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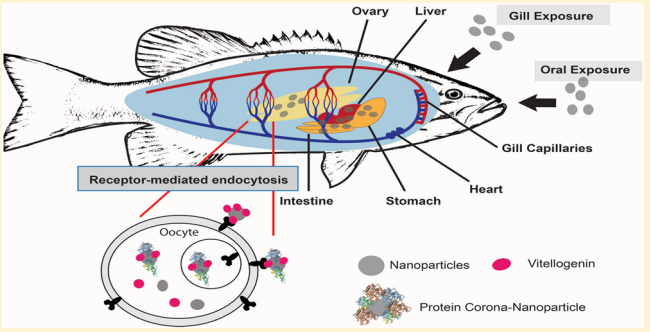
Protein Corona Analysis of Silver Nanoparticles Exposed to Fish Plasma

Jiejun Gao,[†] Lu Lin,[‡] Alexander Wei,*[,‡] and Maria S. Sepúlveda*[,†]

[†]Department of Forestry and Natural Resources and Bindley Biosciences Center and [‡]Department of Chemistry, Purdue University, West Lafayette, Indiana 47907, United States

Supporting Information

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 of PC formation following the incubation of polyvinylpyrrolidone-coated AgNPs (PVP-AgNPs, 50 nm) in the plasma of smallmouth bass (*Micropterus dolomieu*). The level of PC formation increases with exposure time and is also affected by gender, with AgNPs incubated in male plasma having PCs slightly thinner than and ζ potentials less negative than those of AgNPs incubated in female plasma. Proteomic analysis also revealed gender-specific differences in PC composition: in particular, egg-specific proteins (vitellogenin and zona pellucida) were identified in only 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 level of 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, in part because of 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.[7−10] For example, the PC composition can promote or deter the interaction of NPs with outer membrane receptors for specific cell uptake[11,12] or impact their blood circulation lifetime.[13,14]

PC formation is governed by both protein−NP and protein−protein interactions. Factors that influence PC composition can be intrinsic (size and topology, surface chemistry, and charge density) or extrinsic (protein activities, pH, and ionic strength).[15−17] Exposure time is also important, as the dynamics of surface adsorption and exchange can cause the PC size and composition to evolve substantially over relatively short periods. For example, the thickness of PCs formed on silica and polystyrene NPs of variable size and surface chemistry has been observed to increase over time, with the PC composition remaining roughly constant.[17]

Nearly all PC studies are based on NPs exposed to mammalian serum or plasma.[18−23] Similar phenomena should occur in other vertebrate species such as fish; however, studies involving fish plasma are just now emerging.[33] Here we provide a foundational study of PCs formed upon exposure of NPs to plasma extracted from male and female smallmouth bass (*Micropterus dolomieu*), one that offers valuable insights into how NPs might accumulate in specific organs. 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.

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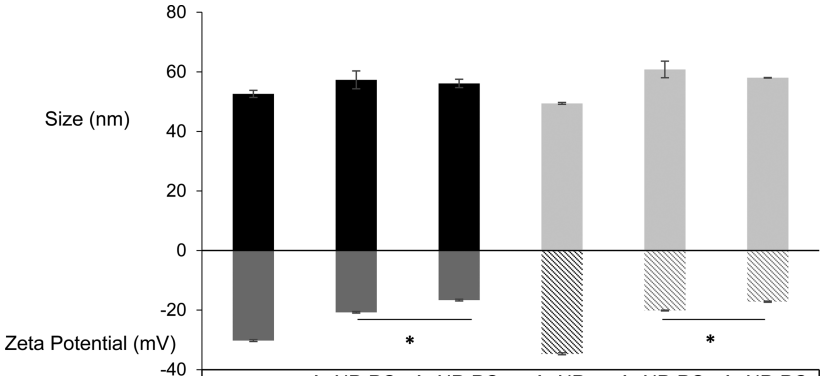

	AgNP, 1h	AgNP-PC-F, 1h	AgNP-PC-M, 1h	AgNP, 24h	AgNP-PC-F, 24h	AgNP-PC-M, 24h
Zeta Potential (mV)	-30.2	-20.7	-16.6	-34.7	-20.1	-17.2
Size (nm)	52.6	57.3	56.1	49.4	60.8	58

Figure 1. Changes in the hydrodynamic size and ζ 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 or 24 h. Untreated PVP-AgNPs were measured at 1 and 24 h to determine changes in size and ζ potential over time. Gender-related differences in ζ potential after protein corona (PC) formation were also established ($*p < 0.05$).

MATERIALS AND METHODS

Smallmouth Bass Plasma Collection. Ten adult fish (six females and four males) were collected from the St. Joseph River (Elkhart, IN) during the peak of their spawning season (middle to late March). Fish were captured uninjured using electrofishing methods. Blood samples (~1 mL) were collected and kept on ice prior to centrifugation (1000*g* for 20 min); the resulting plasma was frozen and stored at −80 °C until it could be further processed. Fish were dissected after bleeding for confirmation of gender.[24]

VTG Analysis. The presence or absence of VTG in plasma was confirmed by Western blotting (Figure S1). The primary antibody used was a polyclonal anti-VTG antibody from Biosense (Bergen, Norway), imaged by a secondary antibody labeled with IRDye 700 (Li-Cor, Lincoln, NE). 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) and a secondary antibody labeled with IRDye 800 (Li-Cor). Plasma samples were pooled by gender, as VTG was found in only female plasma.

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

Isolation and Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (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 or 24 h and then collected by centrifugation (15000*g* for 20 min at 4 °C). Solid pellets were separated from free plasma, then redispersed in a fresh solution, and digested following a published protocol.[25] Aliquots were eluted by 12% SDS−PAGE and visualized with a silver stain kit (Thermo Fisher Scientific, Rockford, IL).

Liquid Chromatography−Mass Spectrometry (LC−MS) Analysis. Protein corona pellets were digested for LC−MS/MS analysis,[26] using a Dionex UltiMate 3000 RSLC Nano System coupled to a Q Exactive HF Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific, Waltham, MA). A database search of nonredundant proteins from chordates (NCBI) was performed using the Mascot MS/MS Ion Server (Matrix Science, Boston, MA). Relative mass fractions of proteins were estimated with the exponentially modified protein abundance index;[27] additional details are provided in the Supporting Information.

Statistical Analysis. All statistical analyses were conducted using SPSS 22.0. One-way analysis of variance (ANOVA) followed by post hoc Tukey's multiple-comparison tests was 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 red-shift upon PC formation (Figure S2). A 5 nm increase in hydrodynamic size is observed after the first hour of incubation with fish plasma, with a further increase (2−3 nm) after a 24 h period (Figure 1). In addition, the ζ potentials for PC-coated AgNPs are less negative after the first hour of incubation and remain essentially the same after 24 h. The changes in size and ζ potential are both expected: with regard to the latter, adsorption of protein to negatively charged NPs has been observed previously to reduce ζ potentials.[17,28,29] It is well-known that the early adsorption of proteins to NP surfaces is kinetically driven and often dominated by hydrophobic species such as apolipoproteins (see below); however, the population of these high-abundance species declines as they are replaced with proteins with lower abundance but higher affinity for the NP surface or components in the inner "hard" corona layer.[8−10] 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-

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

We also observe a significant, gender-based difference in PC formation. Specifically, PVP-AgNPs added to male plasma exhibit a larger change in ζ 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 of gender-related effects in PC formation using 20 nm AgNPs exposed to human plasma, which reported minimal physical or biochemical differences.[29] We attribute the gender-related differential in ζ potentials to variations in PC composition, which will be discussed below.

SDS−PAGE analysis provides additional evidence that PC composition is both gender-specific and time-dependent (Figure 2 and Figure S7). PCs derived from male fish plasma

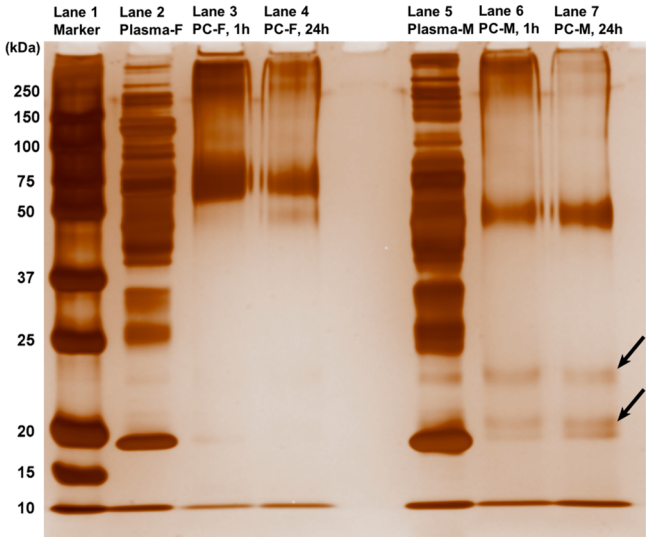

Figure 2. SDS−PAGE gel showing elution of proteins from protein coronas (PCs) formed after incubation of PVP-AgNPs with plasma from adult female (F) or male (M) smallmouth bass, for 1 or 24 h. Compositional differences can be correlated with both gender differences and incubation time. Significantly higher levels of smaller proteins (<25 kDa) were found in PCs isolated from male plasma (black arrows; see Figure S7 for a more heavily stained image). A decrease in the relative abundance of midsized proteins (50−80 kDa) was observed in PCs formed after incubation for 24 h in female plasma. Original female plasma and male plasma are included for comparison.

(lanes 6 and 7) contain significantly higher quantities of smaller proteins (<25 kDa), relative to those from female plasma. 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 contain a larger fraction of low-molecular weight proteins (≤25 kDa, >50% by mass) and a smaller fraction of high-molecular weight proteins (≥100 kDa, <10% by mass) relative to those in bulk plasma (Figure S4). In particular, PCs derived from female fish plasma carry an especially large fraction of <20 kDa proteins, which increased to >50% by mass after incubation for 24 h. The protein sizes and distributions in these PCs are in a range similar to those reported in studies involving citrate- and PVP-coated AgNPs (20 nm) in human plasma, in which the majority of the proteins were <60 kDa.[29] It is worth noting that the 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).[17]

A total of 337 proteins were identified in PC-coated AgNPs by LC−MS/MS proteomic analysis (Figure S5). For PCs derived from female fish plasma, 135 and 147 proteins were identified from AgNPs incubated for 1 and 24 h respectively; for those derived from male plasma, 194 and 193 proteins were identified. Fewer than 18% (60 proteins) were common to all PCs, and fewer than 40% (128 proteins) were shared between genders. These values are much lower than those of PCs formed on 20 nm AgNPs in human plasma, which shared 70% of all proteins between genders.[29] In PCs derived from female fish plasma, roughly two-thirds of the proteins (89 of 135) are common to both 1 and 24 h incubation samples, while the number of proteins unique to either condition is relatively low (n = 19 and 26, respectively). On the other hand, while more than half of the proteins in male-derived PCs (109 or 193) are found in both 1 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 proportions of high-abundance proteins in PCs differs substantially from those in the bulk plasma. In both fish sexes, the populations of parvalbumin, apolipoproteins, and other lipid transport proteins in PCs are far smaller than that found in the bulk, whereas the proportion of immunoglobulins in PCs is significantly higher. The amount of hemoglobin in PCs is also initially higher than that of the bulk during the first hour but has receded by the 24 h mark. Furthermore, levels of fibrinogen and fibronectin in PCs isolated from male plasma are lower than those measured in the bulk and decrease over time; a similar trend is observed for the egg-specific proteins VTG and ZP in PCs isolated from female plasma. No correlations between relative abundance and function are being suggested at this time; however, the mere presence of serum proteins such as VTG and ZP may be sufficient to modulate the uptake and delivery of NPs to specific organs (see below).

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 and complement proteins), followed by those for vascular and oxygen transport (hemoglobin, plasminogen, and fibrinogen/fibronectin). Lipid transport proteins (lipoproteins and apolipoproteins) were also present but to a lesser extent; high-density lipoproteins (HDLs, ApoA) were associated with PVP-AgNPs regardless of gender or length of incubation. Low-density lipoproteins (LDLs) and two apolipoproteins (ApoB-100 and ApoE) were also found in all PCs. Earlier studies with polystyrene and silica NPs in human serum have yielded similar observations,[30,31] leading to hypotheses that lipid transport proteins may be involved in the movement of NPs from the bloodstream into organs and across the blood−brain barrier.[15]

Several other proteins were identified in significant quantities within PCs, some at much higher concentrations relative to that in the bulk plasma (Figure 3b). Ceruloplasmin and plasminogen are particularly noteworthy; other metal-ion regulators such as Ca^{+}/calmodulin-dependent protein kinase II (CaMKII) and transferrin are also present. Acute-phase

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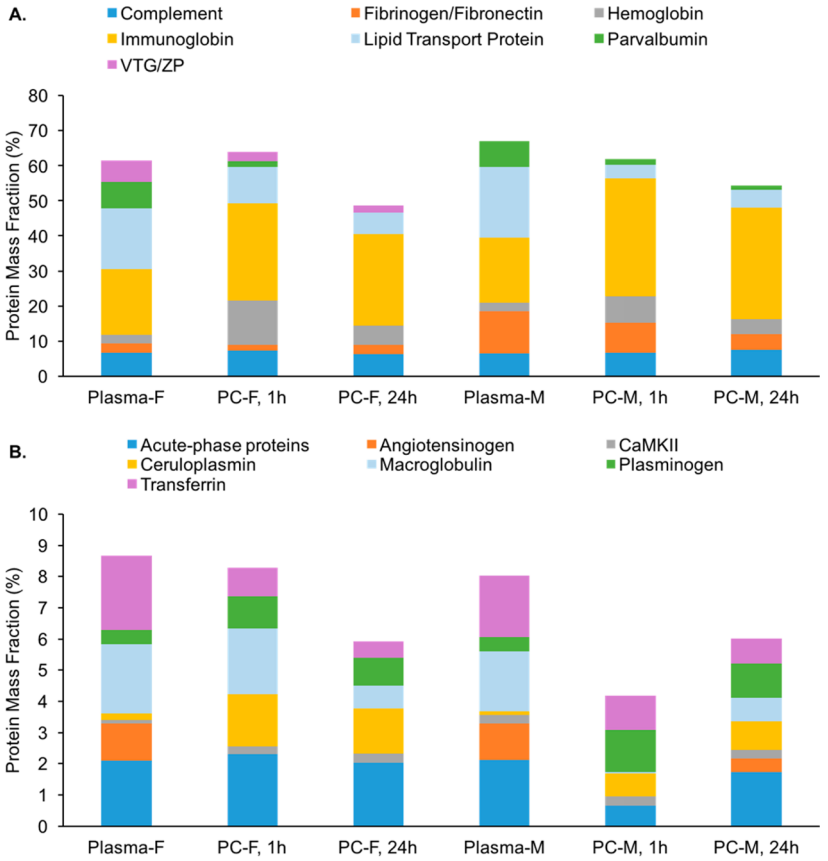

Figure 3. Protein corona (PC) compositions with the relative abundance of proteins by class, derived from PVP-coated AgNPs exposed to adult female (F) or male (M) fish plasma for 1 or 24 h. The composition of bulk plasma is shown for comparison. (A) Highest-percentage proteins within PCs. (B) Other significant proteins found in the PC layer. Abbreviations: CaMKII, calcium/calmodulin-dependent protein kinase; VTG, vitellogenin; ZP, zona pellucida.

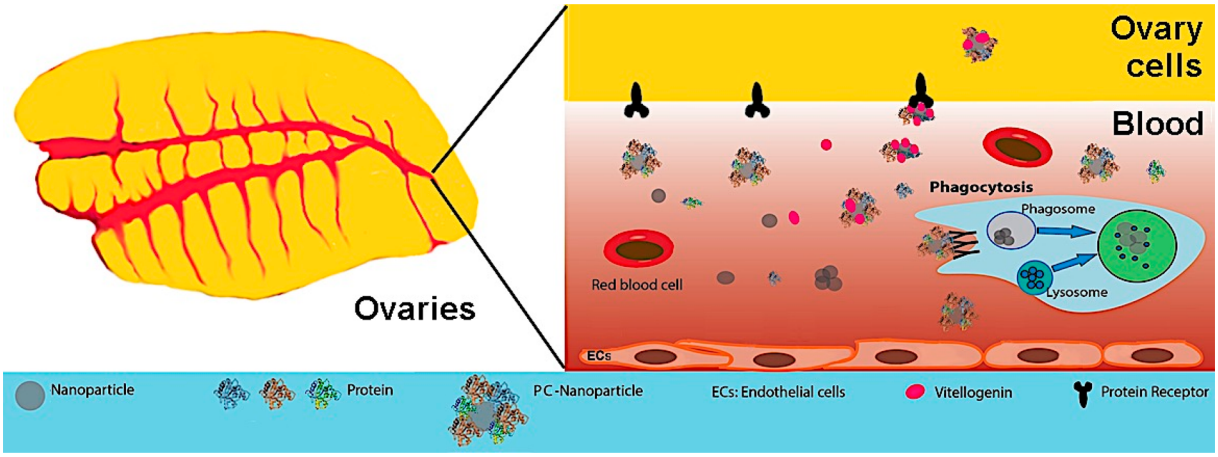

Figure 4. Hypothetical roles of corona proteins in the distribution and transport of silver nanoparticles (AgNPs) in fish plasma. AgNPs labeled with complement proteins and other opsonins are likely taken up into macrophages by phagocytosis, whereas those labeled with egg-specific proteins such as vitellogenin (VTG) may be transported into developing follicles within the ovaries by cognate receptors.

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). 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 levels much lower than those in bulk plasma, and angiotensinogen (a blood pressure regulator) is hardly present at all, especially during the early stages of PC formation (Figure 3 and Figure S6).

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 plays critical roles in oogenesis, is synthesized in the liver and transported to the ovaries via the bloodstream.[32] The significant inclusion of VTG in PCs formed on SiO_2 NPs exposed in zebrafish plasma has also been

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reported very recently, with evidence of sex-specific NP uptake by immune cells.[33] 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).[34,35] These egg-specific proteins are incorporated at an early stage of PC formation, but their levels decrease after a 24 h incubation, which suggests that they reside in the outer "soft" corona layer and are thus readily presented to follicular cells expressing their cognate receptors. This suggests that the biological response to PVP-AgNPs may depend not only on gender but also on the window of exposure during the fish's reproductive cycle.

Previous research has shown that AgNPs can accumulate in fish ovaries, which can lead to abnormal follicular development with subsequent loss of fecundity and reproductive capacity.[36−38] 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 rates of survival.[39−42] 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 about the physiological condition of the host species. Unlike studies involving mammalian sera, gender plays an important role in PC composition, with significant differences in ζ 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 in 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.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.estlett.7b00074.

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

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: mssepulv@purdue.edu. Phone: +1 765 496 3428.
*E-mail: alexwei@purdue.edu. Phone: +1 765 494 5257.

ORCID

Maria S. Sepúlveda: 0000-0003-0473-5760

Notes

The authors declare no competing financial interest.

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