxygen transport (hemoglobin, plasminogen, fibrinogen/fibronectin). Lipid transport proteins (lipoproteins and apolipoproteins) were also present but to a lesser extent; high-density lipoproteins (HDL, ApoA) were associated with PVP-AgNPs regardless of gender or length of incubation. Low-density lipoproteins (LDL) and two apolipoproteins (ApoB-100, ApoE) were also found in all PCs, similar to those derived from human plasma, where they are thought to be involved in the movement of NPs into organs and across the blood–brain barrier.¹⁶

Several other proteins were identified in significant quantities within PCs, some at much higher concentrations relative to the bulk plasma (Figure 3b). Ceruloplasmin and plasminogen are particularly noteworthy; other metal-ion regulators such as Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) and transferrin are also present. Acute-phase proteins associated with the inflammatory response are also represented within the PCs, including amyloid A, antitrypsin, kallikrein, kininogen, and vitamin K-dependent protein (Tables S1 and S2, Supporting Information). Notably, many plasma proteins are incorporated preferentially into PCs while others appear to be excluded; for example, parvalbumin, lipid transport proteins and macroglobulin are present at much lower levels relative to bulk plasma, and angiotensinogen (a blood pressure regulator) is hardly present at all, especially during the early stages of PC formation (Figures 3 and S6, Supporting Information).

The most significant finding in this study is the incorporation of VTG and ZP in PCs derived from female fish plasma. VTG, a precursor to egg yolk that play critical roles on oogenesis, is synthesized in the liver and transported to the ovaries via the bloodstream.³¹ VTG and ZP are produced at elevated levels by female smallmouth bass during the spawning season, and are taken up by developing follicles within fish ovaries by receptor-mediated endocytosis (Figure 4).^{32, 33} These egg-specific proteins are incorporated at an early stage of PC formation but their levels diminish after a 24-hour incubation, which suggests that they reside in the outer “soft” corona layer, and are thus readily presented to follicular cells expressing their cognate receptors.

Previous research has shown that AgNPs are able to accumulate in fish ovaries, which can lead to abnormal follicular development with subsequent loss of fecundity and reproductive capacity.^{34,35,36} Early exposure of fish eggs to AgNPs or silver ions can also result in the defective development of embryos and larvae, resulting in their decreased survival.^{37–40} Given the rapid inclusion of VTG and ZP from female fish plasma into PCs, we postulate that these egg-specific proteins can promote translocation of AgNPs to the ovaries.

In summary, the PC around AgNPs exposed to fish plasma offers a rich source of information on the physiological condition of the host species. Unlike studies involving mammalian sera, gender plays an important role on PC composition, with significant differences in zeta potential, diversity and relative proportions of the constituent proteins, and the incorporation of gender-specific protein markers. The latter may be important in

directing circulating NPs to specific organs and tissues, and promoting their uptake via cell-surface receptors. In particular, the inclusion of VTG and ZP into the PCs of AgNPs in the bloodstream of female fish may provide a mechanism for accelerating their movement to ovaries and developing eggs. Experiments to confirm this hypothesis will be performed in due course.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Changes in the hydrodynamic size and zeta potential (mode $\pm$ standard error; $N = 3$) of PVP-AgNPs, before and after incubation with fish plasma extracted from adult female (F) or male (M) smallmouth bass for 1 h or 24 h. Untreated PVP-AgNPs were measured at $t = 1$ h and 24 h to determine changes in size and zeta potential over time. Gender-related differences in zeta potential after protein corona (PC) formation were also established (*, $p < 0.05$).

Figure 2.

SDS-PAGE gel showing elution of proteins from protein coronas (PCs) formed after incubation of PVP-AgNPs with plasma from adult female (F) or female (M) smallmouth bass, for 1 h or 24 h. Compositional differences can be correlated with both gender differences and incubation time. Significant levels of smaller proteins (< 25 kDa) were found in PCs isolated from male plasma (black arrows). A decrease in the relative abundance of mid-sized proteins (50–80 kDa) was observed in PCs formed after 24-h incubation in female plasma. Original female and male plasma are included for comparison.

Figure 3.

Protein corona (PC) compositions with relative abundance of proteins by class, derived from PVP-coated AgNPs exposed to adult female (F) or male (M) fish plasma for 1 h or 24 h; composition of bulk plasma shown for comparison. (A) Proteins of highest percentage within PCs. (B) Other significant proteins found in PC layer. CaMKII: Calcium/ calmodulin-dependent protein kinase; VTG: Vitellogenin; ZP: Zona pellucida.

Figure 4.

Distribution and transport of silver nanoparticles (AgNPs) coated with plasma proteins (protein corona). AgNPs labeled with complement proteins and other opsonins are taken up into macrophages by phagocytosis, whereas those labeled with egg-specific proteins such as vitellogenin (VTG) may be transported into developing follicles within the ovaries by cognate receptors.