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COMMUNICATION

Identification of silver nanoparticles in *Pimephales promelas* gastrointestinal tract and gill tissues using flow field flow fractionation ICP-MS

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The environmental toxicity of silver nanoparticles (AgNPs) is of increasing concern due to their intensified production, use and subsequent environmental release. To further understand nanoparticle toxicity, more knowledge is needed about the particle fate upon uptake. AgNPs were identified in both the GI tract and gill of fathead minnows using Field-Flow-Fractionation interfaced to inductively coupled plasma mass spectrometry (FFF-ICP-MS).

Silver nanoparticles (AgNPs) are among the most widely used nanoparticles and are included in products such as fabrics, washing machines, and medications. Due to the nature of these products their ultimate fate is waste water treatment plants and/or subsequent release into the aquatic environment.[1] AgNPs are toxic to aquatic organisms due to the effects of ionic silver.[2] Yet, in some experiments, fish exposed to AgNPs have shown differing toxicities than those exposed to ionic silver indicating a unique particle effect.[3] These unique effects could be due to the differential bioaccumulation of AgNPs *in vivo*.[4]

Understanding the relationship that nanoparticles have in toxicity requires an understanding of the particle form as it is taken up into the organism. Particle characterization in a tissue matrix offers a great challenge[5] because many analytical methods require dilution of the sample or an extraction of silver alters results due to transformation of the particles. For example, acid digestion of tissue is used to determine total silver concentrations, however, any particle is destroyed and an understanding of the particle form and physiochemical properties is then impossible.

To meet these challenges, FFF-ICP-MS can be utilized to determine the presence, sizing, and possible heteroaggregation or homoaggregation of AgNPs in tissue of exposed organisms. FFF-ICP-MS was previously successfully employed to characterize polyvinylpyrrolidone (PVP) coated silver nanoparticles-AgNPs in *Lumbriculus variegates,* a freshwater oligochaete, which indicated an increase from 31 to 46 nm in hydrodynamic size of particles taken up by the worms.[6] Based on this success and others[7], we have applied FFF-ICP-MS in the present work, for detection and characterization of Ag-NPs in tissues of a freshwater fish.

Nanosilver release is of particular concern in freshwater ecosystems due the potential dissolution of particles into Ag^+ ions.[8] While the fish gill is the major site of Ag^+ toxicity,[9] unique biological targets of AgNPs toxicity could exist due to differing accumulation patterns.[2] To understand the accumulation pattern of AgNPs in fish, fathead minnows (*Pimephales promelas,* FHM) were exposed to AgNPs and Ag tissue concentrations for gill and GI tract were obtained. FFF-ICP-MS was also performed with tissues to characterize the particles detected in tissue.

Six to eight month old fathead minnows (length range 3.4 – 6.6 cm) were obtained from Aquatic BioSystems (Fort Collins, CO, USA) and cultured according to University of Mississippi IACUC approved conditions. Aqueous BioPure silver nanoparticle suspensions with the nominal size of 20 nm were supplied by Nanocomposix (San Diego, CA, USA). The fish were acclimated to glass chambers containing 1.5 L of moderately hard water (MHW) prepared by U.S. EPA guideline 821-R-02-013 for six days prior to the exposure. During acclimation, fish were fed and water was changed daily. The fish were then exposed to 6 µg/L $AgNO_3$ or PVP-AgNPs or citrate-AgNPs at nominally 200 µg/L for 96 hours (n=5 chambers/treatment; 3 fish/chamber; 1.5 L water/chamber). A

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single feeding occurred at 48 hrs, 30 min before water change. Water was changed and redosed daily at 9 AM. Water parameters were 286±4 µS, 189±3 ppm TDS, 26.6±0.7°C, 136±2 ppm salinity, and pH 8.17±0.3. Water samples were taken at 20-30 min after dosing for concentration confirmation. The fish were euthanized with MS-222 and dissected. Body weight and length were recorded and gills and GI tract removed and frozen at -80°C for FFF-ICP-MS analysis.

To verify differential tissue uptake of total silver, a replicate exposure was performed. Fish were exposed to measured water concentrations of 13.1±0.6, 208±40.7 and 175±5.2 µg/L of $AgNO_3$, PVP-AgNPs and citrate-AgNPs, respectively. Gill and GI tract were then removed and placed separately in 4 mL 50% nitric acid, 2.5 mL 30% hydrogen peroxide, and 0.5 mL water and refrigerated until ready for microwave digestion. An Ethos microwave digestion system (Milestone Inc. Shelton, CT, USA) equipped with a 41-vessel rotor was employed for complete decomposition of the tissue. The digestion program consisted of a 30 min ramp to 120℃ followed by 15 min holding at that temperature. The resultant clear digests were diluted to 50 mL before ICP-MS analysis. Samples were then analyzed on the sector field-ICP-MS (Element XR, Thermo Fisher Scientific, USA) to test for silver accumulation (n=5 tanks; 2 fish per tank).

The results for the total Ag accumulation revealed that fathead minnows accumulated AgNPs in the GI tract more than in the gill. Ratios of concentrations (GI tract:gill) were 23:1, 17:1 and 0.44:1 for fish exposed to PVP-AgNPs, citrate-AgNPs, and $AgNO_3$, respectively. GI tract concentrations were 5.6±4.2 and 8.0±7.1 µg/g, and in the gill they were 0.24±0.05 and 0.48±0.19 µg/g, for PVP-AgNPs and citrate-AgNP, respectively.

In the replicate exposure of fish for the FFF analyses, the measured silver water concentrations were 13.1±0.6, 171±18.6 and 212±5.7 µg/L for $AgNO_3$, PVP-AgNP, and citrate-AgNP, respectively. Particle sizes in the exposure matrix of MHW were determined by FFF following previously described techniques[6,10] to be 26 nm for PVP-AgNPs and 27 nm for citrate-AgNPs. It was expected that the hydrodynamic based FFF method would measure slightly larger sizes compared to the nominal size of 20 nm which was measured by microscopy techniques.[6]

GI tracts and gills extracted from fish for FFF-ICP-MS were placed in 600 µL of deionized water, and the tissue was sonicated at 60% amplitude by a tissue demembraner (Fisher Science) for a total 65 sec with 5 sec pulses between 50 sec rest periods. Samples were then centrifuged at 6339 x *g* for 13 min and 250 µL was analyzed for particle sizing. Particle sizing was performed using an F-1000 symmetrical flow field flow fractionation (FFF) system from Postnova Analytics (Salt Lake City, UT, USA), interfaced to a Perkin Elmer Elan DRC II ICP-MS using a MiraMist pneumatic nebulizer, with both ^{107}Ag and ^{197}Au monitored for metal nanoparticle detection.[10] The UV absorption data was collected using a Prominence UV/VIS detector from Postnova Analytics, primarily for detection of polystyrene bead size standards. UV absorbance data was not collected for the dilute nanosilver particles measured due to the limited absorbance of the silver nanoparticles at the low concentrations (µg/L) studied[10]. The FFF system and analysis has been previously described[6,10] but briefly, consisted of a 10 kDa regenerated cellulose membrane and mobile phase of a 0.01% sodium azide and 0.01% FL-70 surfactant dissolved in deionized water with a resistivity of 18.3 MΩ. Separation of the particles under investigation was achieved using a channel flow of 1.0 mL/min and a cross flow of 0.5 mL/min. The channel flow conditions allow direct connection of the FFF effluent to the ICPMS nebulizer without a flow splitter.

AgNPs were identified in the GI tract of fish exposed to both PVP-AgNPs and citrate-AgNPs (Fig. 1) using FFF-ICP-MS. For

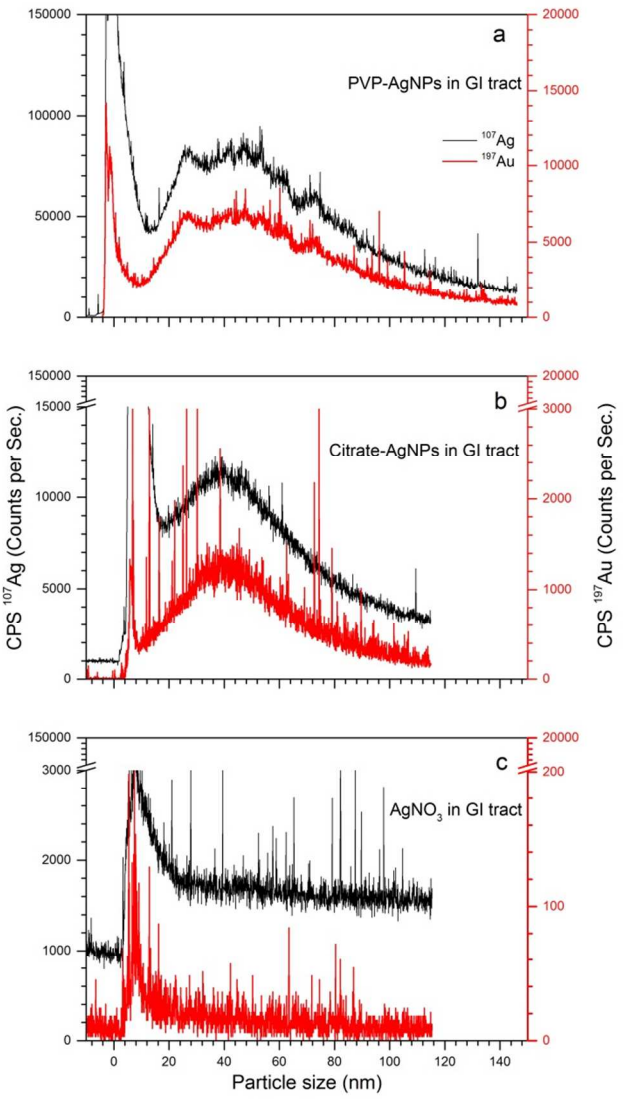

Fig. 1. FFF-ICP-MS fractograms of the GI tract of FHMs after exposure to (a) PVP-AgNPs, (b) citrate-AgNPs, or (c) $AgNO_3$.

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PVP-AgNP exposures (Fig. 1a) the particles agglomerated more with some particles found near the original size of 25 nm but others at 40-70 nm. Fish exposed to citrate-AgNPs displayed a single peak in the fractogram (Fig. 1b) with AgNPs ranging from 40-55 nm in size. As expected, GI tracts from fish in the $AgNO_3$ group displayed no peaks in the fractogram after the void peak (Fig. 1c). The agglomeration pattern in both PVP-AgNPs and citrate-AgNPs could be due to the relative stability of each particle coating in the water column prior to uptake and after ingestion. Sterically stabilized PVP-AgNPs are generally more stable in complex environmental media than the charge-stabilized citrate-AgNPs.[11] PVP-AgNPs could be maintaining their particle form but with the formation of a protein corona or heteroaggregating with other biological media resulting in a greater range of (hydrodynamic) particle sizes.[12] Citrate-AgNPs are more likely to homoaggregate first forming larger silver particles that result in less interaction with biological media and reduced diversification in particle size.

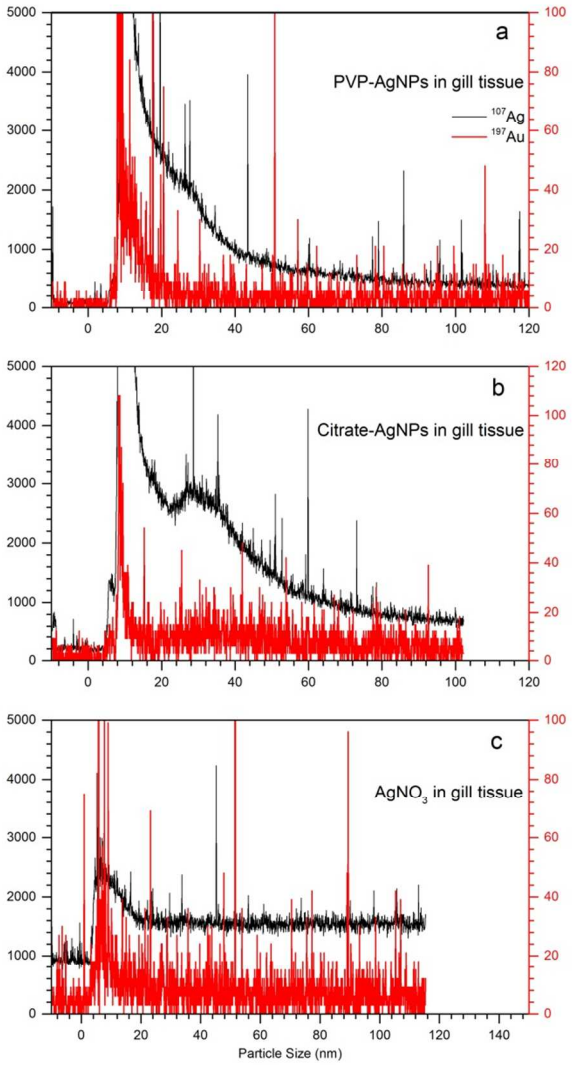

Fig. 2. FFF-ICP-MS fractograms of the gill of FHMs after exposure to (a) PVP-AgNPs, (b) citrate-AgNPs, or (c) $AgNO_3$.

AgNPs were also identified in the gill tissue of fish exposed to both PVP-AgNPs and citrate-AgNPs (Fig. 2) using FFF-ICP-MS. After PVP-AgNPs exposure, the particles did not agglomerate in gill displaying only a single peak sizing at 27 nm (Fig. 2a). Fish exposed to citrate-AgNPs (Fig. 2b) also only had a single peak with AgNPs at 30 nm in size. AgNPs in gill tissue were less aggregated than those found in GI tissue, probably due to fewer interactions with biological substances and overall lower AgNP concentrations. Furthermore, AgNPs found in gill tissue were likely indicative of more recent (e.g. within the last 24 hr of the 96 hr exposure) as compared to particles in the GI which may have accumulated throughout the exposure. The mucus produced by the gill can provide a mechanism by which AgNPs are sloughed off thus limiting gill bioaccumulation. This observation was supported by relatively lower AgNP concentrations in gill relative to GI tract. Conversely, AgNPs in GI tissue can be absorbed over time due to stress induced drinking.[2]

The synthesis of AgNPs often employs the use of gold NP 'seeds' as indicated by the gold trace apparent in the FFF-ICP-MS analysis in Figures 1 and 2. Because the AuNP is resistant to dissolution, unlike AgNPs, it can be used as a particle tracking mechanism. The gold signal indicates that the NPs remain intact and the silver trace is not merely ionic silver associated with large organic moieties or debris. The gold 'tracer' can be clearly seen in Figure 1a and b, where substantial amounts of AgNPs are observed. However, where the NP signature is 1-2 orders of magnitude less in Figures 2a and b, the gold signature is at or near the detection limit in gill tissue exposures.

FFF-ICP-MS can be a useful tool in understanding particle fate after uptake. Some limitations exist, however, because the nanoparticles must be in high enough concentrations. A detection limit of 10 µg/L has been reported for FFF-ICP-MS analysis of water.[6,10] FFF analysis of *Lumbriculus* that averaged 4.4±1.9 µg/g total Ag had no detectable particle signal.[13] The total Ag concentration range in *Lumbriculus* was comparable to GI tract concentrations reported here, however the fish samples were extracted in only 600 µl compared to the 10 mL used for *Lumbriculus* prior to FFF analysis. There is an analytical trade off in that minimal dilution of samples results in a viscous sample for injection into FFF-ICP-MS, whereupon significant fouling of the FFF membrane with biomolecules can occur and interfere with particle elution.

Conclusions

This research was able to confirm that AgNPs can be characterized in the GI tract and gill tissue of exposed fish using FFF-ICP-MS. This method could be a useful in understanding the bioaccumulation and speciation of nanoparticles following *in vivo* or *in vitro* exposures. Gold seeded silver nanoparticles can be an insightful tool because the gold core is important in confirming the presence of a particle form and provides data on the fate of the nanoparticle in such complex systems.

Notes and references

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REFERENCES

1. T. Y. Sun, F. Gottschalk, K. Hungerbühler and B. Nowack, *Environ. Pollut.*, 2014, **185**, 69-76.
2. B. J. Shaw and R. D. Handy, *Environ. Int.*, 2011, **37**, 1083-1097. DOI: 10.1016/j.envint.2011.03.009.
3. R. J. Griffitt, K. Hyndman, N. D. Denslow and D. S. Barber, *Toxicol. Sci.*, 2009, **107**, 404-415. DOI: 10.1093/toxsci/kfn256.
4. R. D. Handy, T. B. Henry, T. M. Scown, B. D. Johnston and C. R. Tyler, *Ecotoxicology*, 2008, **17**, 396-409. DOI: 10.1007/s10646-008-0205-1.
5. F. Von der Kammer, P. L. Ferguson, P. A. Holden, A. Masion, K. R. Rogers, S. J. Klaine, A. A. Koelmans, N. Horne and J. M. Unrine, *Environ. Toxicol. Chem.*, 2012, **31**, 32-49.
6. A. R. Poda, A. J. Bednar, A. J. Kennedy, A. Harmon, M. Hull, D. Mitrano, J. Ranville and J. Steevens, *J. Chromatogr.*, 2011, **1218**, 4219-4225.
7. M.M. Pornwilard and A. Siripinyanond. *J. Anal. At. Spectrom.,* 2014, DOI 10.1039/C4JA00207E.
8. C. Levard, E. M. Hotze, G. V. Lowry and G. E. Brown Jr, *Environ. Sci. Technol.*, 2012, **46**, 6900-6914.
9. C. M. Wood, R. C. Playle and C. Hogstrand, *Environ. Toxicol. Chem.*, 1999, **18**, 71-83.
10. A. J. Bednar, A. R. Poda, D. Mitrano, A. Kennedy, E. Gray, J. Ranville, C. Hayes, F. Crocker and J. Steevens, *Talanta*, 2013, **104**, 140-148.
11. S. J. Yu, Y. G. Yin and J. F. Liu, *Environ. Sci. Processes & impacts*, 2013, **15**, 78-92.
12. S. Lin, Y. Cheng, J. Liu and M. R. Wiesner, *Langmuir*, 2012, **28**, 4178-4186.
13. J.G. Coleman, A. J. Kennedy, A. J. Bednar, J. F. Ranville, J. G. Laird, A. R. Harmon, C. A. Hayes, E. P. Gray, C. P. Higgens, G. Lotufo, and J. A. Steevens, *Environ. Toxicol. Chem*., 2013, **32**, 2069-2077.

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