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# Effects of particle size and coating on toxicologic parameters, fecal elimination kinetics and tissue distribution of acutely ingested silver nanoparticles in a mouse model

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## Abstract

Consumer exposure to silver nanoparticles (AgNP) via ingestion can occur due to incorporation of AgNP into products such as food containers and dietary supplements. AgNP variations in size and coating may affect toxicity, elimination kinetics or tissue distribution. Here, we directly compared acute administration of AgNP of two differing coatings and sizes to mice, using doses of 0.1, 1 and 10 mg/kg body weight/day administered by oral gavage for 3 days. The maximal dose is equivalent to 2000× the EPA oral reference dose. Silver acetate at the same doses was used as ionic silver control. We found no toxicity and no significant tissue accumulation. Additionally, no toxicity was seen when AgNP were dosed concurrently with a broad-spectrum antibiotic. Between 70.5% and 98.6% of the administered silver dose was recovered in feces and particle size and coating differences did not significantly influence fecal silver. Peak fecal silver was detected between 6- and 9-h post-administration and <0.5% of the administered dose was cumulatively detected in liver, spleen, intestines or urine at 48 h. Although particle size and coating did not affect tissue accumulation, silver was detected in liver, spleen and kidney of mice administered ionic silver at marginally higher levels than those administered AgNP, suggesting that silver ion

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may be more bioavailable. Our results suggest that, irrespective of particle size and coating, acute oral exposure to AgNP at doses relevant to potential human exposure is associated with predominantly fecal elimination and is not associated with accumulation in tissue or toxicity.

### Keywords

ICP-OES; *in vivo*; mouse; nanomaterials; nanoparticle; nanotoxicology; silver; toxicology

## Introduction

Silver nanoparticle (AgNP) utilization in consumer products has increased regulatory concern over exposure via ingestion (Bergin & Witzmann, 2013; Drake & Hazelwood, 2005; Hadrup & Lam, 2014; Nowack et al., 2011; O'Brien & Cummins, 2010; Varner et al., 2010; Wijnhoven et al., 2009). These potential exposures include AgNP dietary supplements, AgNP-coated food containers, water contamination or accumulation in food fish and other aquatic organisms (Sharma et al., 2014; Shaw & Handy, 2011; Volker et al., 2013; Walters et al., 2014). The proposed use of silver as an alternative to growth-promoting antibiotics in poultry and livestock carries additional potential for consumer exposure (Ahmadi & Kordestany, 2011; Ahmadi & Kurdestany, 2010; Ahmadi & Rahimi, 2011; Fondevila et al., 2009). Inhalation exposure during manufacture also leads ultimately to oral exposure since particles cleared via the mucociliary escalator are swallowed and cleared through the gastrointestinal tract (Lansdown, 2010; Varner et al., 2010).

Most current regulatory guidelines for nanoparticles are based on the material used for synthesis (e.g. silver), not the nanoparticle's physicochemical properties (U.S. Code, 2011). Analysis of AgNP toxicity and tissue accumulation as a function of physicochemical properties is warranted, since size and nanoparticle coating are often manipulated in AgNP manufacture (Albanese et al., 2012; Yin Win & Feng, 2005). The inverse-squared relationship between nanoparticle size and total surface area for a given mass implies that smaller AgNP may release more ionic silver into the immediate microenvironment (Hamilton et al., 2014). Furthermore, increased surface area has been correlated with increased bioavailability (Dehner et al., 2011; Hillyer & Albrecht, 2001). Particle coating may also influence AgNP properties. Citrate and polymer coatings such as polyvinylpyrrolidone (PVP) may differentially affect stability. In particular, PVP-coated nanoparticles are generally more stable than those coated with citrate (Huynh & Chen, 2011; Tejamaya et al., 2012). However, it is not clear whether these results apply to *in vivo* environments, particularly the molecularly complex gastrointestinal tract. Size or particle coating effects on the kinetics of ingested AgNP elimination or tissue retention are not known.

Another aspect that has not been evaluated extensively is the potential for synergistic activity with antibiotics. AgNP are marketed as health supplements and are likely to be ingested by individuals receiving other agents, including antibiotics. Recent work has suggested a synergistic effect on antibiotic efficacy *in vitro* and in animal models of infection (Morones-Ramirez et al., 2013). Antibiotics can have adverse effects on intestinal

health by eliminating normal intestinal microbes (Antonopoulos et al., 2009). Silver is known to have antimicrobial properties (McQuillan et al., 2012; Morrill et al., 2013). It is unknown whether concurrent AgNP ingestion will enhance antimicrobial effects of a typically well-tolerated antibiotic dose, causing potential adverse effects due to disruption of normal gastrointestinal microbes (e.g. diarrhea and intestinal ileus).

In this study, we evaluated acute ingestion of well-characterized AgNPs of two differing sizes (20 and 110 mm) and coatings (PVP, citrate) using traditional toxicologic pathology markers (body weights, organ weights, histopathologic effects) in a mouse model. In addition, we evaluated the same parameters when particles were administered concurrently with oral antibiotics. Tissue accumulation and fecal elimination were evaluated with respect to particle coating or size after a single orally administered dose. The highest dose utilized in this study was 10 mg/kg body weight/day (bw/d), which is 2000× the EPA oral reference dose (oral RfD: 0.005 mg/kg bw/d) (ATSDR, 1990; U.S. EPA, 1996; Varner et al., 2010) for daily exposure to silver. Silver acetate (AgOAc) was used as an ionic silver control, based on the hypothesis that most dietary silver is absorbed in the form of silver ion (Hadrup et al., 2012). AgNP exposure levels in this study could be potentially anticipated in certain circumstances (overzealous dietary supplementation). We predicted that overall tissue accumulation would be low and significant adverse effects unlikely, regardless of the presence of antibiotics. We further hypothesized that smaller particles with either coating may have a longer time to fecal elimination due to greater potential absorption from the gastrointestinal lumen.

## Materials and methods

### Acute oral toxicity study

**Mice—**Male C57BL/6NCrl mice (6 weeks old; Charles River Breeding Labs, Stone River, NY) that were SPF for 15 common murine viral, bacterial or parasitic agents were utilized. For the pathology study, mice were housed three per cage in static microisolator caging and were fed an irradiated diet (PicoLab Laboratory Rodent Diet 5LOD, LabDiet, St Louis, MO) and water, both provided *ad libitum*. The animal housing room was maintained on a 12:12-h light:dark cycle with constant temperature (72 ± 2 °F). All mice were acclimated to standard housing for at least 7 days. Animals were euthanized by $CO_2$ inhalation. All the procedures were approved by the University of Michigan's IACUC.

**Nanomaterials—**Silver nanoparticles used in this study were supplied by the NIEHS Centers for Nanotechnology Health Implications (NCNHIR) consortium and originally purchased from NanoComposix (San Diego, CA). Particles were provided as PVP- or citrate-stabilized colloidal suspensions of particles with nominal median hydrodynamic diameters of 20 and 110 nm, synthesized over a 5-nm Au core. The citrate-capped particles were supplied as 1 mg/ml suspensions in 2mM citrate buffer, while the PVP-stabilized AgNPs were supplied at the same concentration suspended in water. Vehicle controls consisted of 2mM citrate, prepared in sterile endotoxin free water (MoBio, #17013) from a stock solution (Sigma #71402) and a solution of 100 μg/ml PVP prepared in sterile

endotoxin free water using 10 kDa PVP for the 20-nm particle control or 40 kDa PVP for the 110-nm particle control.

**Characterization of materials—**Silver nanoparticles used in this study were supplied by the NCNHIR consortium and their physicochemical characterization has been previously described (Wang et al., 2014). In brief, baseline physicochemical characterization of materials was performed by the supplier (Nanocomposix) and by the Nano Characterization Laboratory (NCL) at the National Cancer Institute. Stock silver concentrations were confirmed by inductively coupled plasma mass spectrometry (ICP-MS), hydrodynamic size and size distribution by dynamic light scattering and transmission electron microscopy, and surface charge by zeta potential. (Wang et al., 2014). Additionally, endotoxin levels were assessed by kinetic turbidity and gel-clot Limulus Amoebocyte Lysate (LAL) assays. About 20 nm Ag-Citrate, 110 Ag-Citrate and 110 nm Ag-PVP particles were found to have endotoxin levels <0.5 EU/ml and 20 nm Ag-PVP particles had an average endotoxin level of 1.1 EU/ml. Methodology for these assays can be found at http://ncl.cancer.gov/working_assay-cascade.asp. Consortium-supplied AgNP were also characterized in-house (Supplementary Table 1). Stock composition of silver was confirmed using ICP-MS. Particle size distribution was characterized using Nanoparticle Tracking Analysis (NanoSight™ LM10; NanoSight Ltd., Amesbury, UK) and DLS (Zetasizer 3600; Malvern Instruments, Westborough, MA). Size measurements were confirmed using TEM (JEOL, 3011, High Resolution Electron Microscope, Jeol Ltd, Tokyo, Japan). Results confirmed NCL measurements of both the silver concentration and particulate size distrubutions and showed particle stability in diluted solutions for up to 5 days (Axson et al., in press).

**Study groups and dosing regimen—**A total of 384 mice were utilized. Animals were dosed by oral gavage once daily for three consecutive days (days 0, 1 and 2) followed by sacrifice on days 3 or 9 (representing 24 h and 7 days, respectively, after the last dose). Mice were randomly divided into the following groups: (1) sterile water (negative control), (2) ionic silver control: silver acetate (AgOAc, Sigma–Aldrich, #216674, St. Louis, MO), (3) 20 nm citrate-capped AgNP, (4) 110 nm citrate-capped AgNP, (5) 20 nm PVP-coated AgNP, (6) 110 nm PVP-coated AgNP, (7) 2mM citrate buffer (vehicle control), (8) 10 kDa PVP in sterile water (vehicle control) and (9) 40 kDa PVP in sterile water (vehicle control). AgNPs (groups 3–6) were dosed at 0.1, 1.0 or 10 mg/kg bw/d. Each group contained six animals for each dose and for each endpoint (days 3 and 9). The experiment was run in two arms, consisting of three animals per group in each arm. AgNP were used at stock concentrations (1 mg/ml) for the 10-mg/kg dose and dilutions (for 0.1 and 1 mg/kg doses) were made in sterile, endotoxin-free water on the day of dosing (≤2 hours before dosing). AgOAc (group 2) was dosed at 10 mg/kg bw/d from a 1-mg/ml stock in sterile, endotoxin-free water made on the day of dosing. All dosing was performed at 0.1 ml/10 g bw and between 9AM and 12 noon.

In addition, the effects of concurrent antibiotic administration were evaluated using replicate groups 3–9 (AgNPs and respective vehicles). There were six animals per dose and endpoint, as in the non-antibiotic arm. Antibiotic administration consisted of 0.5 mg/ml of cefoperazone (cefoperazone sodium salt, Alpha Aesar, #J65185, Ward Hill, MA)

administered in drinking water (Gibco Laboratories, cat# 15230, North Andover, MA) for 3 days (water changed every other day) prior to administration of AgNP and continuing during the 3 days of AgNP dosing (total 6 days). The AgNP dosing regimen and endpoints were the same as described earlier.

**Gross observations, body weight and euthanasia:** Health status was observed daily. Animals were weighed on the first day of dosing (day 0), and on days 1, 2, 3 and 9 (last timepoint was necropsy). Animals were sacrificed on days 3 or 9, representing 24 h or 7 days, respectively, after the final dose. Mice were euthanized via $CO_2$ inhalation.

**Organ weights and histopathology:** Organs were collected for gross observations, weighing and histopathology. The following organs were weighed at necropsy: brain, liver, spleen, thymus, gonads (paired), left kidney and adrenal, right kidney and adrenal, brain, heart, lung and GI tract (esophagus to rectum). Additional organs harvested at necropsy (but not weighed) included: salivary gland, quadriceps muscle, sternum (bone marrow), esophagus, urinary bladder, skin, mammary gland and mesenteric lymph node. All the organs were fixed in 10% neutral buffered formalin for a minimum of 48 h. Organs were processed to paraffin by the University of Michigan In Vivo Animal Core (IVAC) histology facility on an automated tissue processor using standardized protocols. Tissues were embedded, cut at 4 μm thickness on a rotary microtome and stained with hematoxylin and eosin. Light microscopic evaluation was performed by a board-certified veterinary pathologist (ILB).

**Statistics:** Graph pad Prism version 4.0 by GraphPad Software (San Diego, CA) was used for statistical analyses of toxicologic, distribution and fecal elimination data. Data were analyzed for normality by visual evaluation since small sample size ($n \leq 6$) per group prohibited use of formal normality testing. One-way ANOVA with Tukey's multiple comparisons tests were employed for comparison of control and Ag-dosed groups. Differences were considered statistically significant for $p < 0.05$ (after multiple comparisons correction).

## Distribution and elimination arm

**Mice and nanomaterials—**For the silver distribution arm, a total of 54 male C57BL/6NCrl mice were used and housed singly for 48 h in metabolic caging (Harvard Apparatus, Metabolic Cage for Mice, catalog #526731, Holliston, MA). Mice were fed a gel diet (Diet Gel 31M, Clear $H_2O$, Portland, ME) and water *ad libitum*. Gel diet was fed to avoid contamination of fecal or urine matter with particulate food waste. The source, health status and housing conditions of the mice were otherwise as described under the acute study description above. AgNP were from the same source and batch as those for the acute oral toxicity study.

**Study groups and study design: distribution and fecal elimination arm—**Mice were randomly divided into nine groups as described earlier. All the groups contained six animals except for the citrate vehicle control ($n = 3$) and the 20-nm citrate-capped AgNP group ($n = 9$). AgNP and silver acetate were dosed at 10 mg/kg. Doses were prepared as

described earlier. Animals were dosed by oral gavage once at time 0 and collection of all urine and feces was performed at times 0, 3, 6, 9, 12, 18, 24 and 48 h (cumulative collection at each timepoint). At 48 h, the mice were sacrificed by $CO_2$ inhalation and tissues were collected for silver quantitation.

**Quantitation of silver—**Silver was quantified in urine, feces and selected tissue samples by the Spectrometry Core laboratory at Research Triangle Institute International in Durham, N.C. by inductively coupled plasma-optical emission spectroscopy (ICP-OES) using methods previously established by this laboratory for preparation of biological tissues and analysis of trace elements (Poitras et al., 2015).

## Results

### Toxicology study

**In-life observations and body weight—**Overall, the acute (3 days) administration of AgNPs of two different sizes (20 and 110 nm) and two different coatings (citrate, PVP) to mice was well-tolerated at all doses (0.1, 1 and 10 mg/kg bw/d) in groups with and without concurrent administration of broad-spectrum antibiotics in drinking water (cefoperazone, 0.5 mg/ml). Animals did not exhibit illness, and none died or were euthanized for test substance-related causes prior to the study endpoint. One out of 192 animals in the 24-h endpoint and 4 of 192 animals in the 7-day endpoint died or were euthanized for unrelated causes (i.e. bite wound or gavage-related injury) prior to endpoint. There were no significant differences in the percent baseline body weight at 24 h after the last dose, regardless of antibiotic administration (Figure 1). There were also no significant differences in percent baseline body weight at the washout timepoint of day 7 after the last dose, regardless of antibiotic administration (data not shown).

**Organ weights and pathology findings—**No gross pathological changes were observed at necropsy for any groups at 24 h or 7 days after the final dose of AgNPs, with or without concurrent antibiotic administration.

Relative organ weights (% body weight) at the 24-h endpoint for the no antibiotics group are shown in Table 1. For these animals, only the liver weights were significantly different from controls for any group. Specifically, in the 20-nm PVP-AgNP group, animals receiving the medium dose (1 mg/kg bw/d) had a significantly lower relative liver weight ($p = 0.0266$) at 24 h than the vehicle (coating) control, but not the high- and low-dose groups. In the 110-nm Citrate-AgNP group, the high-dose group (10 mg/kg bw/d) had significantly lower relative liver weights ($p = 0.0377$) than the low-dose (0.1 mg/kg bw/d) group, but not the vehicle or medium-dose groups. Since neither effect was dose-dependent or correlated with histological alterations, these differences were considered inter-animal variation and not test article-related effects. There were no significant differences in liver weights for other AgNP groups, nor for brain, thymus, spleen, kidneys, testes, heart/lungs and gastrointestinal tract in any other non-antibiotic groups at the 24-h timepoint (Table 1).

Relative organ weights (% body weight) at the 24-h endpoint for animals receiving concurrent antibiotics are shown in Supplementary Table 2. For animals receiving

antibiotics, only the GI weight was significantly different than controls for any group at the 24-h timepoint. In the 110-nm Citrate-AgNP group, animals receiving the high-dose (10 mg/kg bw/d) had a lower GI weight at 24 h than the coating control ($p = 0.0277$) or the medium-dose group ($p = 0.0455$), but not the low-dose group. This finding was not correlated with body weight changes, clinical distress or histological alterations. Due to the lack of correlation, and because quantity of ingesta can vary, this was considered inter-animal variation and not a test article-related effect. There were no significant differences at 24 h after the final dose for brain, thymus, spleen, liver, kidneys, testes or heart/lungs in any antibiotic-dosed groups at the 24-h timepoint (Supplementary Table 2).

At the washout timepoint of 7 days after the final dose, there were no significant dose-dependent differences in any relative organ weights for any AgNP or AgOAc-dosed group in either the no antibiotics arm or the antibiotics group (data not shown).

Histological alterations were evaluated in the vehicle (coating) and high-dose AgNP groups and in water and AgOAc groups at 24 h after the last dose. Low- and medium-dose groups were evaluated only for organs with detectable alterations in the high-dose group. No AgNP or AgOAc-related histological alterations were found in any tissues at the 24-h timepoint for either the no antibiotic or the antibiotic-treated arms (Supplementary Table 3A–D). Tissues evaluated consisted of brain, liver, spleen, thymus, gonads (paired), left kidney and adrenal, right kidney and adrenal, lung, heart, salivary gland, quadriceps muscle, sternum (bone marrow), esophagus, stomach, small intestine, large intestine, colon, urinary bladder, skin, mammary gland and mesenteric lymph node. Small infiltrates of neutrophils and macrophages were seen in the livers of one to two animals per dose, including controls, for the first trial ($n = 3$ per dose) of the 110 PVP AgNP, no antibiotics group, but were ascribed to background in this particular cohort of mice, since they were not reproducible in the second trial ($n = 3$ per dose) for the same material, nor were they seen in any other group. (Supplementary Table 4). Random infiltrates of this nature are a common background occurrence in mice (Thoolen et al., 2010).

## Silver fecal elimination and tissue distribution

Fecal silver following single-dose administration of each AgNP was measured at timepoints up to 48 h (Figure 2A and B). Urinary silver was also measured but 353 of 357 samples were below detection limits (data not shown). The timecourse of fecal elimination was similar for all the AgNPs. Fecal silver was first detected at 3-h post-dosing, however the peak was at 6 and 9 h. Fecal silver began to decline at 12 h for all the AgNPs and was at baseline levels by 48 h. Inter-animal variation caused large SDs, particularly for the 20-nm citrate AgNP group. AgOAc had a similar timecourse but was slightly delayed in comparison to the AgNP, with first detection at 6 h and peak fecal silver at 9 and 12 h. Like the AgNP, fecal silver for the AgOAc group was back to baseline by 48 h.

Table 2 depicts the cumulative recovery of silver in feces over the entire 48-h period for each group as both a concentration (μg/g feces) and as a percent of the administered dose. There were no significant differences in the (cumulative) mean fecal silver between groups. There were no significant differences in the percent recovery of silver in feces over the entire 48-h period. Overall recovery ranged from 70.5% ± 18.4 for 110 nm PVP-AgNP to

98.6% ± 32.5 for 20 nm citrate-AgNP group (98.6% ± 32.5) (Table 2). The higher overall recovery (although not statistically significant) in the 20-nm citrate-AgNP group may owe in part to two outliers in the upper end of the data set for this group.

The majority of silver was detected in feces, however silver content was also measured at 48 h in the gastrointestinal tract (esophagus, stomach, small intestine, cecum, large intestine), liver, spleen and kidney (Tables 3 and 4). Of these organs, most silver was detected in the gastrointestinal tract, particularly the cecum and colon. Nevertheless, total gastrointestinal tract silver at 48 h represented only between 0.02% and 0.27% of the amount dosed (Table 3). Silver in other organs was minimal, with the liver having <0.01% of total dosed silver for all the groups except silver acetate (0.14 ± 0.09%) (Table 4). Silver in spleen and kidney for AgNP groups was essentially equivalent to background levels in vehicle controls (Table 4). AgOAc-dosed groups had significantly higher silver detection and recovery in liver, spleen and kidney in comparison to AgNP groups, again suggesting that ionic silver has potentially higher bioavailability. Nevertheless, the cumulative silver recovery in any tested tissue (gastrointestinal tract≫liver> spleen, kidney) was <0.5% for all the AgNP and for AgOAc at 48 h.

## Discussion

In this study, AgNP of two differing sizes and coatings were administered acutely to mice and compared with respect to toxicity, elimination kinetics and tissue distribution. Although coating and particle size have been postulated to play a significant role in toxicity and toxicokinetics (Behra et al., 2013; Hadrup & Lam, 2014; Tejamaya et al., 2012), particles of differing size or coatings have seldom been compared in the same study.

### Acute ingestion of AgNP is well-tolerated at high doses, irrespective of size or coating

Acute ingestion of AgNP had no significant effects on body or organ weights or on tissue histology at any dose at either 24 h or 7 days after the last administration. The doses utilized in this study (0.1, 1 and 10 mg/kg bw/d) were equivalent to, respectively, 20×, 200× and 2000× the EPA oral reference dose (RfD, 0.005 mg/kg bw/d) for silver (ATSDR, 1990; U.S. EPA, 1996; Varner et al., 2010). These doses were considered to reasonably represent the upper range of potential acute exposure in humans. Previous estimates of colloidal silver doses associated with clinically evident argyria range between 40× and 700× the oral RfD, although these typically represent repeated exposures (minimum reported time to occurrence of argyria of 10 months) (Chung et al., 2010; Wadhera & Fung, 2005). Previous acute administration of AgNP (10–20 nm) by gavage at doses up to 5000 mg/kg/ BW was not associated with any detectable toxicity in mice (Maneewattanapinyo et al., 2011). In another study, acute oral administration of both AgNP (13 nm) and microparticle (2–3 μm) silver at doses of 2500 mg/kg bw (single dose) in mice were associated with mild lymphocytic infiltrates in the liver, but the numbers of animals affected and dose-dependence was not reported (Cha & Myung, 2007). For ionic silver, previously reported oral $LD_{50}$ ranged from 280 to 800 mg/kg bw in rats and rabbits, respectively (Hadrup & Lam, 2014).

An LOAEL of 1 mg/kg bw was previously reported in mice gavaged with 42 nm diameter uncoated AgNP for 14 days. The adverse effect, however, was confined to increased serum

alkaline phosphatase (cholestasis marker) and increased pro-inflammatory cytokines in blood, without histological or clinical correlates.(Park et al., 2010). Increased serum alkaline phosphatase was reported after 90 days administration of 56 nm AgNP in carboxymethylcellulose suspension, but at a much higher LOAEL of 125 mg/kg bw (Kim et al., 2010). Only one study was identified that directly compared ingestion of particles of differing coating – here neither PVP-complexed AgNP nor uncoated particles induced adverse effects when administered to rats at 90 mg/kg bw for 28 days (van der Zande et al., 2012). Thus, while some rodent models may show toxicity (or at least increased physiologic stress) to the biliary excretion system with repeated AgNP administration, the doses at which this may occur range from 200× (for 1 mg/kg) to 25–000× (for 125 mg/kg) the current oral RfD for silver.

### Acute ingestion of AgNP is well-tolerated with concurrent antibiotic administration

In this study, concurrent antibiotic and AgNP administration did not affect body or organ weights or tissue histology, regardless of AgNP size or coating. AgNP have recently been reported as having synergistic antimicrobial effects with antibiotics (Morones-Ramirez et al., 2013; Seth et al., 2011). Additionally, AgNP antimicrobial effects have been demonstrated *in vitro* against food-borne pathogens (Zarei et al., 2014) or as a tuberculocidal (Seth et al., 2011). The antibiotic used in our study (cefoperazone) is a broad-spectrum cephalosporin previously shown to detrimentally alter gut microbial communities in mice (Antonopoulos et al., 2009; Theriot et al., 2014). Limited information is available, however, concurrent intraperitoneal injection of gentamicin and 6 mg/kg bw of silver nitrate (ionic silver) was recently reported as well-tolerated in mice with enhanced antimicrobial efficacy against biofilms (Morones-Ramirez et al., 2013). Our study found no toxicity with concurrent administration of antibiotics and AgNP at 2000× the oral RfD (10 mg/kg bw).

### Fecal elimination of orally administered AgNPs is minimally affected by particle size or coating

We found no size or coating differences in AgNP fecal elimination kinetics. Smaller nanoparticles have demonstrated greater solubility in various media (Behra et al., 2013; Liu et al., 2012) and AgNP absorption has been correlated with dissolution to silver ions (van der Zande et al., 2012). Our results for AgOAc support that ionic silver content facilitates absorption, since liver, spleen and kidney values were marginally higher and fecal elimination was slightly delayed in comparison to AgNPs, implying absorption and excretion into bile via enterobiliary recirculation. However, smaller AgNPs had no differences in excretion kinetics between or within coatings, suggesting that either increased solubility for smaller AgNP does not occur *in vivo* or, if present, does not impact elimination kinetics. A previous study evaluating PVP AgNP or uncoated particles (<20 nm) administered to rats also found no coating-dependent toxicokinetic differences (van der Zande et al., 2012). The explanation for this is not clear. Citrate-coated AgNPs have been demonstrated to agglomerate *in vitro* at low pH (as in the gastric compartment), while PVP-coated particles are reportedly more stable in solution (Behra et al., 2013; Huynh & Chen, 2011; Tejamaya et al., 2012). Agglomeration or particle stability may render particles less susceptible to surface Ag dissolution within the GI tract, i.e. less likely to permit Ag absorption by enterocytes. Evaluation of this hypothesis may be facilitated by modeling of

these or other potential coating-dependent AgNP interactions in a synthetic or *ex vivo* environment.

### Ingested AgNP are minimally absorbed from the intestinal tract

Between 70% and 100% of administered Ag was recovered in feces by 48-h post-dosing. This is remarkably similar to the previously reported finding of 63 ± 23% fecal excretion in rats administered a similar dose (9 mg/kg bw/d) of 14 nm PVP-AgNP but for a longer period (28 days) (Loeschner et al., 2011). Oral bioavailability was previously determined as low (4.2%) for a single 10 mg/kg bw dose of 7.9 nm AgNP-citrate in rats (Park et al., 2011). Our study, while not specifically designed to measure bioavailability, suggests similar poor AgNP absorption and low tissue accumulation, since silver was near or below the detection limit in the kidney and spleen for all the AgNP dosed. In the liver, spleen and kidney, silver was detected in significantly higher amounts for the AgOAc-dosed group than for the AgNP groups, suggesting that ionic silver was better absorbed via the portal circulation. Nevertheless, the total amount detected in liver, even for ionic silver, was minimal in comparison to fecal excretion.

### Relevance to human health

The human health relevance of these rodent findings is supported by recent studies in human volunteers which showed no measurable effects on hematologic, metabolic, urinalysis or imaging (MRI) parameters at doses close to the oral RfD (ie. up to 480 μg/day, equivalent to ~0.007 mg/kg/day) of polydisperse AgNP (<100 nm diameter) for 14 days (Munger et al., 2014, 2015). For non-experimental exposures, there have been two case reports of significant neurological adverse health effects involving individuals ingesting amounts in significant excess of typical exposure levels, however these materials and the ingested doses have not been well-characterized and the individuals also had concurrent health conditions (Mirsattari et al., 2004; Ohbo et al., 1996). The most commonly reported effect of high levels of colloidal silver ingestion is argyria, a blue skin hyperpigmentation that is of cosmetic concern but is not associated with dysfunction (Bowden et al., 2011; Brandt et al., 2005; Chung et al., 2010; Kim et al., 2009; Wadhera & Fung, 2005). Typically, argyria occurs only with high levels of ingestion over long periods of time, resulting in accumulation of ~1 g of silver in tissue (Hadrup & Lam, 2014; Varner et al., 2010). A recent review concluded that the current oral RfD provides at least a 5-fold margin of safety for the occurrence of argyria (Hadrup & Lam, 2014).

## Conclusion

In summary, this and previous studies suggest that ingested AgNP, irrespective of size or coating, are well-tolerated in rodents even in markedly high doses, whether ingested acutely, as in this study, or over longer periods, as in the studies reported earlier. No differences in fecal silver or tissue accumulation could be detected based on initial differences in size or coating, suggesting that these parameters are not critical for the biological effects of ingested silver at levels up to 2000× the current oral RfD.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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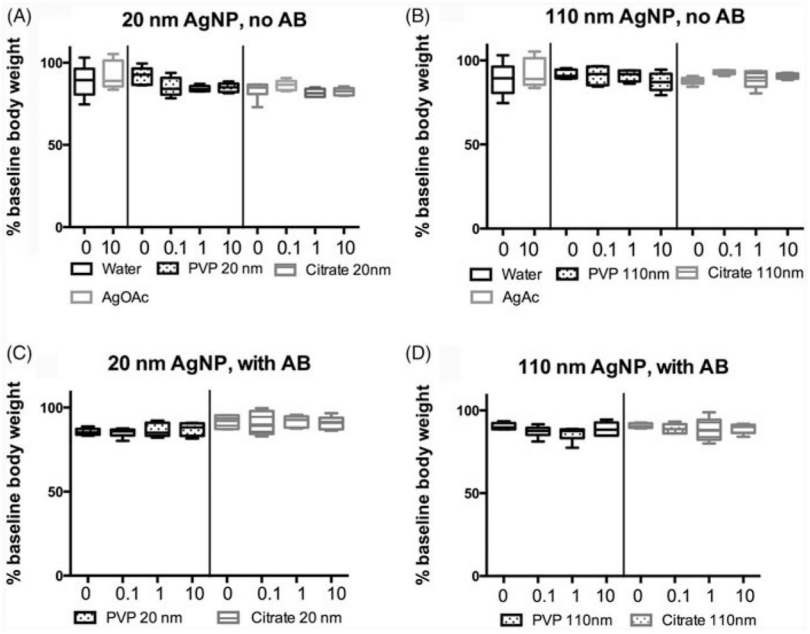


**Figure 1.**

Median percent baseline body weight, all materials, at 24 h after last dose. There were no significant differences between AgNP or AgOAc-dosed groups in comparison to water or coating controls in the no antibiotics groups (A and B) or the groups concurrently dosed with antibiotics (C and D) ($p>0.05$, ANOVA with Tukey's multiple comparisons test). Dose is represented in the *X* axis. Median (horizontal line), interquartile range (box) and total range (whiskers) are shown.

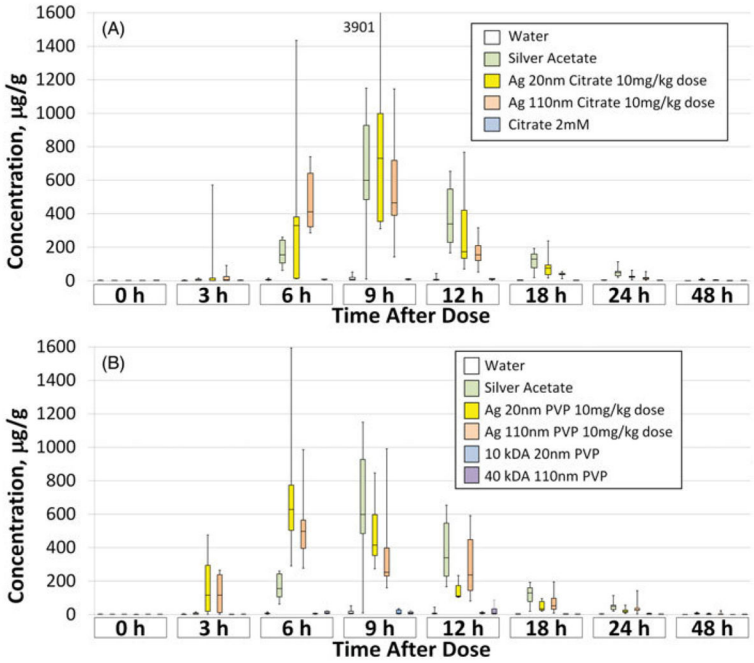


**Figure 2.**

Silver content in feces measured by ICP-OES at timepoints up to 48 h after single-dose oral gavage of 10 mg/kg of citrate-capped AgNP or AgOAc. (A) Citrate-capped AgNP groups. (B) PVP-coated AgNP groups. The water and acetate controls were the same for each group. Box-and-whiskers plots represent median (horizontal line), interquartile range (box) and total range (whiskers).

**Table 1**

Mean relative organ weights (g) ± SD at 24 h after last dose, with no antibiotics.[a]

| | $H_2O$[b] | $AgOA_c$[b] | Vehicle | 20 nM AgNP | | | Vehicle | 110 nM AgNP | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Dose (mg/kg)** | **0** | **10** | **0** | **0.1** | **1** | **10** | **0** | **0.1** | **1** | **10** |
| CITRATE-capped | | | | | | | | | | |
| Brain | 2.04 ± 0.27 | 2.13 ± 0.40 | 2.21 ± 0.41 | 2.12 ± 0.43 | 2.35 ± 0.13 | 2.27 ± 0.31 | 2.61 ± 1.2 | 2.18 ± 0.12 | 2.21 ± 0.16 | 2.02 ± 0.36 |
| Heart/lung | 2.98 ± 0.34 | 2.88 ± 0.25 | 3.12 ± 0.35 | 2.86 ± 0.15 | 3.10 ± 0.31 | 3.23 ± 0.40 | 2.90 ± 0.53 | 2.70 ± 0.24 | 2.46 ± 0.72 | 3.07 ± 0.20 |
| GI tract | 15.3 ± 1.6 | 13.3 ± 0.79 | 13 ± 2.3 | 11.6 ± 1.4 | 12.7 ± 0.40 | 12.6 ± 1.1 | 15.0 ± 4.3 | 13.8 ± 4.1 | 14.8 ± 3.6 | 15.8 ± 0.50 |
| L kidney/adrenal | 0.89 ± 0.13 | 0.89 ± 0.05 | 0.81 ± 0.36 | 0.84 ± 0.24 | 0.85 ± 0.05 | 0.84 ± 0.13 | 0.93 ± 0.18 | 0.89 ± 0.08 | 0.79 ± 0.12 | 0.91 ± 0.05 |
| R kidney/adrenal | 0.90 ± 0.14 | 0.91 ± 0.10 | 1.01 ± 0.35 | 0.81 ± 0.18 | 0.87 ± 0.06 | 1.14 ± 0.58 | 0.82 ± 0.10 | 0.89 ± 0.05 | 0.81 ± 0.14 | 0.82 ± 0.06 |
| Liver | 6.42 ± 0.59 | 6.38 ± 0.51 | 6.22 ± 0.56 | 6.03 ± 0.52 | 5.80 ± 0.58 | 5.62 ± 0.42 | 6.01 ± 0.29 | 6.81 ± 0.75 | 5.68 ± 0.28 | 5.57 ± 0.58[c] |
| Spleen | 0.37 ± 0.08 | 0.38 ± 0.11 | 0.361 ± 0.1 | 0.25 ± 0.05 | 0.27 ± 0.04 | 0.27 ± 0.10 | 0.26 ± 0.06 | 0.39 ± 0.30 | 0.28 ± 0.06 | 0.32 ± 0.03 |
| Testes (paired) | 0.19 ± 0.04 | 0.20 ± 0.03 | 1.51 ± 0.10 | 1.30 ± 0.20 | 1.32 ± 0.30 | 1.17 ± 0.47 | 1.18 ± 0.18 | 1.01 ± 0.26 | 1.12 ± 0.28 | 1.23 ± 0.45 |
| Thymus | 0.22 ± 0.17 | 0.42 ± 0.55 | 0.13 ± 0.04 | 0.21 ± 0.06 | 0.18 ± 0.04 | 0.14 ± 0.06 | 0.21 ± 0.07 | 0.21 ± 0.05 | 0.17 ± 0.03 | 0.26 ± 0.11 |
| PVP-coated | | | | | | | | | | |
| Brain | 2.04 ± 0.27 | 2.13 ± 0.40 | 2.11 ± 0.33 | 2.35 ± 0.07 | 2.35 ± 0.23 | 2.39 ± 0.16 | 2.24 ± 0.16 | 2.25 ± 0.13 | 2.18 ± 0.19 | 2.28 ± 0.25 |
| Heart/lung | 2.98 ± 0.34 | 2.88 ± 0.25 | 2.69 ± 0.32 | 2.84 ± 0.17 | 2.87 ± 0.68 | 2.72 ± 0.41 | 2.98 ± 0.36 | 3.04 ± 0.28 | 3.10 ± 0.27 | 3.14 ± 0.26 |
| GI tract | 15.3 ± 1.6 | 13.3 ± 0.79 | 14.3 ± 1.4 | 13.6 ± 2.4 | 11.8 ± 0.87 | 12.3 ± 2.0 | 15.7 ± 1.3 | 16.2 ± 3.2 | 17.6 ± 2.5 | 16.4 ± 3.2 |
| L kidney/adrenal | 0.89 ± 0.13 | 0.89 ± 0.05 | 0.82 ± 0.09 | 0.94 ± 0.07 | 0.95 ± 0.07 | 0.89 ± 0.21 | 0.92 ± 0.16 | 0.84 ± 0.04 | 0.93 ± 0.10 | 0.95 ± 0.11 |
| R kidney/adrenal | 0.90 ± 0.14 | 0.91 ± 0.10 | 0.89 ± 0.05 | 0.94 ± 0.07 | 0.99 ± 0.13 | 0.90 ± 0.15 | 0.93 ± 0.13 | 0.87 ± 0.04 | 0.91 ± 0.09 | 0.86 ± 0.18 |
| Liver | 6.42 ± 0.59 | 6.38 ± 0.51 | 6.72 ± 0.85 | 6.18 ± 0.49 | 5.44 ± 0.78[d] | 5.85 ± 0.46 | 6.38 ± 1.0 | 6.71 ± 0.20 | 5.69 ± 0.42 | 5.63 ± 0.87 |
| Spleen | 0.37 ± 0.08 | 0.38 ± 0.11 | 0.26 ± 0.08 | 0.36 ± 0.06 | 0.37 ± 0.17 | 0.35 ± 0.07 | 0.49 ± 0.57 | 0.29 ± 0.03 | 0.30 ± 0.20 | 0.31 ± 0.01 |
| Testes (paired) | 1.42 ± 0.25 | 1.45 ± 0.39 | 1.61 ± 0.44 | 1.82 ± 0.44 | 1.57 ± 0.46 | 1.67 ± 0.4 | 1.33 ± 0.30 | 1.11 ± 0.36 | 1.30 ± 0.25 | 1.45 ± 0.41 |
| Thymus | 0.22 ± 0.17 | 0.42 ± 0.55 | 0.19 ± 0.10 | 0.14 ± 0.04 | 0.15 ± 0.03 | 0.22 ± 0.05 | 0.26 ± 0.12 | 0.21 ± 0.04 | 0.62 ± 0.78 | 0.17 ± 0.11 |

[a] Statistical significance was determined by comparison of AgNP groups to coating controls and AgOAc groups to water (ANOVA, Tukey multiple comparison's test, significance defined as $p<0.05$).

[b] The same water and silver acetate groups were used as controls for both citrate and PVP-coated AgNPs.

[c] Statistically significant versus low dose ($p = 0.0377$ after multiple comparisons correction). See text for interpretation.

[d] Statistically significant versus vehicle (coating) control ($p = 0.0266$ after multiple comparisons correction). See text for interpretation.

**Table 2**

Cumulative Ag in feces from 0 to 48 h after oral gavage with 10 mg/kg bw/d AgNP or AgOAc.[a]

| | | | Total Ag in feces (μg Ag/g feces) | | Ag in feces (% recovery of administered dose) | |
|---|---|---|---|---|---|---|
| **Dosing group** | **Vehicle (coating)** | **Ag dose (mg/kg)** | **Mean** | **SD** | **Mean** | **SD** |
| AgNP 20 nm | 2 mM citrate | 10 | 111.2 | 56.5 | 98.6 | 32.5 |
| AgNP 110 nm | 2 mM citrate | 10 | 80.5 | 26.5 | 84.0 | 16.6 |
| AgNP 20 nm | 10 kDa PVP | 10 | 95.7 | 33.4 | 81.1 | 22.9 |
| AgNP 110 nm | 40 kDa PVP | 10 | 76.9 | 42.0 | 70.5 | 18.4 |
| AgOAc | Sterile $H_20$ | 10 | 107.0 | 19.3 | 84.0 | 17.0 |
| na | 2 mM citrate | 0 | 2.5 | 0.3 | na | na |
| na | 10 kDa PVP | 0 | 2.5 | 0.3 | na | na |
| na | 40 kDa PVP | 0 | 2.2 | 0.4 | na | na |
| na | Sterile $H_20$ | 0 | 2.1 | 0.5 | na | na |

[a] No significant differences between the Ag-dosed groups (one-way ANOVA, Tukey's multiple comparisons test, significance $p<0.05$).

na, not applicable.

**Table 3**

Ag in gastrointestinal tract (stomach, cecum, esophagus and intestines) at 48 h after oral gavage with 10 mg/kg AgNP or AgOAc.[a]

| | | | Ag in GI tract (μg/g) | | Ag in GI tract (% recovery of administered dose) | |
|---|---|---|---|---|---|---|
| **Dosing group** | **Vehicle (coating)** | **Ag dose (mg/kg)** | **Mean[a]** | **SD** | **Mean[a]** | **SD** |
| AgNP 20 nm | 2 mM citrate | 10 | 0.030 | 0.029 | 0.05 | 0.05 |
| AgNP 110 nm | 2 mM citrate | 10 | 0.015 | 0.008 | 0.02 | 0.01 |
| AgNP 20 nm | 10 kDa PVP | 10 | 0.032 | 0.026 | 0.04 | 0.04 |
| AgNP 110 nm | 40 kDa PVP | 10 | 0.152 | 0.257 | 0.21 | 0.34 |
| AgOAc | Sterile $H_2O$ | 10 | 0.177 | 0.153 | 0.27 | 0.24 |
| na | 2 mM citrate | 0 | 0.002 | 0.000 | na | na |
| na | 10 kDa PVP | 0 | 0.002 | 0.001 | na | na |
| na | 40 kDa PVP | 0 | 0.002 | 0.001 | na | na |
| na | Sterile $H_2O$ | 0 | 0.002 | 0.001 | na | na |

[a]No significant differences between the Ag-dosed groups (one-way ANOVA, Tukey's multiple comparison's test, significance $p<0.05$).

**Table 4**

Ag in tissues (liver, spleen and kidney) at 48 h after oral gavage with 10 mg/kg bw/d AgNP or AgOAc.[a]

| | | | Ag in liver (ng/g)[b] | | Ag in liver (% recovery administered dose) | | Ag in spleen (ng/g)[b] | | Ag in spleen (% recovery administered dose) | | Ag in kidney (ng/g)[b] | | Ag in kidney (% recovery administered dose) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Dosing group** | **Vehicle (coating)** | **Ag dose (mg/kg)** | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** | **Mean[a]** | **SD** |
| AgNP 20 nm | 2 mM citrate | 10 | 16.87 | 11.43 | 0.008 | 0.005 | 22.94 | 9.78 | 0.0110 | 0.0050 | 2.62 | 0.81 | 0.0010 | 0.0003 |
| AgNP 110 nm | 2 mM citrate | 10 | 11.19 | 7.45 | 0.005 | 0.003 | 24.45 | 11.13 | 0.0114 | 0.0053 | 2.12 | 0.20 | 0.0010 | 0.0001 |
| AgNP 20 nm | 10 kDa PVP | 10 | 12.88 | 4.83 | 0.006 | 0.002 | 26.44 | 9.34 | 0.0128 | 0.0045 | 2.21 | 0.14 | 0.0011 | 0.0001 |
| AgNP 110 nm | 40 kDa PVP | 10 | 8.11 | 6.65 | 0.004 | 0.003 | 33.17 | 18.11 | 0.0164 | 0.0091 | 2.43 | 0.20 | 0.0012 | 0.0001 |
| AgOAc | sterile $H_2O$ | 10 | 301.32### | 162.50 | 0.144### | 0.810 | 94.44## | 60.37 | 0.045‡ | 0.029 | 12.54### | 6.77 | 0.0060### | 0.0034 |
| na | 2 mM citrate | 0 | 1.04 | 0.03 | na | na | 15.34 | 0.45 | na | na | 2.23 | 0.23 | na | na |
| na | 10 kDa PVP | 0 | 0.98 | 0.07 | na | na | 22.78 | 9.92 | na | na | 2.28 | 0.40 | na | na |
| na | 40 kDa PVP | 0 | 1.00 | 0.06 | na | na | 12.33 | 6.87 | na | na | 2.22 | 0.30 | na | na |
| na | sterile $H_2O$ | 0 | 0.92 | 0.09 | na | na | 22.94 | 11.46 | na | na | 2.17 | 0.17 | na | na |

[a] Significantly different from AgNP groups

### $p<0.0001$,

## $p = 0.002$,

‡ $p = 0.003$; one-way ANOVA, Tukey's multiple comparisons test.

[b] Note that mass recovery of Ag is expressed in ng/g tissue.