Cellular Internalization of Silver Nanoparticles in Gut Epithelia of the Estuarine Polychaete Nereis diversicolor

ARTICLE *in* ENVIRONMENTAL SCIENCE & TECHNOLOGY · MAY 2011

Impact Factor: 5.33 · DOI: 10.1021/es2005122 · Source: PubMed

8 AUTHORS, INCLUDING:

Superb K Misra
University of Birmingham
45 PUBLICATIONS **1,305** CITATIONS
SEE PROFILE

Philip S Rainbow
Natural History Museum, London
242 PUBLICATIONS **9,693** CITATIONS
SEE PROFILE

Samuel N Luoma
University of California, Davis
216 PUBLICATIONS **10,657** CITATIONS
SEE PROFILE

Eugenia Valsami-Jones
Natural History Museum, London
79 PUBLICATIONS **2,072** CITATIONS
SEE PROFILE

Environmental Science & Technology

ARTICLE

pubs.acs.org/est

Cellular Internalization of Silver Nanoparticles in Gut Epithelia of the Estuarine Polychaete *Nereis diversicolor*

Javier García-Alonso,[*,§] Farhan R. Khan,[§] Superb K. Misra,[‡] Mark Turmaine,[∥] Brian D. Smith,[§] Philip S. Rainbow,[§] Samuel N. Luoma,[§,†] and Eugenia Valsami-Jones[*,‡]

[‡]Department of Mineralogy, Natural History Museum of London, London, England

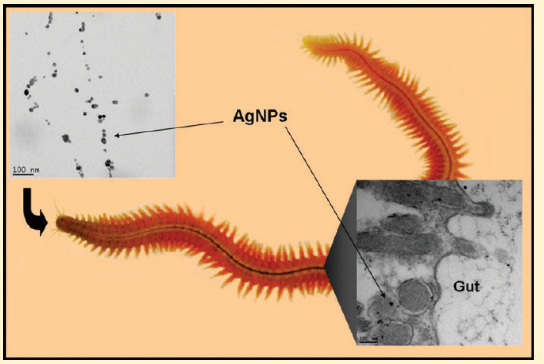
[§]Department of Zoology, Natural History Museum of London, Cromwell Road SW7 5BD, London, England

[∥]Department of Anatomy and Developmental Biology, University College of London, London, England

[†]John Muir Institute of the Environment, University of California at Davis, Davis, California, United States

Supporting Information

ABSTRACT: Silver nanoparticles (AgNPs) are widely used which may result in environmental impacts, notably within aquatic ecosystems. As estuarine sediments are sinks for numerous pollutants, but also habitat and food for deposit feeders such as *Nereis diversicolor*, ingested sediments must be investigated as an important route of uptake for NPs. *N. diversicolor* were fed sediment spiked with either citrate capped AgNPs (30 ± 5 nm) or aqueous Ag for 10 days. Postexposure AgNPs were observed in the lumen of exposed animals, and three lines of evidence indicated direct internalization of AgNPs into the gut epithelium. With TEM, electron-dense particles resembling AgNPs were observed associated with the apical plasma membrane, in endocytotic pits and in endosomes. Energy dispersive X-ray analysis (EDX) confirmed the presence of Ag in these particles, which were absent in controls. Subcellular fractionation revealed that Ag accumulated from AgNPs was predominantly associated with inorganic granules, organelles, and the heat denatured proteins; whereas dissolved Ag was localized to the metallothionein fraction. Collectively, these results indicate separate routes of cellular internalization and differing *in vivo* fates of Ag delivered in dissolved and NP form. For AgNPs an endocytotic pathway appears to be a key route of cellular uptake.



INTRODUCTION

Among the metal nanoparticles, silver NPs (AgNPs) are the most widely used (www.nanotechproject.org). Owing to their antibacterial properties they are currently found within a wide range of consumer products (clothing, toiletries, eating utensils, and food storage containers as well as electrical appliances) and biomedical applications, and this use is expected to continue.[1] Release of AgNPs to waste streams from both manufacture and use of such products is likely. For example, Benn and Westerhoff[2] demonstrated the leaching of silver (colloidal and ionic forms) into wash water from socks embedded with AgNPs. Therefore the impact and potential toxicity of AgNPs within the aquatic environments are of concern.

The toxicity of AgNPs in the environment will be influenced by the novel properties of the nanoparticle. A first step leading to toxic effects at the cellular, tissue, and whole body level is uptake. Before it can be toxic, a particle must be bioavailable, or accessible to the cell, or at least facilitate access of any ionic Ag from the nanoparticle to cell processes. Studies to date with aquatic organisms are inconclusive as to the relative contributions to subsequent toxicity of the uptake of AgNPs themselves as compared to the uptake of dissolved Ag originating from the NPs.[3,4] In this study we address how intracellular uptake of citrate-capped AgNPs occurs after dietary exposure to AgNPs in the estuarine polychaete *Nereis diversicolor*, as a first step in evaluating dietary toxicity.

The ragworm *N. diversicolor* is an excellent biomonitor of bioavailable trace metals (including Ag) and an indicator of environmental disturbance.[5,6] It is a common species in estuarine intertidal mudflats where the sediments are a sink for trace contaminants. Like many other chemical forms of trace metals, AgNPs are also likely to associate with estuarine sediments because of their propensity to aggregate and settle in the higher salinity waters typical of estuaries.[7] For nereid polychaetes like *N. diversicolor*, as with other sediment ingesting invertebrates, diet is an important route of metal uptake and bioaccumulation from sediments.[8,9] As deposit-feeding animals ingest sediment containing nutritious organic matter they also ingest sediment-associated trace metals.[6,10] Wang et al.[11] showed that 65%−95%

Received: February 14, 2011
Accepted: April 8, 2011
Revised: April 7, 2011
Published: April 25, 2011

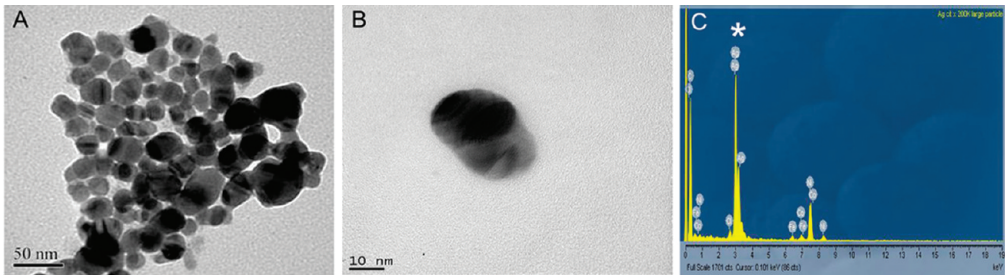


Figure 1. TEM images and Energy dispersive X-ray (EDX) of citrate-capped AgNPs. The particle size as determined by TEM for the AgNPs were 30 ± 5 nm. As depicted in the TEM image (A), the AgNPs had a broad size distribution ranging from 20−30 nm. (B) A single and isolated silver citrate nanoparticles (AgNPs). (C) EDX of this small NP, showing elemental Ag composition.

of Ag uptake by the polychaete *Nereis succinea* in the estuarine environment would be from sediment ingestion, and Rainbow and colleagues[9] showed that 20−54% of Ag uptake by *N. diversicolor* itself was from ingested sediment. If nanoparticles associate with sediments, their ingestion is also likely.[12] Nevertheless dietborne exposure to nanoparticles remains poorly known for nanoparticles in general[13] and remains unstudied for nanosilver.

In the present study we introduced citrate-capped AgNPs via a sedimentary diet to *N. diversicolor* over the course of 10 days and compared that to a sedimentary diet spiked with aqueous Ag. To describe mechanisms of internalization of AgNPs, the gut epithelia were imaged by Transmission Electron Microscopy (TEM). As a second test of nanoparticle uptake we applied a protocol previously described,[14,15] which separates tissues into operationally defined subcellular fractions with differing ecotoxicological relevance, to test if fractionation differed among treatments. To our knowledge this is the first time this protocol has been used in the study of nanoparticles. We demonstrate that it can be an important tool for determining possible responses in the organism but also, and perhaps more importantly, a way to operationally differentiate between metal that remains in nanoparticle form following uptake and metal that has been dissociated into dissolved forms.

■ EXPERIMENTAL SECTION

Synthesis and Characterization of Silver Nanoparticles (AgNPs). Citrate-capped AgNPs were synthesized at 25 °C by adding $NaBH_4$ (10 mM) to a mixture of 1.25 mM sodium citrate and 10 mM $AgNO_3$.[16] The solution was then left stirring for 3 h and thereafter stored in the dark for further use. To image the fresh particles, a drop of the AgNP suspension was left to dry at room temperature on carbon coated copper grids, which were then examined using a Hitachi TEM (Hitachi 700, 100 kV, Figure 1). The stability of the nanoparticle suspension was monitored by suspending them in diluted seawater (16 salinity) and measuring the zeta potential using Zetasizer Nano ZS (Malvern Instruments, UK).

Animals and Exposure Study. *Nereis diversicolor* were collected from the shore in the outer Thames estuary, UK (051° 31.92N, 00° 37.62E) in November 2009. Animals of similar size (244 ± 87 mg) were kept in diluted artificial seawater Tropic Marin (16‰ salinity) at 10 °C and under light controlled conditions (12:12, L:D). Individuals were allowed to clear their guts for 3 days before the start of the feeding study. In three treatments, *N. diversicolor* were exposed to sediment from the site of origin (control) and the same sediment spiked with either AgNPs or aqueous Ag (as $AgNO_3$). Five grams of dried sediments was spiked with 725 μL of 2 ppm AgNPs or aqueous Ag solutions (expected final concentrations in both treatments of 250 ng Ag g^{-1} sediment), stirred for 5 min with a spatula, and left for 24 h at room temperature. Then, the water was added to the containers after which the worms were introduced. As previously described, no Ag^+ was released from sediment to the water.[8] Each individual worm was exposed to 5 g of spiked sediments covered by 60 mL of water (salinity 16) for 10 days at 10 °C. Ten replicates were made for each treatment. Postexposure, animals were allowed to purge their gut contents by keeping them in diluted seawater for 3 days. In each treatment, four individuals were used for sectioning and TEM analysis (small sample of the worms containing a couple of segments), and then the rest of these worms were analyzed for total Ag concentrations. The remaining worms ($n = 6$) were used to determine the subcellular distribution of the Ag. The total concentration of Ag in control and exposed worms were recorded and used to calculate the recovery from the subcellular distribution protocol. In preparation for analysis, samples were digested in concentrated nitric acid (Aristar, BDH #450043X) at 100 °C and diluted with Milli-Q water, and the Ag concentration was measured by ICP-MS (Varian, Agilent Technologies).

TEM and EDX Analysis. Worms were sacrificed (by refrigeration), and a few segments were dissected from each worm and fixed in PBS (pH 7.4) containing 2% glutaraldehyde and 2% formaldehyde. The fixed samples were washed with 0.1 M PBS (pH 7.4), osmicated for 1 h with 1% osmium tetroxide, and washed with PBS. Dehydration was accomplished stepwise on the washed sample: 1 × 5 min in each of 25, 50, 70, and 90% ethanol followed by five 10 min treatments in absolute ethanol. Samples were finally placed in 3 × 10 min treatments with propylene oxide and embedded in an Agar100 resin, a mixture of Agar, dodecenyl succinic anhydride (DDSA), and methyl nadic anhydride (MNA) at a proportion 12:8:5, w-w:w respectively, with benzyldimethylamine (BDMA) subsequently added. A Reichert ultramicrotome was used to produce sections about 78 nm thick. The sections were collected on a grid and transmission electron micrographs (TEM) were taken using a Hitachi H7 100. Energy dispersive X-ray (EDX) analysis was performed on both isolated AgNPs and sample sections. A 5 μL aliquot suspension of the AgNPs was pipetted onto hydrophilic carbon coated nickel grids, the excess liquid was drained, and the grids were allowed to dry completely. Spectra were obtained using an Oxford Instruments Inca X-stream EDS attached to a Jeol 2010 TEM (Figure 1).

Subcellular Distribution. In order to compare the distribution of Ag in worms exposed to AgNPs or aqueous Ag, the

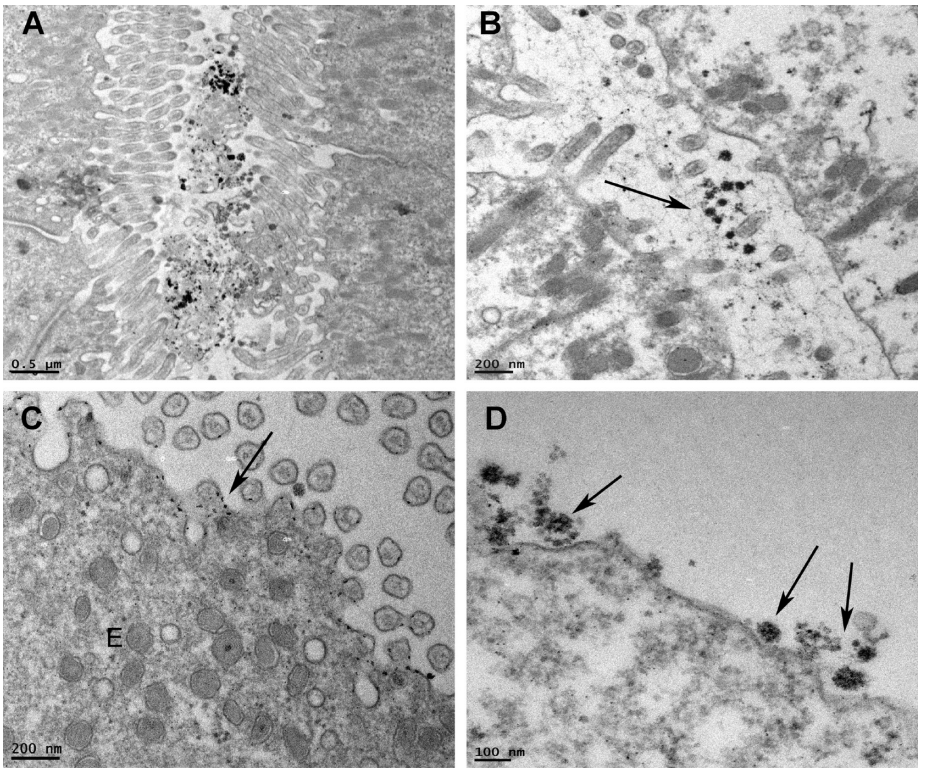


Figure 2. TEM images of gut epithelial cells of *Nereis diversicolor* exposed to AgNPs. (A) and (B) AgNPs retained in the gut lumen surrounded by epithelial cell microvilli and embedded in a glycocalyx matrix. (C) Nonaggregated AgNPs associated with plasma membrane (arrow) in cells with a cytoplasm covered by endosomes, E. (D) Agglomeration of AgNPs associated with endocytotic pits. Arrows indicate the aggregated AgNPs.

differential centrifugation scheme of Wallace et al. (ref 14, SI Figure S1) was used to produce five operationally defined subcellular fractions. Six postexposure worms from each of the three different treatments were fast frozen in liquid nitrogen and stored at −80 °C until fractionated. A tissue weight of about 200 mg (fw) (215 ± 95) was homogenized (1:10 w/v ratio) in 20 mM Tris-base buffer (pH 7.6) using a glass homogenizer on ice. Between centrifugation steps all samples were kept on ice. Homogenates were centrifuged at 1450*g* for 15 min at 4 °C (Beckman J2-21). The supernatant from this initial step was ultracentrifuged at 100000*g* for 60 min at 4 °C (Beckman L8-80M) which resulted in a pellet containing membrane bound vesicles including mitochondria, lysosomes, and microsomes (termed the organelle fraction) and the cell cytosol as supernatant. The cytosol was heated for 10 min at 80 °C and then cooled on ice for 60 min, after which the cytosol was centrifuged at 30000*g* (10 min at 4 °C). The pellet from this centrifugation consisted of heat denatured proteins (HDP), while proteins remaining in the supernatant are heat stable which is a property of the metal binding protein metallothionein (MT). Thus, the heat stable cytosol is termed the MT-like protein (MTLP) fraction. From the pellet of the initial homogenate centrifugation, metal rich granules (MRG) and cellular debris fractions were separated by first resuspending the pellet in 0.5 mL of Milli-Q water and then heating for 2 min. A volume of 0.5 mL of 1 M NaOH was added to the heated mixture and again heated at 80 °C for 10 min. The NaOH digest was centrifuged at 5000*g* (10 min) from which the inorganic MRGs formed the pellet and digested material (organic cellular debris) remained in the supernatant. The same protocol was applied to nanoparticles added to buffer alone and to nanoparticles added to homogenates of unexposed animals, in order to test behavior of Ag NPs unaffected by digestive assimilation (see the Supporting Information).

Following fractionation, all samples were digested with pure HNO_3 (Aristar grade) as described for total metal analysis and Ag was determined by ICP-MS. Fraction Ag burdens are expressed as $\mu g\ g^{-1}$ (fw) *N. diversicolor*. Different controls were carried out to assess the behavior of NPs during this operational procedure (SI, controls in subcellular distribution procedure, Figure S1).

Statistical Analyses. All data are reported as mean ± standard deviations (SD). Differences in Ag concentrations among the fractions of each treatment and between exposed and control worms at each fraction were compared using a one way ANOVA test, followed by a Tukey HSD postcomparison test. Differences were considered statistically significant at $p \leq 0.05$ and very highly significant at $p < 0.001$. All data were analyzed using the STATISTICA 9 software package.

RESULTS

AgNPs Associated with Epithelial Cell Membranes. Almost no electron-dense structures were visualized in the gut lumen of control worms or in their gut epithelia (SI Figure S2). However, in the tissues of AgNP-exposed worms (Figures 2 and 3), electron-dense particles with the expected appearance of the AgNPs were present in the gut lumen, within the gut lumen close to the microvilli, and in the extracellular matrix (glycocalyx, Figure 2A,B). In addition, at the base of the microvilli there are endocytotic pits. Small particles associated with the gut epithelial apical membrane were observed in areas with apparent high

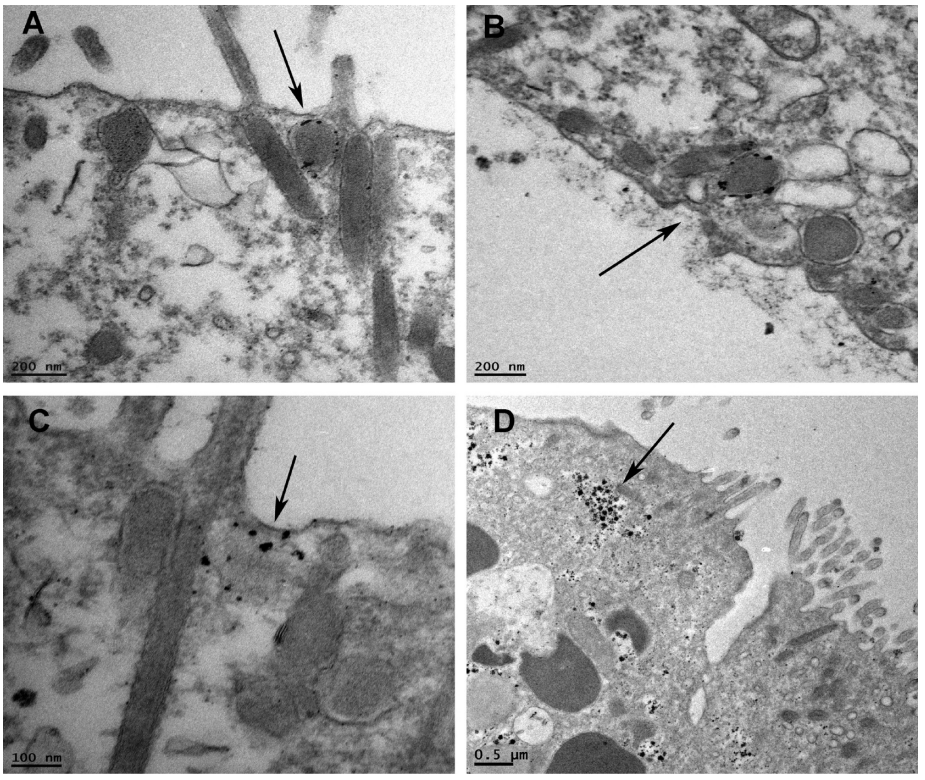


Figure 3. TEM images of gut epithelial cells of *Nereis diversicolor*. (A), (B), and (C) AgNPs already internalized and located in endosomes. (D) AgNPs distributed in the cytoplasm and it appears lysozomes could be breaking down their membranes, but further study could help clarify this. Arrows indicate the presence of AgNPs.

endocytotic activity denoted by a high number of endosomes and small vesicles near the cellular membrane (Figure 2C). Under higher magnification, endocytotic pits were also visible which contained clustered or agglomerated particles (Figure 2D), suggesting a mechanism for cellular internalization of AgNPs. Inside the cells, endosomes were present in the gut epithelium that contained electron dense materials of similar or smaller size than the AgNPs administered (Figure 3A−C). In addition, some gut epithelial cells presented dense particles distributed along the cytoplasm near the apical face (Figure 3D).

In control worms no signal was detected in the analytical spectrum of EDX in intracellular parts of the gut epithelial cells (Figure 4A,B). In worms exposed to sediment with AgNPs, however, there were small peaks in the region of the EDX spectrum typical of Ag (Figure 4C,D; SI Figure S3). Peaks in this region were unique to exposed animals, although they occurred only in some animals and some parts of the gut epithelial cells with high number of electron dense particles.

Accumulation of Ag in the Cells. After 10 days exposure, *N. diversicolor* bioaccumulated silver in its body from sediment spiked with aqueous Ag and with AgNPs. No accumulation was found in the control. After background concentrations in the worm were subtracted, *N. diversicolor* accumulated 79.35 ± 31.0 ng Ag g^{-1} (fw) when exposed to sediment spiked with aqueous Ag, and 93.77 ± 28.16 ng Ag g^{-1} (fw) when exposed to AgNPs in sediment (Table 1; Figures S2, S3). The recoveries of Ag following differential centrifugation were 78.5 ± 6.7% and 88.4 ± 6.1% for aqueous Ag and AgNPs, respectively, which are similar to recoveries observed by Wallace et al.[14] Among the intracellular fractions, Ag concentrations were significantly different from control concentrations only in the metallothionein fraction (MTLP) (ANOVA, MS = 4041; $F = 17.8$, $p < 0.0001$) in worms exposed to sediments spiked with aqueous Ag (Figure 5). In worms fed AgNP spiked sediment, Ag concentrations exceeded controls in the metal rich granule and organelle fractions as well as the heat denatured proteins (HDP) but not in the MTLP fraction (ANOVA, MS = 3635; $F = 16.8$, $p < 0.0001$). This was the same distribution found when NPs were added to buffer or to homogenates of unexposed animals (SI Figure S3). Because of the operational nature of the fractionation procedure, the exact partitioning of the AgNPs within the cells cannot be unambiguously differentiated by this procedure. But the fractionation both after and in the absence of assimilation as well as the lack of Ag in the MT fraction in animals exposed to AgNPs are consistent with the observations in the TEM that AgNPs were present in the worm tissues (e.g., gut epithelium). Not enough dissolution of Ag in the AgNP exposures occurred to result in detectable Ag in the MTLP fraction during the short time period of this experiment.

DISCUSSION

Studying dietary exposure of NPs represents a realistic exposure route for animals in nature and is an important consideration when analyzing the bioavailability of toxicants and estimating their potential negative effects in environmentally relevant conditions. AgNPs capped with citrate are negatively charged (zeta potential) particles. Therefore in contrast to the Ag^{+} cations, they will associate with positively charged ligands in the environment, many of which could occur on natural particles or in sediments. In addition, as salinity increases, the zeta-potential (surface charge properties) of AgNPs changes.[12] This

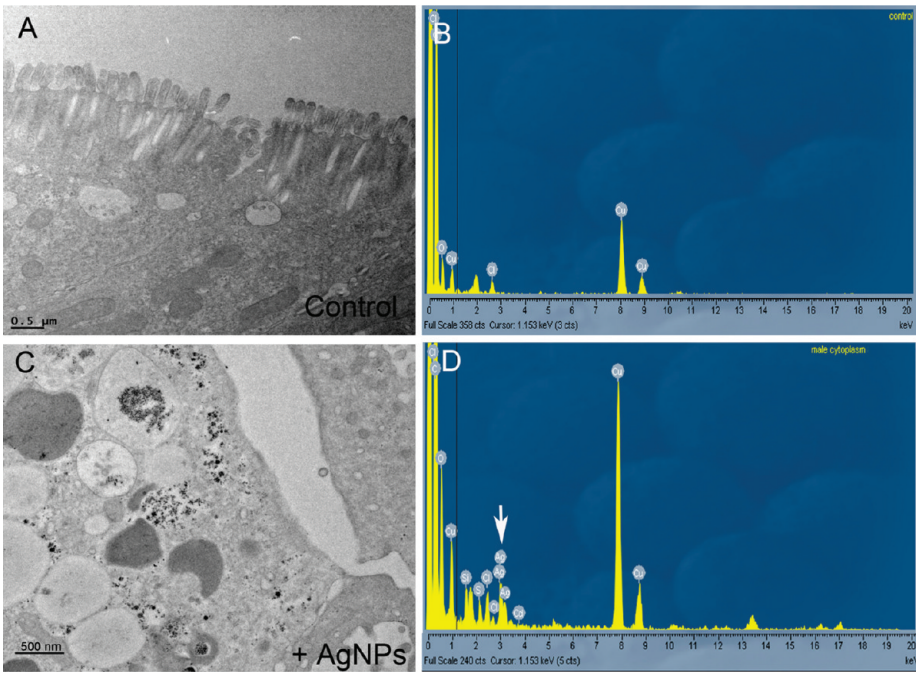


Figure 4. Images of gut epithelium in *N. diversicolor*. (A) TEM image of gut epithelium in control worms. (B) EDX analysis in controls, where no peaks corresponding to Ag were observed. (C) TEM image from a worm exposed to AgNPs showing the cytoplasm of gut epithelial cells with lysosomes with some AgNPs in adjacent areas and (D) the corresponding EDX spectrum, indicating the presence of Ag (arrow) which appears overlapping with a background generated from other elements presented in the cells.

Table 1. Partial and Total Concentrations of Ag ($\mu g\ g^{-1}$ Fresh Weight) in *Nereis diversicolor*[a]

treatments	debris	MTLP	MRG	Org	HDP	sum of fractions	total expected	recovery (%)
control	22.6 ± 7.6	18.0 ± 13.0	19.5 ± 12.2	20.9 ± 12.4	11.7 ± 8.5	98.6 ± 19.0	105.0 ± 7.1	93.9 ± 11.6
aqueous Ag ($AgNO_3$)	17.7 ± 8.2	78.7 ± 21.1	18.8 ± 6.0	41.1 ± 9.8	21.5 ± 21.9	178.0 ± 31.4	226.7 ± 11.2	78.5 ± 6.7
AgNPs	25.9 ± 12.8	26.4 ± 12.9	40.6 ± 15.1	85.6 ± 21.3	36.7 ± 8.1	192.4 ± 38.2	218.7 ± 14.8	88.4 ± 9.1

[a] Concentrations measured in worms exposed to sediments spiked with dissolved silver or with citrate-capped silver nanoparticles (AgNPs) and sediments with no Ag spike as well as concentrations in different cell fractions and the percentage recovery in the fractionation procedures. MTLP, metallothionein-like proteins; MRG, metal rich granules, Org, organelles; HDP, heat denatured proteins.

enhances the likelihood of agglomeration and of NPs settling and/or interacting with available binding sites in the surrounding environment. Consequently, spiking sediments and studying the resultant dietary exposure of endobenthos like *N. diversicolor* is a relevant approach for investigation of the potential ecotoxicological impact of AgNPs in estuaries.

Electron microscopy is the predominant method used to describe cellular intake of NPs.[12] In our observations under the TEM, the AgNPs appeared in aggregates and/or clusters, perhaps associated with other molecules in the extracellular glycocalyx of *N. diversicolor* (Figure 2A,B). Choi et al.[17] found aggregation of AgNPs when they were in contact with planktonic and biofilm cells, due to ionic strength and association with extracelullar polymeric substances. Sometimes, particles were near to or associated with endocytotic pits (Figure 2D) and endosomes within the epithelium. Smaller particles were also present attached to the gut epithelial cell membrane (Figure 2C). Mechanistically, endocytosis has been proposed as a method by which AgNPs might enter cells.[1,18] One of the most common endocytotic pathways is the clathrine-mediated endocytosis, which is indicated by a typical fuzzy coated appearance of the pits.[19] However, the AgNPs are negatively charged, and it seems that this method of internalization typically favors positively charged NPs rather than negative ones;[20] although changes in charge could occur in the gut lumen after the NPs mix with the complex suite of organic molecules in that milieu. Another possibility is that cellular uptake of nanoparticles was influenced by the surface chemistry of the particles,[21,22] for example the use of citrate as a capping agent. Citrate is an organic trivalent anion that is one of the major substrates for energy production by cells and can be used for fatty acid synthesis. Citrate itself is taken up through different plasma membrane transporters;[23] thus it is possible that AgNPs could enter cells through a specific citrate membrane transport pathway or such a pathway facilitated by endocytosis.

Endosomes and lysosomes are involved in isolation, intracellular transport, and degradation of materials sequestered by endocytosis, protecting the rest of the cell from potential toxic chemicals.[18,24] In the digestive systems, lysosomes have been identified as target sites for the toxic effects of many environmental xenobiotics as well as trace metals.[25] When the accumulation of some toxins in the lysosomes exceeds a threshold, they empty their contents into the cytoplasm and cause death of the cell in a process called autolysis.

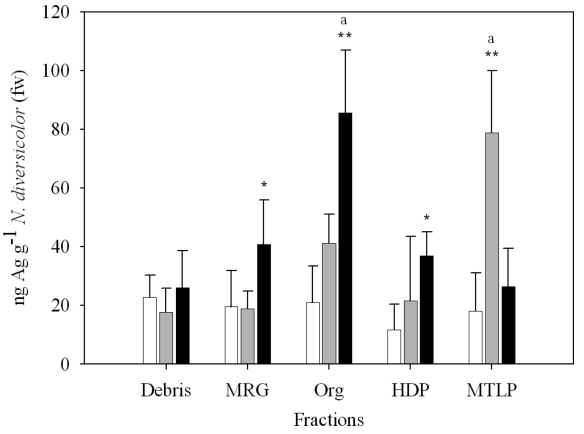


Figure 5. Concentrations of Ag (mean ± SD, $n = 6$) in different subcellular fractions of *Nereis diversicolor* exposed to dissolved Ag or AgNPs. Clear bars are background concentrations in unexposed animals; gray bars are animals exposed to sediments spiked with aqueous Ag; black bars are animals exposed to sediments spiked with citrate capped AgNPs. One asterisk denotes significant ($p < 0.05$) and two asterisks highly significant ($p < 0.001$) differences between exposed and control worms for a specific fraction. Letter "a" denotes highly significant ($p < 0.001$) different in the distribution of Ag among the fractions in a specific treatment. MRG, metal rich granules, Org, organelles; HDP, heat denatured proteins; MTLP, metallothionein-like proteins.

In the present study, TEM images showed not only that AgNPs were associated with structures typical of endocytotic processes, but that they then localized in endosomes or lysosomes within the cells (Figure 3A,B). It is known that high affinity Ag-specific transport proteins are found in lysosome membranes.[26] Some cells also presented an unexpected amount of electron-dense material in the cytosol after exposure to AgNPs, perhaps due to the effect on lysosomal membrane stability (LMS) as noted above. If the accumulation of nanosilver decreased lysosomal membrane stability as has been suggested,[27] this could promote apoptosis processes. Lysosomal rupture is an early event wherein apoptosis and cell death are induced by oxidative stress factors.[28] It has been shown that both Ag NPs and other metal-based NPs induce lysosome rupture.[24,29]

Although TEM-EDX was able to describe and identify the isolated AgNPs, identification of composition was more ambiguous for the electron-dense particles in the sections of the worms. The area of each particle is small in relation to the rest of the cell section that is taken for EDX analyses (the minimal area), causing poor resolution for specific targets such as the NPs. Although EDX was confounded by the diversity of peaks in the tissues and the small size of the particles relative to the EDX "beam", Ag was only detected by EDX in electron micrographs from animals in which electron dense particles appeared (not controls) and which had been fed AgNPs.

The tissue fractionation results provided an additional line of evidence that AgNPs were present in the tissues. First Ag was found in the fractions expected from the preliminary experiments (SI Figure S3): organelles, heat sensitive proteins, and metal-rich granules. In addition, Ag from sediments spiked with dissolved Ag accumulated mainly in the MTLP fraction, but no Ag beyond that in controls was detectable in the MTLP fraction when the animals were exposed to AgNP spiked sediments (Figure 5). The binding of silver to metallothioneins has been well described,[30] and the accumulation of Ag in the MT-enriched fraction confirms the importance of the MT detoxification process for Ag.[30] The absence of MTLP Ag in the animals exposed to AgNPs raises the possibility that dissolution of the NPs, either externally or internally, was not important over the time scale studied here.

In conclusion, Ag is taken up by the gut epithelium of *N. diversicolor* from ingested sediments spiked with either aqueous Ag or with AgNPs. The TEM images indicate that at least some of the AgNPs are internalized directly into the epithelial cells. There is strong evidence to show that endocytosis is occurring, with clusters of electron dense particles appearing in apparent endosomes and/or lysosomes. Furthermore, the subcellular distribution of Ag after exposure to AgNP independently suggested that AgNPs, rather than dissolved Ag (dissolution of Ag from the nanoparticles), were the predominant form accumulated in the cells. This was further substantiated by appearance of Ag in these animals in the same fractions in which Ag appears when NPs are spiked into buffer or tissue homogenates.

■ ASSOCIATED CONTENT

Supporting Information. Methodologies and results detailing the properties of the AgNPs, TEM descriptions of the gut epithelial cells from unexposed *N. diversicolor*, and the additional sample sets (AgNPs in 20 mM TRIS-Base buffer and *N. diversicolor* homogenize spiked with AgNPs) for subcellular fractionation protocol. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: 0044-02079426685. Fax: 0044-02079425054. E-mail: j.garcia-alonso@nhm.ac.uk (J.G.-A.). Phone: 0044-2079425334. Fax: 0044-2079425537. E-mail: evj@nhm.ac.uk. (E.V.-J.).

■ ACKNOWLEDGMENT

We are grateful to Stanislav Strekopitov (Natural History Museum) and Ann Lowry (The University of Hull) for helping on ICP-MS and EDX analyses, respectively. This work was funded by The Leverhulme Trust project grant no F/00 696/N.

■ REFERENCES

(1) Luoma, S. N. Silver Nanotechnologies and the Environment: Old Problems or New Challenges. Project on Emerging Nanotechnologies. Publication 15. Woodrow Wilson International Center for Scholars and PEW Charitable Trusts, Washington, DC, 2008.

(2) Benn, T. M.; Westerhoff, P. Nanoparticle silver released into water from commercially available sock fabrics. *Environ. Sci. Technol.* **2008**, *42*, 4133–4139.

(3) Navarro, E.; Piccapietra, F.; Wagner, B.; Marconi, F.; Kaegi, R.; Odzak, N.; Sigg, L.; Behra, R. Toxicity of Silver Nanoparticles to *Chlamydomonas reinhardtii*. *Environ. Sci. Technol.* **2008**, *42*, 8959–964.

(4) Wijnhoven, S. W. P.; Peijnenburg, W. J. G. M.; Herberts, C. A.; Werner, I.; Hagens, A. G.; Oomen, E. H. W.; Heugens, B. R.; Bisschops, J.; Gosens, J.; Van De Meent, D.; Dekkers, S.; De Jong, W. H.; van Zijverden, M.; Sips, A. J. A. M.; Geertsma, R. E. Nano-silver – a review of available data and knowledge gaps in human and environmental risk assessment. *Nanotoxicology* **2009**, *3*, 109–138.

(5) Bryan, G. W.; Hummerstone, L. G. Indicators of heavy metal contamination in the Looe Estuary (Cornwall) with particular regard to silver and lead. *J. Mar. Biol. Assoc. U. K.* **1977**, *57*, 75–92.

(6) Gray, J. S.; Elliott, M. Ecology of marine sediments: From Science to management, second ed.; Oxford University Press: Oxford, 2009.

(7) Bradford, A.; Handy, R. D.; Readman, J. W.; Atfield, A.; Muling, M. Impact of Silver Nanoparticle Contamination on the Genetic Diversity of Natural Bacterial Assemblages in Estuarine Sediments. *Environ. Sci. Technol.* **2009**, *43*, 4530–4536.

(8) Rainbow, P. S.; Smith, B. D.; Luoma, S. N. Differences in the trace metal bioaccumulation kinetics among populations of the polychaete *Nereis diversicolor* from metal-contaminated estuaries. *Mar. Ecol.: Prog. Ser.* **2009a**, *376*, 173–184.

(9) Rainbow, P. S.; Smith, B. D.; Luoma, S. N. Biodynamic modelling and the prediction of Ag, Cd and Zn accumulation from solution and sediment by the polychaete *Nereis diversicolor*. *Mar. Ecol.: Prog. Ser.* **2009b**, *390*, 145–155.

(10) Luoma, S. N.; Rainbow, P. S. *Metal Contamination in Aquatic Environments: Science and Lateral Management*. Cambridge University Press: Cambridge, 2008.

(11) Wang, W.-X.; Stupanoff, I.; Fisher, N. S. Bioavailability of dissolved and sediment-bound metals to a marine deposit-feeding polychaete. *Mar. Ecol.: Prog. Ser.* **1999**, *178*, 281–293.

(12) Farré, M.; Gajda-Schrantz, K.; Kantiani, L.; Barceló, D. Ecotoxicity and analysis of nanomaterials in the aquatic environment. *Anal. Bioanal. Chem.* **2009**, *393*, 81–95.

(13) Croteau, M.-N.; Dybowska, A. D.; Luoma, S. N.; Valsami-Jones, E. A novel approach reveals that zinc oxide nanoparticles are bioavailable and toxic after dietary exposures. *Nanotoxicology* **2010**. Early online, 1-2 (doi:10.3109/17435390.2010.501914).

(14) Wallace, W. G.; Lee, B.-G.; Luoma, S. N. Subcellular compartmentalization of Cd and Zn in two bivalves. I. Significance of metal sensitive fractions (MSF) and biologically detoxified metal (BDM). *Mar. Ecol.: Prog. Ser.* **2003**, *249*, 183–196.

(15) Wallace, W. G.; Luoma, S. N. Subcellular compartmentalization of Cd and Zn in two bivalves. II. Significance of trophically available metal (TAM). *Mar. Ecol.: Prog. Ser.* **2003**, *257*, 125–137.

(16) Doty, R. C.; Tshikhudo, T. R.; Brust, M.; Fernig, D. G. Extremely Stable Water-Soluble Ag Nanoparticles. *Chem. Mater.* **2005**, *17*, 4630–4635.

(17) Choi, O.; Yu, C.-P.; Fernández, G. E.; Hu, Z. Interactions of nanosilver with Escherichia coli cells in planktonic and biofilm cultures. *Water Res.* **2010**, doi:10.1016/j.watres.2010.06.069.

(18) Moore, M. Do nanoparticles present ecotoxicological risks for the health of the aquatic environment? *Environ. Int.* **2006**, *32*, 967–976.

(19) Bozzola, J. J.; Russell, L. D. Electron Microscopy: Principles and techniques for biologist, Second ed.; Jones and Bartlett Publishers: Toronto, Canada, 1998.

(20) Harush-Frenkel, O.; Debotton, N.; Benita, S.; Altschuler, Y. Targeting of nanoparticles to the clathrin-mediated endocytic pathway. *Biochem. Biophys. Res. Commun.* **2007**, *353*, 26–32.

(21) Chithrani, B. D.; Chan, C. W. Elucidating the mechanism of cellular uptake and removal of protein-coated gold nanoparticles of different sizes and shapes. *Nano Lett.* **2007**, *7*, 1542–1550.

(22) Brunner, T. J.; Wick, P.; Manser, P.; Spohn, P.; Grass, R. N.; Limbach, L. K.; Bruinink, A.; Stark, W. J. In vitro cytotoxicity of oxide nanoparticles: comparison to asbestos, silica and the effect of particle solubility. *Environ. Sci. Technol.* **2006**, *40*, 4374–4381.

(23) Mycielska, M. E.; Patel, A.; Rizaner, N.; Mazurek, M. P.; Keun, H.; Patel, A.; Ganapathy, V.; Djamgoz, M. B. A. Citrate transport and metabolism in mammalian cells Prostate epithelial cells and prostate cancer. *BioEssays* **2009**, *31*, 10–20.

(24) Ringwood, A. H.; McCarthy, M.; Bates, T. C.; Carroll, D. L. The effects of silver nanoparticles on oyster embryos. *Mar. Environ. Res.* **2010**, *69*, S49–51.

(25) Moore, M. N. *Environmental stress signals: cellular reactions to marine pollution*. In Graumann, W., Drukker, J. Eds.; Histo- and cytochemistry as a tool in environmental toxicology. Fisher: New York, 1991; pp 1−19.

(26) Havelaar, A. C.; de Gast, I. L.; Snijders, S.; Beerens, C. E. M. T.; Mancini, G. M. S.; Verheijen, F. W. Characterization of a heavy metal ion transporter in the lysosomal membrane. *FEBS Lett.* **1998**, *436*, 223–227.

(27) Moore, M. N. Do nanoparticles present ecotoxicological risks for the health of the aquatic environment? *Environ. Int.* **2006**, *32*, 967–976.

(28) Yuan, X.-M.; Li, W.; Dalen, H.; Lotem, J.; Kama, R.; Sachs, L.; Brunk, U. T. Lysosomal destabilization in p53-induced apoptosis. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 6286–6291.

(29) Hamilton, R. F., Jr.; Wu, N.; Porter, D.; Buford, M.; Wolfarth, M.; Holian, A. Particle length-dependent titanium dioxide nanomaterials toxicity and bioactivity. *Part. Fibre Toxicol.* **2009**, *6*, 35.

(30) Amiard, J.-C.; Amiard-Triquet, C.; Barka, S.; Pellerin, J.; Rainbow, P. S. Metallothioneins in aquatic invertebrates: Their role in metal detoxification and their use as biomarkers. *Aquat. Toxicol.* **2006**, *76*, 160–202.