Differential Effects of Silver Nanoparticles and Silver Ions on Tissue Accumulation, Distribution, and Toxicity in the Sprague Dawley Rat Following Daily Oral Gavage Administration for 13 Weeks

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ABSTRACT

There are concerns within the regulatory and research communities regarding the health impact associated with consumer exposure to silver nanoparticles (AgNPs). This study evaluated particulate and ionic forms of silver and particle size for differences in silver accumulation, distribution, morphology, and toxicity when administered daily by oral gavage to Sprague Dawley rats for 13 weeks. Test materials and dose formulations were characterized by transmission electron microscopy (TEM), dynamic light scattering, and inductively coupled mass spectrometry (ICP-MS). Seven-week-old rats (10 rats per sex per group) were randomly assigned to treatments: AgNP (10, 75, and 110 nm) at 9, 18, and 36 mg/kg body weight (bw); silver acetate (AgOAc) at 100, 200, and 400 mg/kg bw; and controls (2 mM sodium citrate (CIT) or water). At termination, complete necropsies were conducted, histopathology, hematology, serum chemistry, micronuclei, and reproductive system analyses were performed, and silver accumulations and distributions were determined. Rats exposed to AgNP did not show significant changes in body weights or intakes of feed and water relative to controls, and blood, reproductive system, and genetic tests were similar to controls. Differences in the distributional pattern and morphology of silver deposits were observed by TEM: AgNP appeared predominantly within cells, while AgOAc had an affinity for extracellular membranes. Significant dose-dependent and AgNP size-dependent accumulations were detected in tissues by ICP-MS. In addition, sex differences in silver accumulations were noted for a number of tissues and organs, with accumulations being significantly higher in female rats, especially in the kidney, liver, jejunum, and colon.

Key words: silver; nanoparticle; silver acetate; exposure assessment; accumulation; distribution; *in vivo*; oral administration; rodent; transmission electron microscopy

Silver nanoparticles (AgNPs) are among the most widely used nanomaterial in consumer products, and their use in the food industry is a growing public concern in regards to safety, toxicity, and health risk (Chen and Schluesener, 2008; Wijnhoven

et al., 2009). Silver metal has been used for centuries as an anti-bacterial agent, and the release of silver ions, in particular Ag⁺, at the surface of the metal is considered the source of its anti-bacterial activities. Silver ions are known to have toxic effects

in a number of biological species, including bacteria, viruses, fungi, and some aquatic organisms (Liu and Hurt, 2010; Rai et al., 2014). In contrast, silver exhibits relatively low toxicity in humans, due to the fact that human body fluids contain large amounts of chloride and sulfide ions that form insoluble salts with silver ions and protect against silver toxicity (Zhang et al., 2013). Still, cases of argyria (the irreversible gray or bluish gray pigmentation of the skin) and irreversible neurologic toxicity and death have been reported upon long-term ingestion of colloidal silver (Hadrup and Lam, 2014; Mirsattari et al., 2004). The mechanisms that apply to the fate and transport of bulk and colloidal silver may not apply to nanoscale silver.

The antibacterial properties of AgNP are related both to the small size of the particles and to the propensity to form silver ions (Doty et al., 2005; Lok et al., 2007). The antibacterial properties of AgNP tend to increase with decreasing particle size; smaller particles offer increased surface-area-to-mass ratios and higher surface reaction activities (Lok et al., 2007). Dispersed AgNP readily form silver ions on their surfaces in the presence of oxygen; however, AgNP tend to aggregate in the presence of electrolytes, resulting in decreased ion formation and antibacterial activities (Moskovits and Vlckova, 2005). A capping agent is often used in the synthesis of AgNP to stabilize their size, shape, and solubility and to limit their aggregation, reactivity, and formation of ions (Liu and Hurt, 2010). A recent appraisal of the scientific literature revealed that sodium citrate is the most commonly used capping agent for this purpose (Tolaymat et al., 2010).

In view of their potential widespread distribution, consumers will likely be exposed to AgNP via multiple routes. Reports indicate that in animals exposed orally, AgNP are distributed to multiple organs (Loeschner et al., 2011). Studies have shown that the uptake of ingested nanoparticles occurs via M-cells and enterocytes in the intestinal mucosa and that the fraction of the administered dose that is absorbed ranges from 0.4% to 10% depending on the species (Florence, 1997, 2005). The degree to which AgNP are absorbed may be related to particle size, surface charge, or the presence of oxygen. It is generally accepted that absorption increases with decreasing nanoparticle size and that maximal absorption occurs with particles <100 nm in size (Florence, 2005; Jani et al., 1990).

The aggregation and/or agglomeration of AgNP in the lumen of the intestinal tract results in decreased absorption, and this may depend on the presence of electrolytes in the matrix. Positively charged particles appear to be absorbed more efficiently through the gastrointestinal tract than neutral or negatively charged particles (Hussain et al., 2001). The commonly used methods for the synthesis of AgNP usually produce negatively charged AgNP (Tolaymat et al., 2010). Studies have shown that the surfaces of AgNP adsorb Ag^+ , so suspensions of AgNP likely contain 3 species that can affect the absorption kinetics: silver metal, free Ag^+ , and surface-adsorbed Ag^+ . In addition, thermodynamic and kinetic measurements indicate that the release of Ag^+ from AgNP is a cooperative oxidation process involving protons and dissolved oxygen, and that the AgNP will not persist under some conditions but will proceed to full dissolution and disappearance of the particle phase (Liu and Hurt, 2010).

The aim of this study was to investigate the differential accumulation, distribution, and potential toxicity of silver in rats following daily oral exposure to discrete sizes (10, 75, and 100 nm) of citrate-capped AgNP or silver acetate (AgOAc) for 13 weeks.

MATERIALS AND METHODS

Test Materials

Three sizes of citrate-coated AgNP (10, 75, and 110 nm) were purchased (>99.5% pure; 1.0 mg/ml nominal concentration in 2 mM sodium citrate buffer) from nanoComposix (San Diego, California) and were received as weekly batch shipments of each particle size. The AgNP were spherical in shape and the synthesis and characterization procedures used by the manufacturer qualified these particles as representative nanomaterials in the Organisation for Economic Co-operation and Development (OECD) testing program. A unique lot number was pre-assigned by the manufacturer to each size of AgNP within a batch shipment; multiple containers of the same particle size were assigned the same lot number. Upon arrival, the stock suspensions were stored in their original containers in the dark at 4°C–8°C. Prior to submission of subsamples for characterization, stock suspensions of AgNP were mixed by inversion and ultrasonic bath sonication (Model 8510, Branson Ultrasonic Corp., Danbury, Connecticut) for 30 min at 100% output and ambient temperature (~25°C). The multiple containers of each size of AgNP within a batch were then combined into a single vessel, followed by magnetic stirring (Model PC103; Corning Life Sciences, Tewksbury, Massachusetts) for a minimum of 10 min prior to subsample collection.

In order to determine the amount of silver in the AgNP stock suspensions that was in ionic form, filtrates of the AgNP suspensions were prepared by centrifugation through a micro-centrifuge cellulose filter that had a molecular weight cut-off of 3 kDa (Amicon® Ultra-4 centrifugal filter units; Millipore Corporation, Billerica, Massachusetts). Subsamples (1 ml) of the pre-mixed AgNP stock suspensions were applied to the Amicon filter units and centrifuged at 4000 × g for 30 min in an Eppendorf 5810R centrifuge that was equipped with an Eppendorf A-4-81 swinging bucket.

AgOAc was purchased 99% pure as a single lot from Gelest, Inc. (Morrison, Pennsylvania), and batches of stock aqueous solutions (nominal concentration 10.4 mg/ml) were prepared in 18 megaohm and autoclave-sterilized water on an 'as needed' or bi-weekly basis and stored at room temperature. Samples were submitted for inductively coupled mass spectrometry (ICP-MS) analysis to determine the actual silver mass concentrations. The AgOAc solutions remained clear and colorless throughout the useful life of the solution of ~2 weeks, indicating that the silver did not precipitate or become reduced.

Characterization of AgNP Stock Suspensions and AgOAc Solutions

Test material characterization was conducted weekly at the National Center for Toxicological Research (NCTR) on triplicate subsamples of each of the AgNP stock suspensions and on freshly prepared AgOAc solutions.

The stability and homogeneity of the particle size for each of the AgNP were determined on a single batch shipment of AgNP stock suspensions. For the stability study, total silver concentrations were determined by ICP-MS, initially for the AgNP stock suspensions and filtrates, and subsequently for filtrates collected from the same stock suspensions after storage in the dark at 4°C–8°C for 1 and 90 days. The size distributions and morphological characteristics of the particles were determined in subsamples from the same AgNP stock suspensions collected initially and after storage in the dark at 4°C–8°C for 1, 5, 7, 14, 29, 60, and 90 days by dynamic light scattering (DLS) and transmission electron microscopy (TEM). Homogeneity testing was conducted on AgNP stock suspensions from the same shipment as

the stability study; however, triplicate subsamples were collected from the top, middle, and bottom of each vessel and analyzed in triplicate.

Prior to measurement by ICP-MS, aliquots of AgNP stock suspensions and filtrates were mixed in an ultrasonic bath, and triplicate (25–50 μ l) samples were weighed and transferred into separate 20-ml digestion vessels. The samples were digested with ultrapure concentrated nitric and hydrochloric acids in a microwave accelerated reaction system (MARS-X system; CEM Corp., Matthews, North Carolina). After digestion, the samples were cooled and quantitatively transferred into 50-ml polypropylene centrifuge tubes. The samples were diluted to 50 ml with an acid mixture of 1N each of nitric and hydrochloric acids. Subsamples of the AgOAc stock solutions were treated similarly; however, samples did not require digestion prior to analysis.

The average total silver concentrations by mass of the AgNP and AgOAc samples were determined with a Thermo Scientific XSERIES 2 Quadrupole ICP-MS (Franklin, Massachusetts), using ^{107}Ag , ^{109}Ag , and $^{103}\text{Rhodium}$ (Rh) at 50 ng/ml and $^{115}\text{Indium}$ (In) at 100 ng/ml as internal standards. The concentration of Ag was quantified against an external calibration curve (NIST traceable silver samples). The limit of quantification (LOQ) for the AgOAc and AgNP suspensions and filtrates was estimated to be 40 ng/ml. The percentage of ionic silver in the stock AgNP suspensions was calculated by dividing the silver content (ng/ml) in the filtrates by the silver content (ng/ml) in the unfiltrated AgNP stock suspensions.

The hydrodynamic diameter and zeta potential of particles in the AgNP stock suspensions and AgOAc stock solutions were determined using DLS analysis (Zetasizer-nano ZS analyzer, v 6.12, Malvern Instruments Ltd, Malvern, United Kingdom). Prior to sample measurement, the instrument was equilibrated to a temperature of 25°C for 2 min, and the samples were diluted 10-fold with 18 megaohm water. Volumes of 40 μ l were transferred to a micro-cuvette and 3 measurements were performed on triplicate samples using an average of 3 runs of 50 s each. The laser power and the measurement position within the cuvette were determined automatically by the instrument. The size distribution by intensity of the AgNP was assessed using the z-average (d.nm) parameter. Electrophoretic mobility (converted to zeta potential) measurements were performed on triplicate (100 μ l) samples of the AgNP suspensions that were diluted 10-fold in 18 megaohm water and transferred to a clear disposable zeta cell (Malvern, United Kingdom). After equilibration to a temperature of 25°C for 2 min, 3 measurements were performed with the number of runs determined by the instrument.

TEM was used as an additional technique to confirm the AgNP size and to obtain information on the morphological features of particles in the stock suspensions. TEM determinations were conducted using a JEM-2100 200 keV instrument (JEOL USA, Inc., Peabody, Massachusetts) that was operated at an accelerated voltage of 80 keV. The TEM instrument was equipped with a 4k $\times$ 4k pixel charged couple device (CCD) camera (US4000, Gatan, Inc., Warrendale, Pennsylvania). The AgNP suspensions (two microliters) were distributed onto copper electron microscopy grids (EMS) in triplicate and diluted two-fold in isopropyl alcohol. Particles within an image were selected using EMAN software (Baylor College of Medicine, Houston, Texas) and analyzed with the public domain Java image processing program, ImageJ (version 1.47, NIH, available for download at <http://rsb.info.nih.gov/ij/download.html>). The median number of particles evaluated per sample was 201 (range 88–429).

The identity of AgOAc was confirmed by ^1H nuclear magnetic resonance spectroscopy on an AVANCE III spectrometer equipped with a BBFO Plus Smart Probe (Bruker Instruments, Billerica, Massachusetts) operating at 500 MHz. The samples were dissolved in D_2O and the acquisition was conducted at room temperature using a standard ^1H acquisition sequence.

Dose Formulation and Characterization

Materials. Carboxymethyl cellulose (CMC) and trisodium citrate dihydrate (CIT) were purchased from Sigma Aldrich (St. Louis, Missouri), and methyl cellulose (MC) was purchased from Fisher Scientific (Fair Lawn, New Jersey). The water used in dose formulations was 18 megaohm and autoclave-sterilized.

Dose preparation. Dose formulations were prepared weekly and were based on the total silver mass concentrations obtained by ICP-MS for the batch stock AgNP suspensions and AgOAc solutions. The high-dose formulation (0.9 mg/ml) for each size of AgNP was prepared in the CIT/CMC vehicle (2 mM sodium citrate/0.1% CMC; final concentration; wt/wt), and the high dose formulation of AgOAc (10 mg AgOAc/ml or approximately 6.46 mg Ag/ml) was prepared in water/0.1% MC (final concentration; wt/wt). The total silver concentrations of the high-dose formulations were confirmed by ICP-MS and, subsequently, the AgNP high-dose formulations were serially diluted in the CIT/CMC vehicle to achieve the 0.45 and 0.225 mg/ml concentrations for the mid- and low-dose formulations, respectively. Similarly, the total silver concentration of the AgOAc high-dose formulation was confirmed by ICP-MS and then serially diluted in water/0.1% MC to achieve the 3.23 and 1.62 mg Ag/ml concentrations for the mid- and low-dose AgOAc formulations, respectively.

Dose characterization. The total silver concentration by mass of each of the prepared dose formulations (high-, mid-, and low-dose) and the controls (CIT/CMC and water/MC) was determined by ICP-MS in acid- and microwave-digested samples. The hydrodynamic sizes of particles in the dosed solutions were determined by DLS, and the core diameters and aspect ratios of the AgNP were evaluated by TEM. The median number of particles evaluated by TEM per sample was 214 (range 44–498). Characterization analyses were conducted on triplicate samples. Dose concentration acceptability was set at $\pm 10\%$ targeted values.

Rationale for Dose and Route of Exposure

Ingestion is the primary route of exposure in humans for silver, silver compounds, and colloidal silver (Silver, 2003). Daily intakes for silver in humans are estimated in the range 0.4–27 $\mu\text{g/day}$ (Clemente et al., 1977; Gibson and Scythes, 1984; Hamilton and Minski, 1972/1973); however, with more than 1300 manufactured nano-containing consumer products in the marketplace, actual exposures may be greater (Munger et al., 2014). The Integrated Risk Information System (IRIS) Database of the Environmental Protection Agency lists the reference dose for silver as 0.5 $\mu\text{g/kg/day}$ or $\sim 35 \mu\text{g/day}$ for an average 70 kg adult (EPA, 2014).

In a prospective study of commercial 10 and 32 mg/kg bw AgNP-containing solutions, healthy human subjects ingested 100 $\mu\text{g/day}$ of the 10 mg/kg bw solution for 3, 7, and 14 days or 480 $\mu\text{g/day}$ of the 32 mg/kg bw solution for 14 days. At baseline and end of the 14-day dosing periods, subjects underwent a complete physical examination. Ingestion of silver in the diet was not controlled. The oral exposure *in vivo* to these commercial AgNP solutions did not elicit any relevant clinical changes

in human metabolic, hematologic, urine, physical findings, imaging morphology, or cytochrome P450 enzyme inhibition or induction activity, suggesting that toxicity thresholds were not reached even at 480 µg/day of AgNP (Munger et al., 2014, 2015).

The aim of this study was to investigate the potential toxicity of AgNP at doses selected to provide the maximal concentrations that could be achieved with the citrate stabilize particles and that would also ensure stability of the nanoparticles. The nominal concentrations (1 mg/ml) of the AgNP stock suspensions and the maximum gavage dose volume of 20 ml/kg bw per dosing episode (OECD, 1998) set the high dose level for AgNP at 36 mg/kg bw (0.9 mg/ml × 40 ml). Oral gavage, rather than drinking water or dosed feed, was the selected method of dose administration to ensure that the suspensions of AgNP were dispersed at the time of introduction to the animals and to ensure the precision of the dosed amount.

The surface-area-to-mass ratio of AgNP and release of ionic silver at their surface are considered important factors for potential toxicity of AgNP (Oberdorster et al., 2005; Wijnhoven et al., 2009). AgOAc, an ionic form of silver, served as a positive control in this study and as a marker for silver ion toxicity. Limited studies were found that evaluated the toxicity of AgOAc. In studies with mice, Horner et al. (1983) determined that the oral lethal dose, 90% (LD₉₀) of AgOAc was 2505 mg/kg bw. In studies with rats to evaluate AgOAc for developmental toxicity, the lowest observed adverse effect level by the oral route was 30 mg/kg bw/day AgOAc, and the no observed adverse effect level for development toxicity was 100 mg/kg bw/day (NTP, 2002). Based on the wide range of doses in toxicity tests and concern that data on AgNP would yield mostly negative findings, a low dose of 100 mg AgOAc/kg bw/day (64.6 mg Ag/kg bw/day) was selected.

Animal Source, Housing, and Treatment

This study was conducted in accordance with FDA regulations for Good Laboratory Practices in Non-Clinical Studies (CFR, 2010), the OECD guidelines for testing chemicals in toxicity studies in rodents (OECD, 1998), and the NTP specifications for the conduct of studies in laboratory animals (NTP, 2011). The animal care and all experimental procedures were performed in accordance with an animal study protocol that was approved by the NCTR Institutional Animal Care and Use Committee.

In preliminary studies, the pharmacokinetic properties of AgNP and AgOAc were examined to determine whether or not particle size or the administered mass particles (dose, mg/kg) affected oral bioavailability. Groups of 7-week-old male and female Sprague Dawley/CD-23 rats (2 males and 2 females per group) were administered by oral gavage a single dose of AgNP (10, 75, and 110 nm) or AgOAc at 10 mg/kg, and tail vein blood was sampled (100 µl/time point) at 0, 5, 15, and 30 min, and 1, 2, 4, 6, 8, 12, 24, 48, and 72 h after administration and stored at -70°C until analyzed for Ag content by ICP-MS. Animals were euthanized humanely by carbon dioxide asphyxiation after the 72 h blood sample collection.

For the main study, 3-week-old male and female Sprague Dawley/CD-23 rats with specific pathogen-free health status were obtained from the NCTR breeding colony. At 6 weeks of age, the rats were weight-ranked and randomly assigned to treatment groups. Male and female rats were housed conventionally in separate animal rooms with 2 animals per cage. The environment of the animal rooms was set to maintain a 12-h light cycle, temperature of 22 ± 4°C, relative humidity of 40%–70%, and air changes of 10–15 per hour. The animals were provided NIH-41 gamma-irradiated pellets and Millipore-filtered

drinking water *ad libitum*. Rats were dosed initially at 7 weeks of age.

Groups of rats (10 males and 10 females) were exposed daily by oral gavage to dose formulations of AgNP (10, 75, or 110 nm) at 9, 18, and 36 mg/kg bw; AgOAc at 100, 200, and 400 mg/kg bw; or to the respective control formulations (CIT/CMC or water/MC) for a period of 13 weeks. Gavage dosing was conducted using computer-controlled MicroLab® 500 series dispensers (Hamilton Co., Reno, Nevada) equipped with gastight syringes and capable of dispensing 1 µl to 50 ml. The syringes were fitted with flexible plastic gavage needles, and the rats were provided equal volume doses based on the daily body weight of the individual rats. The MicroLab dispensers were programmed to administer the total daily dose in 2 daily gavage administrations per day, with half of the dose administered at the start of the light cycle and half of the dose administered just prior to start of the dark cycle. The dose volumes did not exceed 20 ml/kg bw (OECD, 1998). Animals were dosed 7 days each week and the study period was 13 weeks.

Throughout the study, health checks of animals were conducted twice daily, and weekly clinical observations on individual animals were recorded in the animal records database (NCTR Multi-generation Computer Support System; MGSS). Feed and water consumptions were measured and recorded weekly in MGSS, and body weights recorded daily in MGSS for dose administration. Blood was separately collected via tail vein into Microtainer® tubes (Becton Dickinson Co., Franklin Lakes, New Jersey), with K₂EDTA, at weeks 1, 4, 8, and 12 for the determination of blood silver concentrations and micronuclei assays.

Terminal sacrifices were conducted on over-night fasted rats. At terminal and moribund sacrifices, rats were weighed (necropsy weight), anesthetized by exposure to carbon dioxide, and cardiac blood was withdrawn until exsanguination for hematology and clinical chemistry evaluations. After verification of death, a complete necropsy was performed on all animals.

Tissue collection. At necropsy, organs and tissues were examined for grossly visible lesions, and protocol designated tissues and organs were weighed and preserved in 10% neutral buffered formalin (NBF), with the exception that modified Davidson's fixative was used for the right testes and eyes. Femur bone marrow was collected for histopathology (left) and ICP-MS (right). The testes and epididymides were collected for sperm analysis (left) and histopathology (right). A section of the ileum and scrapping of the ileal mucosa were collected for intestinal microbiota and immune response evaluations (Williams et al., 2015). Sections of the proximal ileum, jejunum, proximal colon, and mesenteric lymph nodes, the right kidney, median and left lateral liver lobes, and spleen were preserved for 2 weeks at 4°C in Karnovsky's fixative and stored in 10% NBF at 4°C for TEM evaluations. Additional sections of these organs and tissues, along with the heart and uterine horn, were collected, flash frozen in liquid nitrogen, and stored at -80°C for total silver analysis by ICP-MS.

Tissue preparation for TEM and energy-dispersive X-ray spectroscopy (EDS). Tissue sections of rats exposed only to the highest dose (36 mg/kg bw) of AgNP or the lowest dose (100 mg/kg bw) of AgOAc were used for TEM and EDX evaluations. Fixed tissue sections that were stored in 10% NBF at 4°C were transferred subsequently to 4% glutaraldehyde in 0.1 M Sorensen phosphate buffer (SPB, pH 7.4) for 2 h, rinsed 3 times in SPB, and post-fixed in 1% w/v osmium tetroxide (OsO₄) in SPB for 1 h. The

specimens were dehydrated in graded series of ethanol, transferred to propylene oxide, and embedded in Epon™ epoxy resin (Hexion Inc., Columbus, Ohio) in accordance with standard procedures (Maunsbach and Afzelius, 1999).

Epon™-embedded tissues were ultra-thin sectioned (≤ 70 nm) with an EM UC6 microtome (Leica Microsystems, Inc., Buffalo Grove, Illinois) and 4–6 serial sections were collected on copper grids with and without carbon coating. Sections on copper grids without carbon coating were left unstained, while sections on copper grids with carbon coating were stained with uranyl acetate and lead citrate and used as a reference to localize the deposition of AgNP and AgOAc in the sections. The sections were examined with a JEM-1400 TEM (JEOL USA Inc.) operated at an accelerating voltage of 80 kV and equipped with an Orius ($4k \times 4k$ pixels) CCD camera (Gatan, Inc.). Digital images were recorded and processed with the Digital Micrograph Software (Gatan, Inc.).

Analysis of the elemental composition of silver deposits in unstained sections was performed with a JEM-2100 200 keV instrument operated at an accelerated voltage of 80 kV and equipped with a Genesis XM2 EDS (EDAX Inc., Mahwah, New Jersey). Elemental data obtained from EDS analyses were plotted using Origin Software (v. 8.5, OriginLab Corporation, Northampton, Massachusetts) and cross-verified with the original graphs. The diameters and aspect ratios of granule deposits within tissue sections were determined using the ImageJ program (version 1.47; NIH), and the size frequency of particles ($n \sim 60$) for each tissue was charted.

In vivo assessment of micronuclei, vaginal cytology, and sperm count, motility, and morphology. The genotoxic effects of AgNP exposure or AgOAc exposure in male and female rats were examined with a micronucleus assay, using flow cytometric analysis of peripheral blood (Witt et al., 2008). Blood samples obtained from rats at weeks 1, 4, and 12 of the study were fixed in ultracold methanol and stored at -80°C until analyzed for the frequency of micronucleated cells in 20 000 reticulocytes per sample.

The effect of AgNP exposure or AgOAc exposure on estrous cycling in female rats was conducted by vaginal lavage each of 16 consecutive days prior to terminal sacrifice. A sterile glass medicine dropper was used to gently flush the vagina (2 or 3 times) with physiological saline, and the vaginal lavages were placed on glass slides, air dried, and fixed by immersion in methanol. The slides were stained with 0.5% toluidine blue and the cells were evaluated by light microscopy for stage of estrous cycle (diestrus, proestrus, or estrus).

The left testis and the caudal epididymis of male rats were used to determine sperm counts, motility, and morphology. For testis sperm count, the tunica albuginea was removed, and the parenchyma was weighed and homogenized in distilled water. A sample of the testis suspension (100 μl) was added to a vial, and the total sperm count evaluated with a sperm analyzer (12.3 Tox Integrated Visual Optical System, Hamilton Thorne Biosciences, Beverly, Massachusetts). Sperm motility and morphology were determined on the caudal epididymis. The caudal portion of the epididymis was placed in buffer (1% bovine serum albumin in phosphate-buffered saline), and the distal portion of the cauda was pierced 3 times to allow sperm to migrate. The onset and completion times were recorded for the migration period. Aliquots of the caudal suspension were loaded onto a chamber of the sperm analyzer (Hamilton Thorne Biosciences), and sperm motility was determined by the instrument automatically in 5 scanned image fields. For caudal sperm count, a sample of the caudal suspension (100 μl) was added to a vial,

and the total sperm count evaluated with the sperm analyzer (Hamilton Thorne Biosciences). Morphological features of sperm were evaluated on the same suspensions used for sperm motility. Aliquots of the suspensions were placed on glass slides, dried, and stained with a 5% eosin aqueous solution. Morphology was examined manually by light microscopy in a total count of 200 normal sperm.

Histopathology. Protocol designated tissues and organs and grossly visible lesions were trimmed, processed, embedded in paraffin (Formula R, Leica Microsystems, Inc.), sectioned at $\sim 5 \mu\text{m}$, and stained with hematoxylin and eosin. The study pathologist examined the specimens and microscopic findings were recorded. A complete microscopic examination was conducted for all organs and tissues of control animals, the highest dose groups with 60% survival, and all higher dose groups. When applicable, non-neoplastic findings were graded for severity.

Statistical analyses. A Cox proportional hazard model was used to analyze survival data and to test the effect of treatment relative to control. All tests were conducted as 2-sided with significance at the .05 probability level.

Pharmacokinetic parameters were determined through the use of PK Solutions 2.0 software (Summit Research Services, Montrose, Colorado). The parameters included maximum observed concentration (C_{max}), the area-under-the concentration-versus-time curve (AUC), absorption/distribution half-life ($t_{1/2 \text{ A/D}}$), and elimination half-life ($t_{1/2 \text{ E}}$). The AUC was estimated to the last sampling time using the trapezoidal rule and further extrapolated to infinity ($\text{AUC}_{0-\infty}$).

Body weights, feed consumption, water intake, and blood were analyzed using a 1-way repeated measures, mixed model ANOVA for each sex, with terms for dose, week, and all interactions. Week was treated as the repeated measure. For body weight analyses, the last body weight obtained for each dose week was used as the weekly body weight. Within-group correlations were modeled using a heterogeneous first-order autoregressive correlation structure, and pairwise comparisons of the 2 control groups and each of the treatment groups to the appropriate control group were performed with Bonferroni adjustments.

The statistical analysis of hematology and clinical chemistry data was performed using a non-parametric method with mid-ranks (Brunner et al., 2002). Pairwise comparisons were performed with Bonferroni adjustments. All tests were conducted as 2-sided with significance at the .05 probability level.

The organ weights were analyzed using a 1-way analysis of covariance with necropsy body weights as a covariate. Pairwise comparisons were performed with Bonferroni adjustments.

Sperm motility, testes count, cauda count, and sperm morphology (number of normal/abnormal of 200 sperm) were analyzed for treatment differences using a 1-way ANOVA, and pairwise comparisons were performed with Bonferroni adjustments.

Estrous cycle length was assessed in female rats for 16 days prior to sacrifice, and the proportion of days spent in each estrous phase (diestrus, proestrus, and estrus) was analyzed for treatment differences using the arcsine-square root transformed proportion with a 1-way ANOVA.

The statistical analysis of the total silver concentration in blood was performed using a 2-way, unbalanced, repeated-measures analysis of variance for treatment and sex differences. For blood and all other endpoints, a non-parametric

method with mid-ranks (with the average of the left and right ranks as boundaries) and an unstructured covariance was applied, with values below the LOQ given a lower rank than measured values (Brunner *et al.*, 2002). Linear dose trend tests for each particle size and pairwise comparisons were performed with Bonferroni adjustments. Sex comparisons were performed for each time and for each treatment group. All tests were conducted as 2-sided with significance at the .05 probability level.

Micronuclei analysis was conducted for each sex using a 2-way fixed effect ANOVA to determine the effect of treatment using the arcsin square root transformation for percent data. Pairwise comparisons of the 2 control groups and each of the treatment groups to the appropriate control group were performed with Bonferroni adjustments. All tests were conducted as 2-sided with significance at the .05 probability level.

The Poly-3 test (Bailer and Portier, 1988), with variance correction (Bieler and Williams, 1993) and the NIEHS continuity-correction (Peddada and Kissling, 2006), was used to compare age-adjusted non-neoplastic lesion incidence among treatment groups.

RESULTS

Homogeneity and Stability of the AgNP Suspensions

The results of homogeneity and stability tests are shown in Table 1 and indicate that the mixing procedure provided evenly dispersed particles throughout the vessel. Stability testing by DLS and TEM was conducted on the same combined lot for each particle size suspension and by ICP-MS on filtrates for ionic silver determinations for 90 days. The hydrodynamic size of the particles determined by DLS and the core size of the particles determined by TEM remained stable over the testing period;

although, the TEM determinations of particle core diameters were smaller (9.29 ± 0.13 nm) for the 10 nm AgNP when compared with measurements obtained by DLS (17.43 ± 0.07 nm). ICP-MS analysis of filtrate samples showed only a very slight increase in soluble silver formed over the 90-day period (Table 1).

Characterization of AgNP Stock Suspensions, AgOAc Solutions, and Dose Formulations

Shipments of AgNP suspensions were received on a weekly basis, and total silver concentration determinations were obtained initially for the stock suspensions to prepare the high-dose formulations, again for the high-dose formulations to prepare mid- and low-dose formulations, and finally for all dose levels in order to obtain dose certification analyses prior to animal administration.

The results of the characterization for each of the individual lots of AgNP suspensions and AgOAc solutions are summarized in Table 2. The concentrations of total silver and ionic silver were determined for the AgNP stock suspensions by ICP-MS. The mean total silver concentration in the 10, 75, and 110 nm AgNP stock suspensions, as determined by ICP-MS of nitric acid and microwave digested samples, closely approximated the nominal concentration of 1.0 mg/ml. The average total silver concentration in the AgOAc stock solution was 6.73 ± 0.048 mg/ml and approximated the solubility (10.4 mg/ml) of AgOAc in water; AgOAc is composed of 64.6% silver by weight. The proportion of silver in the AgNP stock solutions that was present in dissolved ionic form was determined by means of ultrafiltration over a 3-kDa membrane. TEM examination of filtrates confirmed that AgNP did not cross the membrane, and ICP-MS determinations of the pre-filtered samples, filtrates, and re-suspensions of the concentrated solutes and filtrates indicated

TABLE 1. Homogeneity and Stability of AgNP Stock Suspensions

Nominal Diameter (nm)	Analysis	Homogeneity Study ^a	Stability Study	
			Day 0	Day 90
10	Hydrodynamic size (nm) $\pm$ SD		17.43 ± 0.07	16.43 ± 0.055
	Core size (nm) $\pm$ SD		9.29 ± 0.13	8.19 ± 2.17
	Aspect ratio $\pm$ SD		1.10 ± 0.08	1.1 ± 0.06
	ICP-MS (mg/ml) $\pm$ SD	0.941 ± 0.011	0.933 ± 0.010	
		0.928 ± 0.007		
		0.931 ± 0.008		
	Filtrate (ng/ml) ^b		55	130
75	Hydrodynamic size (nm) $\pm$ SD		74.35 ± 0.62	74.03 ± 0.103
	Core size (nm) $\pm$ SD		75.27 ± 4.81	71.92 ± 10.43
	Aspect ratio $\pm$ SD		1.12 ± 0.07	1.11 ± 0.07
	ICP-MS (mg/ml) $\pm$ SD	0.978 ± 0.007	0.947 ± 0.023	
		0.936 ± 0.005		
		0.937 ± 0.021		
	Filtrate (ng/ml)		42	106
110	Hydrodynamic size (nm) $\pm$ SD		104.3 ± 12.75	104.3 ± 0.208
	Core size (nm) $\pm$ SD		106.8 ± 12.74	97.53 ± 21.5
	Aspect ratio $\pm$ SD		1.13 ± 0.09	1.12 ± 0.08
	ICP-MS (mg/ml) $\pm$ SD	1.08 ± 0.03	1.07 ± 0.02	
		1.05 ± 0.01		
		1.08 ± 0.02		
	Filtrate (ng/ml)		23	142

A single lot of each size of AgNPs was selected for homogeneity and stability studies. Silver concentration was determined by IC-MS analyses, the shape, and size of AgNP was determined by TEM, and size distribution of particles in solutions was determined by DLS.

^aHomogeneity values represent mean $\pm$ SD of triplicate samples collected from the top, middle, and bottom of a single lot for each size of AgNP suspensions (mg/ml).

^bFiltrate values represent the ionic silver content (ng/ml) from the same combined lot of AgNP suspensions. The average limit of quantitation for silver is 40 ng/ml.

an average recovery >98%, irrespective of AgNP size (data not shown). The mean concentration of ionic silver in the AgNP suspensions was similar across nanoparticle sizes and averaged 0.01%–0.02% of the AgNP stock suspensions (Table 2).

The size, morphology, and distribution parameters of the AgNP stock suspensions were determined by TEM and DLS. AgNP size derived from TEM determinations indicated that mean core sizes for the 10, 75, and 110 nm particles were 8.1 ± 1.8 , 72.7 ± 7.2 , and 101.5 ± 15.8 nm, respectively (Table 2). Representative micrographs of the AgNP and histograms of their associated size distributions are shown in Figure 1. These results closely corroborate the data provided by the manufacturer with each shipment of the AgNP suspensions. The morphological characteristics of the AgNP were also evaluated by TEM, and the mean aspect ratios confirmed that the AgNP particles were mostly spherical in shape (Table 2).

The hydrodynamic size was determined for the AgNP by DLS (z-average), as shown in Table 2. Figure 2 illustrates the DLS size distribution graphs by intensity. In the examples, the largest peak in the graph represents 100% intensity, with z-average values of 16.81, 74.04, and 103.7 nm for the 10, 75, and 110 nm AgNP, respectively (Figure 2). The size determinations by DLS were larger than those measured by TEM for the same particle suspension (Table 2).

TEM and DLS provide different types of information about particle size and distribution. TEM gives information regarding the size and morphology of individual naked particles; whereas DLS measures the size distribution of dynamic particles in aqueous suspensions. The discrepancy arises because DLS measures the average hydrodynamic particle diameter, which accounts for both the core and coating of polydispersed nanoparticles in an aqueous solution; whereas TEM measures the size and shape of the electron-dense core diameter of individual nanoparticles that are dehydrated and immobilized on a solid surface (Cumberland and Lead, 2009; Ito et al., 2004). The AgNP used in this study were stabilized with the citrate capping agent, likely accounting for the larger hydrodynamic size when measured by DLS, suggesting that the actual size of the particles reside somewhere within the upper TEM and lower DLS range of values.

A summary of the characterization results for the dose formulations is provided in Table 3. The concentrations of total silver for the AgNP and AgOAc dose and control formulations were determined by ICP-MS. The levels of total silver for each of the control formulations were below the limit of quantitation

(40 ng/ml) by ICP-MS throughout the study. Dose formulations were prepared on a weekly basis at 3 concentrations levels for each size of AgNP (0.225, 0.45, and 0.9 mg/ml) and AgOAc (1.62, 3.23, and 6.46 mg Ag/ml), and the total silver concentration of each formulation was determined by ICP-MS prior to issuance of the dose formulations to animals on the study. The ratio of the determined total silver concentration and the targeted silver concentration is reported for each dose as a percentage of the targeted dose (Table 3). The mean percentage of targeted dose for all doses over the study period was 102 ± 1 , 104 ± 2 , and $104 \pm 2\%$ for the 10, 75, and 110 nm AgNP formulations, respectively, and $104 \pm 3\%$ for the AgOAc formulations.

Dose formulations for the AgNP and AgOAc were prepared in their respective control formulations that contained either 0.1% CMC or 0.1% MC. Both CMC and MC were used in this study as bulking agents to inhibit somewhat the gastrointestinal passage of the mostly aqueous dose formulations. These compounds are not toxic and do not promote allergic reactions in humans or rodents (Frawley et al., 1964; Shelanski and Clark, 1948). The hydrodynamic size of particles were higher in the dose formulations, when compared with the stock solutions, and the CMC and MC were likely responsible for the increase in particle size, as determined by DLS (Table 3). The mean core diameters and aspect ratios of the particles were similar to those of the stock solutions, suggesting that little change occurred in the particle size and shape during the preparation of dose formulations (Table 3).

Pharmacokinetics of Silver

The blood concentration-time curves of silver following a single oral gavage administration of 10 mg/kg bw of AgNP (10, 75, and 110 nm) or AgOAc are shown in Figure 3 for female (A) and male (B) rats, respectively. The calculated half-times for distribution (4–5 h) and elimination (~24 h), and the time to reach maximum concentration levels (12.0 h) were similar for both male and female rats. In contrast, the analysis of the AUC revealed differences due to sex, form of silver, and particle size. As illustrated in Figure 3, the AUC profiles were similar for female (A) and male (B) rats; however, the AUCs were ~2-fold greater for female rats, irrespective of silver form or particle size. The AUCs were greater for AgOAc than for AgNP, and, among AgNP, the AUCs showed an inverse relationship with particle size: smaller particles had larger AUCs than larger particles.

TABLE 2. Mean Results of Test Material Characterization for the Weekly Shipments of AgNP Stock Suspensions and AgOAc Stock Solutions in the 13-Week Study

Test Material Lot	Total Silver Conc. (mg/ml) $\pm$ SD	Ionic Silver Conc. (ng/ml)	Ionic Silver (%)	Mean Core Diameter (nm) $\pm$ SD	Aspect Ratio $\pm$ SD	Hydrodynamic Size $\pm$ SD	Zeta Potential $\pm$ SD
AgNP 10 nm							
Mean (n = 22)	0.968 ± 0.02	215	0.02	8.10 ± 1.79	1.09 ± 0.07	17.10 ± 0.23	-34.86 ± 1.59
AgNP 75 nm							
Mean (n = 22)	1.117 ± 0.02	158	0.01	72.73 ± 7.20	1.13 ± 0.08	73.97 ± 0.43	-46.13 ± 1.37
AgNP 110 nm							
Mean (n = 22)	1.003 ± 0.02	106	0.01	101.54 ± 15.81	1.13 ± 0.08	103.22 ± 0.59	-43.96 ± 1.15
AgOAc							
Mean (n = 16)	6.73	0.05					

Stock suspensions (nominal concentration 1.0 mg/ml) of 3 sizes of citrate-coated AgNP (10, 75, and 110 nm) were received as weekly batch shipments. Stock aqueous solutions of AgOAc (nominal concentration 10.4 mg/ml) were prepared in 18 megaohm sterilized water on a bi-weekly basis. The total silver and ionic silver concentrations of the test materials were determined by ICP-MS, mean core particle diameters, and aspect ratios were determined by TEM, and the hydrodynamic size and stability of particles in suspension were determined by DLS. Values represent the mean $\pm$ SD of 22 shipments of each size of AgNP or of 16 batch preparations of AgOAc.

FIG. 1. Representative micrographs and size distribution histograms of AgNP stock solutions obtained by TEM. TEM was used to determine the mean core size and aspect ratios of AgNP. Representative micrographs and histograms of particle size distributions are shown for (A) a 10-nm AgNP suspension ($n = 325$), (B) a 75-nm AgNP suspension ($n = 161$); and (C) a 110-nm AgNP suspension ($n = 301$).

Survival, Body Weight, Food Consumption, and Water Intake

Five animals in this study were either moribund or died with severe acute nephropathy. These types of renal changes have been noted in Sprague Dawley rats in previous studies

conducted at NCTR and are considered spontaneous findings and not treatment related. In contrast, morbidity was pronounced in groups of rats exposed to the high (400 mg/kg bw) dose of AgOAc, with a majority (70%) of female and (100%) male

FIG. 2. Representative hydrodynamic size distributions of AgNP stock solutions. DLS analysis was used to determine the hydrodynamic size and zeta potential of AgNP in stock suspensions and hydrodynamic size of particles in dose formulations. Representative distribution graphs showing the average hydrodynamic size of AgNP stock suspensions for (A) a 10-nm AgNP suspension, (B) a 75-nm AgNP suspension, and (C) a 110-nm AgNP suspension.

TABLE 3. Mean Dose Formulation Characterization and Dose Certification Determinations in the 13-Week Study of AgNP and AgOAc

Test Material Lot	Target Dose Concentration (mg/ml)	Total Silver Conc. (mg/ml) $\pm$ SD	Percentage of Targeted Dose ^a $\pm$ SD	Mean Particle Core Diameter (nm) $\pm$ SD	Particle Aspect Ratio $\pm$ SD	Particle Hydrodynamic Size (nm) $\pm$ SD
10 nm AgNP/CMC	0.225	0.228 $\pm$ 0.00	101.28 $\pm$ 1.59	8.70 $\pm$ 1.91	1.09 $\pm$ 0.07	22.95 $\pm$ 2.41
	0.45	0.462 $\pm$ 0.01	102.79 $\pm$ 1.40	8.71 $\pm$ 1.82	1.09 $\pm$ 0.06	22.95 $\pm$ 2.62
	0.9	0.943 $\pm$ 0.01	104.79 $\pm$ 1.21	8.64 $\pm$ 1.84	1.09 $\pm$ 0.06	21.80 $\pm$ 1.52
75 nm AgNP/CMC	0.225	0.228 $\pm$ 0.00	101.38 $\pm$ 1.74	74.38 $\pm$ 6.43	1.12 $\pm$ 0.07	79.66 $\pm$ 2.42
	0.45	0.464 $\pm$ 0.01	103.26 $\pm$ 1.55	74.05 $\pm$ 6.60	1.12 $\pm$ 0.07	79.92 $\pm$ 1.52
	0.9	0.961 $\pm$ 0.01	105.91 $\pm$ 1.49	73.90 $\pm$ 6.61	1.13 $\pm$ 0.07	80.02 $\pm$ 1.04
110 nm AgNP	0.225	0.231 $\pm$ 0.00	102.73 $\pm$ 2.13	104.20 $\pm$ 13.91	1.12 $\pm$ 0.08	112.20 $\pm$ 2.50
	0.45	0.471 $\pm$ 0.01	104.58 $\pm$ 2.08	104.36 $\pm$ 14.31	1.12 $\pm$ 0.07	112.24 $\pm$ 2.26
	0.9	0.943 $\pm$ 0.02	104.82 $\pm$ 2.15	102.12 $\pm$ 15.83	1.13 $\pm$ 0.08	111.27 $\pm$ 1.90
AgOAc	1.62	1.684 $\pm$ 0.05	104.28 $\pm$ 3.02			225.14 $\pm$ 14.45
	3.23	3.327 $\pm$ 0.07	103.20 $\pm$ 2.12			225.77 $\pm$ 14.94
	6.46	6.657 $\pm$ 0.15	103.10 $\pm$ 2.30			226.03 $\pm$ 7.15

Dose formulations were prepared weekly and were based on the total silver mass concentrations determined by ICP-MS for the stock AgNP suspensions and the stock AgOAc solutions. Target dose concentrations were set at 0.225, 0.45, and 0.9 mg/ml to provide animals with doses of 9, 18, and 36 mg Ag/kg bw. Reported values are the mean $\pm$ standard deviation of formulations prepared over the course of the study ($n = 22$).

rats being removed prior to the scheduled terminal sacrifice. Clinical findings suggested severe gastrointestinal symptoms, loss of body weight, and unthrifty appearance among these animals, likely due to the bactericidal activity of the silver ion on the intestinal microbiota. There were no significant differences in survival between the vehicle and water controls or between any AgNP group and the vehicle control group in either the female or male rats.

Body weights did not differ significantly between the vehicle and water controls or between the different doses and sizes of AgNP and the vehicle controls in female rats (Figure 4A). Sporadic statistically significantly higher body weights were observed in male rats administered the 10 and 110 nm AgNP, but body weights never exceeded 113% and were not considered biologically significant (Figure 4B). Kim *et al.* (2010) found a significant decrease in the body weights of male rats administered 60 nm AgNP (500 mg/kg bw) and Shahare and Yashpal (2013) found decreased body weights in male mice dosed orally with AgNP (5–20 mg/kg bw), but the majority of investigations found no effects on body weights following oral administration of AgNP (Espinosa-Cristobal *et al.*, 2013; Kim *et al.*, 2008; van der Zande *et al.*, 2012).

Significantly lower mean body weights were observed in female rats administered AgOAc at doses of 100 and 400 mg/kg bw; the overall mean body weights were 88.5% and 74.4 % of the control group, respectively (Figure 4C). Male rats administered 400 and 200 mg/kg bw AgOAc demonstrated significantly lower mean body weights than the controls, beginning at week 1 and at week 3, respectively (Figure 4D). Hadrup *et al.* (2012) also observed depressed body weight gains in rats administered AgOAc (9 mg silver/kg bw) for 28 days, and Matuk *et al.* (1981) found significantly lower body weights in rats administered a 0.25% silver nitrate solution in lieu of drinking water for >10 weeks. In this study, male rats in the low dose (100 mg/kg bw) AgOAc group were administered ~64 mg silver/kg bw/day; body weights in this group were not significantly affected.

There were no significant differences in feed and water intakes between the vehicle and water controls. When compared with respective control animals, sporadic differences in feed and water intakes were observed in female and male rats administered AgNP or AgOAc. The control dose formulations

contained the same concentration of CMC or MC as did the dose formulations of AgNP or AgOAc, and rats were administered equal volume doses based on body weight; therefore, the amount of bulking agent was the same across formulations. In addition, decreased feed and water intakes occurred similarly at both low and high AgNP dose levels, although the high-dose formulations contained 3 times the amount of silver. There were no significant dose trend effects; therefore, these sporadic differences were not considered of biological significance. Other investigations found no significant differences in the feed consumption and water intake of rats administered AgNP (~60 nm) by gavage (30–1000 mg/kg bw/day) for a period of 28 days to 13 weeks (Kim *et al.*, 2008, 2010).

Hematology and Clinical Chemistry

Hematological and plasma clinical chemistry parameters recorded for rats exposed to AgNP and AgOAc are provided in Supplementary Table S1. There were no significant differences in the values for these parameters between the vehicle and water controls, and values recorded for the AgNP-treated groups did not differ significantly from the vehicle controls, with the exception of a disparate value (63% lower than control) for sorbitol dehydrogenase in the 18 mg/kg bw 75 nm AgNP dose group of male rats.

Sporadic differences in the hematological and biochemistry parameters were found for the AgOAc-treated groups when compared with their respective controls. Mean cell volumes (MCV) were significantly lower for the AgOAc mid- and high-dose-treated female rats and for the AgOAc low- and mid-dose-treated male rats. Red blood cell (RBC) counts were significantly higher ($P < .05$ vs control) for the AgOAc low- and mid-dose-treated female rats and for the low-dose male rats (Supplementary Table S3). The significantly lower MCV values and higher RBC counts by AgOAc-treated rats are suggestive of low iron status; however, minerals other than Ag were not evaluated in blood samples.

Organ Weights

The effects of AgNP and AgOAc on the absolute organ weights and relative organ weights (%; organ weight/necropsy body weight) of rats exposed for 13 weeks to these formulations are shown in Supplementary Table S2. There were no meaningful

FIG. 3. Blood concentration versus time curves for silver. Female (A) and male (B) rats ($n=2$) were administered AgNP (10, 75, 110 nm) or AgOAc at 10 mg/kg bw by oral gavage and blood was collected via tail vein at 0, 5, 15, 30 min and 1, 2, 4, 6, 8, 12, 24, 48, and 72 h. Blood samples were analyzed for total silver content by ICP-MS, and maximum concentration (C_{max}), the area under the curve (AUC), absorption/distribution half-life ($t_{1/2 A/D}$), and elimination half-life ($t_{1/2 E}$) were determined.

treatment-related differences in the absolute or relative organ weights of female and male rats exposed to AgNP when compared with control groups. In male rats treated with AgOAc (100 mg/kg bw), the absolute kidney weights were depressed (control, 1.52 g; AgOAc, 1.34 g), and the relative weights were higher for the left epididymis (control, 0.11%; AgOAc, 0.14%) and heart (control, 0.3%; AgOAc, 0.36%); the relative liver weight was higher in the 100 and 200 mg/kg bw group (control, 2.51%; 100 mg/kg bw, 2.80%; 200 mg/kg bw, 2.94%). In female rats exposed to AgOAc at 200 mg/kg bw, the absolute weights for the left kidney (0.85 g) and thymus (0.22 g) were lower when compared with controls (0.97 and 0.32 g, respectively). At the high dose of AgOAc (400 mg/kg bw), the absolute heart (0.88 g) and thymus (0.13 g) weights were lower when compared with the controls (1.09 and 0.32 g, respectively). Relative organ weights

for the liver were higher than control weights (2.55%) in the 200 mg/kg bw dose group (3.39%). Since the necropsy body weights were significantly lower in these same groups of rats, the significant changes in absolute and relative organ weights likely reflect mild dehydration due to the presence of gastrointestinal distress in these animals.

Disposition and Accumulation of Silver

Total silver concentrations by mass in the organs and tissues of female and male rats administered AgNP or AgOAc are shown in [Supplementary Table S3](#). The analysis of silver concentrations by ICP-MS in tissue and organ samples collected from the control groups showed no significant differences in silver concentrations between the water and vehicle controls for any reported parameter; however, all tissues and organs analyzed

FIG. 4. Body weight growth curves during the 13-week administration by oral gavage of AgNP and AgOAc. Plotted values are mean treatment body weights for weeks 1 through 13. (A) Female rats administered AgNP of 10, 75, and 110 nm at doses of 9, 18, and 36 mg/kg bw. (B) Male rats administered AgNP of 10, 75, and 110 nm at doses of 9, 18, and 36 mg/kg bw. (C) Female rats administered AgOAc at 100, 200, and 400 mg/kg bw. (D) Male rats administered AgOAc at 100, 200, and 400 mg/kg bw.

from rats exposed to AgNP or AgOAc for 13 weeks, with the exception of bone marrow, revealed statistically significant ($P < .05$) dose-dependent increases in silver concentrations (Supplementary Table S3). An abbreviated summary of these data are presented in Figures 5, 6 and 7.

Figure 5 presents the silver concentrations in blood collected at the end of weeks 1 and 12 of the in-life study phase and in femur bone marrow collected at necropsy from female and male rats exposed to AgNP (Figs. 5A and B, respectively) or AgOAc (Figs. 5C and D, respectively). The silver content in blood and bone marrow averaged 3–4 times lower than the silver content of the heart (Supplementary Table S3), which had the lowest content of silver among the major organs analyzed and indicated that the contribution of silver accumulation in the blood and bone marrow was minimal. Furthermore, a repeated measures statistical analysis of these data found no significant differences for silver concentrations in rat blood collected at weeks 1 and week 12, with the exception that week 1 levels were higher in male rats exposed to AgNP-110 nm (36 mg/kg bw) (Figure 5B). As blood levels of silver were not significantly higher after 12 weeks of dosing than levels found after only 1 week of dosing, these results suggest rapid clearance of silver from the blood for all groups irrespective of silver form. In preliminary studies, the half-time for elimination of silver from the blood was ~24 h regardless of sex or form of silver.

Results of pairwise comparison tests indicated that silver concentrations were significantly higher in the blood and bone marrow of rats exposed to AgNP-10 nm, especially at the 2 higher doses, when compared with those of rats exposed to AgNP-75 or 110 nm and suggested that the concentration of silver in these samples was inversely related to particle size (Figs. 5A and B). Silver concentrations were significantly higher in the blood and bone marrow of rats exposed to AgOAc when compared with those of rats exposed to AgNP (Figs. 5C and D). Results indicate that a much higher uptake of silver occurred following the administration of AgOAc, than could be accounted for by the differences in silver content of the dose (Supplementary Table S3). For example, the mean blood concentration of silver at week 1 in female rats exposed to AgNP-10 nm at 36 mg/kg bw was 436 ng/ml and that for female rats exposed to AgOAc at 100 mg/kg bw was 1170 ng/ml. The silver content of the AgOAc dose was ~64 mg/kg bw—a 1.8-fold higher dose of silver than that of the AgNP—yet, the blood concentration was almost 3-fold higher, likely reflecting aggregation and decreased absorption of AgNP from the lumen of the gastrointestinal tract. Previous studies have shown that the uptake of ingested nanoparticles ranges from 0.4% to 10% depending on the species (Florence, 2005; van der Zande et al., 2012). Particle charge may be an important determinant in the absorption and distribution processes. Positively charged particles appear to be

FIG. 5. Silver content in the blood and bone marrow of Sprague Dawley rats in the 13-week study of AgNP and AgOAc. Values are reported as the mean $\pm$ standard error of the mean. Tests were conducted as 2-sided at the .05 significance level; an asterisk (*) indicates statistically significant dose trend effects and a cross (†) indicates statistically significant pairwise comparisons to the appropriate controls.

absorbed more efficiently than neutral or negatively charged particles through the gastrointestinal tract (Florence, 2005; Hussain et al., 2001).

Silver concentrations in the flushed, emptied tissues of the gastrointestinal tract were higher than most tissues (Figure 6), although large differences were observed between treatment groups. Silver accumulations were significantly higher in female rats when compared with male rats, with the exception that sex differences were not observed in the ileum (Figs. 6A and B). A similar profile in silver distribution was observed for rats exposed to AgOAc (Figs. 6C and D). Among the gastrointestinal tract tissues examined, the mesenteric lymph nodes had the highest concentrations of silver irrespective of treatment or form of silver administered to rats.

The concentrations of silver in the major organs of rats are shown in Figure 7. In female and male rats exposed to AgNP or AgOAc, the kidney and spleen represented sites of significant silver accumulation. As observed in the intestinal tract, sex differences in silver concentrations were observed. Rats exposed to AgNP-10 nm demonstrated higher accumulations of silver in the major organs than rats exposed to AgNP-75 or 110 nm. The concentrations of silver in all organs were significantly lower in male rats exposed to AgNP. In addition, the pattern of distribution in the kidneys and spleens of male rats differed from that of female rats. In female rats exposed to AgNP-10 nm, kidney

concentration levels of silver were 4-fold higher than spleen concentration levels of silver (Figure 7A) and were 13–18-fold higher than the kidney concentrations of male rats (Figure 7B). In male rats exposed to AgNP-10 nm, silver concentrations in the spleen were 1.4 to 2.4-fold higher than those in the kidney (Figure 7B), indicating a sex-related difference in both the accumulation and pattern of silver in the kidney and spleen. The concentrations of silver in all organs were several folds higher in rats exposed to AgOAc, and, as observed with rats exposed to AgNP, the concentrations of silver in all organs appeared to be higher in females than males. In contrast to the accumulation of silver by rats exposed to AgNP, the accumulation and pattern of silver distribution was similar in the kidney and spleen of female and male rats administered AgOAc (Figs. 7C and D).

Localization of Silver in Rat Tissues

The low accumulation of silver in tissues of rats administered the 75 or 110 nm AgNP diminished the ability to detect silver in these tissues by electron microscopy. In contrast, tissues from rats administered the 10 nm AgNP (36 mg/kg bw/day) demonstrated significant silver accumulations, and results collected from these samples and those of rats administered AgOAc (100 mg/kg bw/day) are reported in this article.

Figure 8 shows the localization of silver within the intestinal tissues of rats exposed to 10 nm AgNP and AgOAc. In rats

FIG. 6. Silver content in the gastrointestinal tract of Sprague Dawley rats in the 13-week study of AgNPs and AgOAc. The silver content in the ileum, jejunum, mesenteric lymph node, and colon of female (A) and male (B) rats exposed to different sizes AgNP at 9, 18, and 36 mg/kg bw or vehicle control for 13 weeks. The silver content in the ileum, jejunum, mesenteric lymph node, and colon of female (C) and male (D) rats exposed to AgOAc at 100, 200, and 400 mg/kg bw or water control for 13-weeks. Values are reported as the mean $\pm$ standard error of the mean. Tests were conducted as 2-sided at the .05 significance level; an asterisk (*) indicates statistically significant dose trend effects and a cross (†) indicates statistically significant pairwise comparisons to the appropriate controls.

exposed to 10 nm AgNP (36 mg/kg bw), the accumulation of silver appeared mostly as round to slightly oval, but strongly agglomerated, electron dense granules, with smooth, continuous surfaces that were observed primarily within the lamina propria region in the jejunum (Figure 8A), in macrophages within the villar lamina propria of the ileum (Figure 8B), and within enterocytes and the lamina propria region of the colon (Figure 8C). In rats exposed to AgOAc (100 mg/kg bw), the accumulation of silver granules was detected primarily along the epithelial basement membrane of the jejunum (Figure 8D), ileum (Figure 8E), and colon (Figure 8F). In contrast to the smooth, continuous surface of the AgNP, the surface morphology of the accumulated electron dense silver granules in the intestine of rats exposed to AgOAc was irregular and segmented (Figs. 8D–F).

The measured diameters and aspect ratios of accumulated granules in the gastrointestinal tract of rats administered the 10 nm AgNP had a tendency to be smaller in the jejunum than in the ileum and colon (Table 4) and approximated the hydrodynamic size (22.2 nm) of the administered dosed particles that were measured by DLS; however, the deposited granules were larger and more ovoid in shape when compared with the average core diameter (8.6 nm) and aspect ratio (1.1) of the 10 nm

dose formulations determined by TEM for the main study. The discrepancies in the measurements of particle size and shape by TEM can be explained by the differences in attraction forces of the particles to each other and to the components of the dispersion media. TEM measurements were obtained on ~200 individual, evenly dispersed particles in the defined dose formulations; whereas TEM measurements of granules deposited in tissues were complicated by the tendency of particles to agglomerate due to attractive forces between the particles themselves and the attractive forces of chemical molecules, such as thiol and chloride groups, within the vicinity of the particles. Mean diameters were larger for granules accumulated in the intestinal tract of rats exposed to AgOAc when compared with rats exposed to 10 nm AgNP (Table 4).

Silver accumulations in the kidneys of rats are shown in Figure 9. In rats exposed to 10 nm AgNP were localized primarily within the renal tubular epithelium (Figure 9A). As shown in Table 4, the majority of silver granules in the kidneys retained a similar size (8.6 ± 1.8 nm vs 18.7 ± 5.6 nm) and aspect ratio (1.1 ± 0.1 vs 1.5 ± 0.4), respectively, as the administered particles, suggesting that the 10 nm AgNP were absorbed as intact particles. The accumulation of silver was observed along the basement membranes of the glomeruli of kidneys from rats

FIG. 7. Silver content in the major organs of Sprague Dawley rats in the 13-week study of AgNPs and AgOAc. The silver content in the kidney, liver, spleen, heart, and uterus of female (A) and kidney, liver, spleen, and heart of male (B) rats exposed to different sizes AgNP at 9, 18, and 36 mg/kg bw or vehicle control for 13 weeks. The silver content in the kidney, liver, spleen, heart, and uterus of female (C) and kidney, liver, spleen, and heart of male (D) rats exposed to AgOAc at 100, 200, and 400 mg/kg bw or water control. Values are reported as the mean $\pm$ standard error of the mean. Tests were conducted as 2-sided at the .05 significance level; an asterisk (*) indicates statistically significant dose trend effects and a cross (†) indicates statistically significant pairwise comparisons to the appropriate controls.

FIG. 8. TEMs of intestinal tissues from 10 nm AgNP and AgOAc exposed female rats. TEM of ultra-thin sections stained with uranyl acetate and lead citrate showing (A) an overview of an intestinal villus within the rat jejunum and highlighting the morphology of a silver granule of interest from within lamina propria; (B) an overview of an intestinal villus within the rat ileum highlighting the round to slightly oval and continuous and smooth surface of an intra-cytoplasmic silver granule within a macrophage (MΦ); (C) an overview of a section of a rat colon showing silver granules within an enterocyte (EN) and highlighting the shape of a silver granule from the lamina propria region; (D) an overview of an intestinal villus within the rat jejunum and highlighting a silver granule deposited along the basement membrane (BM); (E) an overview of an intestinal villus within the rat ileum featuring a silver granule localized within the BM in the ileum; and (F) an overview of colonic tissue with silver granules lining the basement membrane and highlighting a silver granules of interest from the region.

exposed to AgOAc (Figure 9B). Ham and Tange (1972) similarly found the accumulation of silver granules primarily along the basement membranes of the glomeruli in kidneys from rats exposed to AgOAc. In contrast to the small and smooth shape of granules deposited in the kidneys of 10 nm AgNP-exposed rats,

those deposited in the kidneys of AgOAc-exposed rats were large (mean 71.2 ± 31.9 nm) and irregular in shape, often with surface bulges (Figure 9B).

The distribution pattern of silver granules was similar in the livers of AgNP and AgOAc exposed animals (Figure 10), although

TABLE 4. Mean Physical Parameters of Granules Deposits Within the Tissues of Rats Following Oral Gavage Exposure to 10 nm AgNP (36 mg Silver/kg bw/Day) or to AgOAc (64.6 mg Silver/kg bw/Day) for 13 Weeks

	Number Examined	Area ^a Mean ± Std	Diameter Mean ± Std	Aspect Ratio ^b Mean ± Std
10 nm AgNP				
Jejunum	60	334 ± 183	19.9 ± 5.5	1.5 ± 0.4
Ileum	60	497 ± 250	24.3 ± 6.5	1.6 ± 0.5
Colon	56	520 ± 241	25.0 ± 6.3	1.4 ± 0.3
Kidney	60	300 ± 192	18.7 ± 5.6	1.5 ± 0.4
Liver	57	593 ± 362	26.2 ± 8.4	2.1 ± 2.7
Spleen	60	182 ± 96	14.7 ± 3.9	1.4 ± 0.3
AgOAc				
Jejunum	60	824 ± 1506	26.7 ± 18.3	1.8 ± 0.8
Ileum	60	1818 ± 1596	43.3 ± 21.1	1.5 ± 0.6
Colon	56	1495 ± 1256	39.9 ± 17.7	1.2 ± 0.3
Kidney	60	4786 ± 3419	71.2 ± 31.9	1.6 ± 0.6
Liver	57	1109 ± 683	36.2 ± 10.2	1.4 ± 0.2
Spleen	60	1181 ± 815	36.4 ± 13.4	1.3 ± 0.2

^aValues represent the mean ± the standard deviation of the mean, n = 56–60.

^bThe size and aspect ratio of particles were computed using Image J 1.47v software (NIH, <http://imagej.nih.gov/ij/>).

the intensity of silver appeared greater in rats exposed to AgOAc. Silver granules derived from AgNP were detected in the portal triad region of the liver by TEM (Figure 10A), and the surfaces of granules in these livers were continuous and circular-to-oval in shape. In contrast, silver accumulations in the liver of AgOAc-exposed rats appeared very diffused, and, as shown in Table 4, the size distribution of the granules were quite variable (36.2 ± 10.2 nm). The micrograph shown in Figure 10B demonstrates the irregular shape of granules within a Kupffer cell in the liver of a rat exposed to AgOAc.

The localization of silver within the spleen of exposed rats is shown in Figure 11. In the spleen of rats exposed to 10 nm AgNP, intracellular silver granules were visualized within macrophages in the red pulp (Figure 11A). Most of the silver granules located within the spleen of AgNP-exposed rats were agglomerated, although the smooth borders of the granules were visually well defined. The average size of the granules (14.7 ± 3.9 nm) indicated that the granules retained similar size and surface characteristics to the administered AgNP (Table 4). As with other tissues, TEM revealed differences in the distributional pattern of silver; AgNP appeared predominantly within cells (intracellular) while AgOAc appeared to have an affinity for extracellular membranes. The micrograph shown in Figure 11B illustrates silver granules associated with the surface of a macrophage within the red pulp of the spleen of a rat exposed to AgOAc.

EDS was used to determine the compositional analysis of the granules within rat tissues (Figure 12). Strong energy signals were observed for silver, and the presence of selenium, sulfur, and chloride was noted in association with the silver granules for both AgNP (Figure 12A) and AgOAc (Figure 12B). Signals corresponding to copper and lead were also identified. These originated from the grids that were used in the visualization procedures; an osmium signal was observed and originated from the tissue fixation procedures. There were no qualitative differences in the elemental compositions of granules detected in tissues of AgNP-exposed animals and those of AgOAc-exposed rats; whereas, only signals for lead,

copper, and osmium were detected by EDS in tissues of the vehicle control (Figure 12C) and water control (Figure 12D) animals.

Micronuclei, Vaginal Cytology, and Sperm Analysis

The effect of AgNP exposure or AgOAc exposure on *in vivo* genotoxicity was examined using a flow cytometric-based assay. The results of the micronuclei assay of peripheral blood are reported in Supplementary Table S4 and show that the data were negative for each time point in the peripheral blood of rats treated with AgNP. Male and female rats administered AgOAc at 400 mg/kg bw had a small but significant increase in the frequency of micronucleated reticulocytes at week-4, but not at subsequent time points (Supplementary Table S4). These animals had severe gastrointestinal distress and only 1 female and no male rats survived to week 12.

The results of the male and female reproductive system evaluations are shown in Supplementary Table S5. The evaluation of male rat testes revealed that there were no statistically significant differences in sperm motility, testis sperm count, caudal sperm count, or sperm morphology irrespective of treatment or form of silver (Supplementary Table S5). Similarly, there were no statistically significant differences between control groups or between any treatment and control groups in the proportion of days in each phase of the estrous cycle or in the frequency of transition between normal and abnormal states (Supplementary Table S5).

Histopathology

A single non-treatment-related neoplasm was found in this study—a mammary gland adenocarcinoma in a low-dose (9 mg/kg bw) female rat that was administered AgNP 75 nm. There were no treatment-related histopathological findings in female or male rats administered AgNP (36 mg/kg bw). Therefore, histopathology was not conducted on the mid- or low-dose AgNP-exposed animals.

Non-neoplastic lesions were confined primarily to rats administered AgOAc. With the exception of an increased dose-response in the incidence of histiocytic infiltrates in the lungs of female and male groups, there were no meaningful treatment-related differences from controls in the 100 and 200 mg/kg bw AgOAc dose groups. Increased incidences and severities of lesions were detected in rats exposed to AgOAc (400 mg/kg bw), and included mucosal hyperplasia in the small and large intestine, as well as, thymic atrophy or necrosis—a stress-response to the gastrointestinal disturbances experienced by these animals.

A notable observation by light microscopy was the occurrence of diffuse brown pigmentation in numerous organs and tissues of rats exposed to AgNP or AgOAc. In many respects, the detection of pigmentation was more a measure of silver mobility rather than toxicity, and pigmentation was graded for severity as (1) minimal, (2) mild, (3) moderate, or (4) marked. Table 5 provides a detailed description by site of the incidences, severities, and results of statistical analysis of the pigmentation detected by light microscopy in tissues and organs of rats administered AgNP and AgOAc.

The incidences of pigmentation observed by light microscopy among rats that were administered the AgNP treatments conveyed a similar story as told by the results of silver distribution obtained by ICP-MS. The severity of pigmentation in AgNP treatment groups was graded as minimal, with only a few instances of mild pigmentation. Pigmentation was more prevalent in female rats than male rats, especially in the mesenteric

FIG. 9. TEMs of kidney tissue from 10 nm AgNP and AgOAc exposed female rats. TEM of ultra-thin sections stained with uranyl acetate and lead citrate showing (A) an overview of the localization of silver granules within the brush border of the renal tubular epithelium (RTE) of a rat exposed to 10 nm AgNPs, and highlighting the shape and morphology of a silver granule from this region; (B) an overview of the localization of silver granules at the basement membranes (BM) of renal tubules in a kidney of a rat exposed to AgOAc, and highlighting the convoluted surface of a silver granule from this region.

lymph nodes, the large intestine (except the rectum), the stomach, kidneys, and spleen (Table 5). However, the high incidences (~80%–100%) of pigmentation in most every tissue of female rats tended to mask any differences due to AgNP size. Male rats administered AgNP showed a similar pattern of pigmentation as female rats; however, incidences of pigmentation were much lower and demonstrated a size-dependent prominence, with

the AgNP-10 nm having greater prominence than either the AgNP-75 nm or 110 nm treatments. The results obtained by ICP-MS for female and male rats exposed to AgNP showed size-dependent trends, with AgNP-10 nm demonstrating a significantly higher accumulation of silver; although accumulations were minimal relative to the accumulation of silver by AgOAc. Evaluations by light microscopy were able to discern the

FIG. 10. TEMs of liver tissue from 10 nm AgNP and AgOAc exposed female rats. TEM of ultra-thin sections stained with uranyl acetate and lead citrate showing (A) the accumulation of silver granules within a portion of the cytoplasm of a cell in the portal region of a liver from a 10 nm AgNP exposed rat, and featuring the smooth spherical shape of a granule. Note a small bile duct at the upper left of the micrograph, a portal vein in the upper right of the micrograph, and hepatocytes at the bottom of the micrograph; (B) the accumulation of silver granules within a Kupffer cell in the liver of a rat exposed to AgOAc, and highlighting the irregular shape of a silver granule from this deposit.

presence or absence of silver pigmentation in tissue and organ specimens; however, this method could not quantify differences in the intensity of the pigmentation at the levels of accumulation observed for AgNP-exposed rats.

The incidence and intensity of pigmentation were greatly increased in rats exposed to AgOAc (100 and 200 mg/kg bw) when compared with that for AgNP-treated animals, and results indicate that the pigmentation was dose-dependent (Table 5).

FIG. 11. TEMs of spleen tissue from 10 nm AgNP and AgOAc exposed female rats. TEM of ultra-thin sections stained with uranyl acetate and lead citrate showing (A) an overview of the localization of silver granules within the cytoplasm of a macrophage (MΦ) in the spleen of a rat exposed to AgNPs and highlights the smooth spherical shape and continuous border of a silver granule from this area of interest; (B) an overview of the localization of silver granules associated with the cell membrane of a macrophage in the spleen of a rat exposed to AgOAc and featuring the convoluted border of a granule from this area of interest.

However, the distribution of silver tended to be less prominent in rats exposed to AgOAc (400 mg/kg bw), likely a result of early removal of these animals from the study due to severe gastrointestinal assault.

DISCUSSION

In this study, the distribution profiles of silver in organs and tissues were similar between rats exposed to AgNP or AgOAc—silver accumulations were detected in the same tissues

FIG. 12. EDS spectra of granules within villi of the rat jejunum. EDS showing (A) emission spectra corresponding to silver granules accumulated within the lamina propria region from the jejunum of a rat exposed to 10 nm AgNP; (B) emission spectra corresponding to silver granules accumulated within the basement membrane within the jejunum of a rat exposed to AgOAc; (C, D) emission spectra within the villus from the jejunum of a vehicle and water control rat, respectively.

regardless of the form; however, differences in the absolute concentration of silver in tissues and organs were observed due to silver form, particle size, and dose.

In general, silver concentrations were several folds higher in all tissues of rats exposed to AgOAc when compared with those of rats exposed to AgNP. This may be explained by the 1.8 to 7-fold higher dose of silver from AgOAc (~64–256 mg silver/kg bw/day) compared with the AgNP high-dose formulation (36 mg silver/kg bw/day). Still, as observed in the blood, differences were not due solely to concentration levels, but more likely reflected much lower uptake of AgNP due to particle aggregation in the lumen matrix and higher uptake of silver ions due to surface charge. The greater accumulation of silver in tissues for ionic forms has been reported by others (Loeschner et al., 2011; van der Zande et al., 2012). In a 28-day study, silver nitrate was administered to rats at a 10-fold lower concentration than 20 nm AgNP (9 vs 90 mg/kg bw), yet the absolute tissue levels of silver were significantly higher for the ionic form in the liver, spleen, testis, kidney, brain, and lungs (van der Zande et al., 2012). Loeschner et al. (2011) administered AgOAc and 14 nm AgNP to rats at equivalent doses and found the distribution patterns were similar between silver forms but the absolute silver concentrations in tissues were lower following oral exposure to AgNP.

The absolute concentration of silver in tissues, as determined by ICP-MS, showed significant AgNP size-dependent relationships, with $10 > 75 > 110$ nm. It is generally agreed that the absorption of AgNP is inversely related to particle size (Florence, 2005). Jani et al. (1990) fed 125 I-labeled polystyrene microspheres in the size range 50–300 nm to rats and found that the extent of absorption of 50 and 100 nm particles was 34% and 26%, respectively; whereas particles > 100 nm were minimally absorbed. Therefore, one possible explanation for the minimal accumulation of silver in tissues and organs of the 75 and 110 nm AgNP-exposed rats is that the passage of the larger sized particles through the intestinal barrier may be inferior to that of 10 nm AgNP. In addition, AgNP tend to aggregate in the presence of electrolytes, in particular the chloride ion, and the matrix in the intestinal tract may induce aggregation and/or agglomeration of AgNP in the lumen; thus, accounting for the much higher absorption and accumulation of soluble silver forms, such as AgOAc. This may also explain the diminished ability of TEM to detect granules in the tissues of 75 and 110-nm-exposed rats, even at doses as high as 36 mg/kg bw/day. In addition, the accumulation of silver in tissues and organs showed significant dose relationships, irrespective of silver form or particle size, suggesting that the uptake and deposition of silver was proportional to the administered amount of silver.

TABLE 5. Incidence and Severity of Pigmentation in Female and Male Rats Exposed to AgNP or AgOAc by Oral Gavage for 13 Weeks

Female Rats	Vehicle Control	AgNP-10 nm 36 mg/kg bw	AgNP-75 nm 36 mg/kg bw	AgNP-110 nm 36 mg/kg bw
Intestine Large, Cecum				
Incidence	0/10 (0.0%)	10/10 (100.0%)	10/10 (100.0%)	9/10 (90.0%)
P-value ^a	<.001***	<.001***	<.001***	<.001***
Severity ^b	–	10 0 0 0	10 0 0 0	9 0 0 0
Intestine Large, Colon				
Incidence	0/10 (0.0%)	10/10 (100.0%)	10/10 (100.0%)	9/10 (90.0%)
P-value	<.001***	<.001***	<.001***	<.001***
Severity	–	10 0 0 0	10 0 0 0	9 0 0 0
Intestine Large, Rectum				
Incidence	0/10 (0.0%)	10/10 (100.0%)	2/10 (20.0%)	0/10 (0.0%)
P-value	0.005N**	<.001***	0.237	
Severity	–	10 0 0 0	2 0 0 0	–
Intestine Small, Duodenum				
Incidence	0/10 (0.0%)	10/10 (100.0%)	9/10 (90.0%)	9/10 (90.0%)
P-value	<.001***	<.001***	<.001***	<.001***
Severity	–	5 5 0 0	7 2 0 0	9 0 0 0
Intestine Small, Ileum				
Incidence	0/10 (0.0%)	10/10 (100.0%)	8/10 (80.0%)	3/10 (30.0%)
P-value	0.481	<.001***	<.001***	0.105
Severity	–	10 0 0 0	8 0 0 0	3 0 0 0
Intestine Small, Jejunum				
Incidence	0/10 (0.0%)	10/10 (100.0%)	10/10 (100.0%)	8/10 (80.0%)
P-value	0.003**	<.001***	<.001***	<.001***
Severity	–	10 0 0 0	10 0 0 0	8 0 0 0
Liver				
Incidence	0/10 (0.0%)	10/10 (100.0%)	7/10 (70.0%)	4/10 (40.0%)
P-value	0.377	<.001***	0.002**	0.043*
Severity	–	10 0 0 0	7 0 0 0	4 0 0 0
Pancreas				
Incidence	0/10 (0.0%)	9/10 (90.0%)	1/10 (10.0%)	6/10 (60.0%)
P-value	0.391	<.001***	0.5	0.005**
Severity	–	9 0 0 0	1 0 0 0	6 0 0 0
Stomach, Glandular				
Incidence	0/10 (0.0%)	10/10 (100.0%)	10/10 (100.0%)	9/10 (90.0%)
P-value	<.001***	<.001***	<.001***	<.001***
Severity	–	10 0 0 0	10 0 0 0	9 0 0 0
Parathyroid Gland				
Incidence	0/10 (0.0%)	7/10 (70.0%)	3/10 (30.0%)	3/10 (30.0%)
P-value	0.475N	0.002**	0.105	0.105
Severity	–	7 0 0 0	3 0 0 0	3 0 0 0
Ovary				
Incidence	0/10 (0.0%)	8/10 (80.0%)	9/10 (90.0%)	3/10 (30.0%)
P-value	0.212	<.001***	<.001***	0.105
Severity	–	8 0 0 0	9 0 0 0	3 0 0 0
Lymph Node, Mandibular				
Incidence	0/10 (0.0%)	5/10 (50.0%)	7/10 (70.0%)	2/10 (20.0%)
P-value	0.209	0.016*	0.002**	0.237
Severity	–	5 0 0 0	7 0 0 0	2 0 0 0
Lymph Node, Mesenteric				
Incidence	0/10 (0.0%)	10/10 (100.0%)	10/10 (100.0%)	9/10 (90.0%)
P-value	<.001***	<.001***	<.001***	<.001***
Severity	–	8 2 0 0	9 1 0 0	9 0 0 0
Spleen				
Incidence	4/10 (40.0%)	9/10 (90.0%)	9/10 (90.0%)	10/10 (100.0%)
P-value	0.002**	0.029*	0.029*	0.005**
Severity	1 3 0 0	0 9 0 0	3 6 0 0	9 1 0 0
Brain, Brain Stem				
Incidence	0/10 (0.0%)	6/10 (60.0%)	7/10 (70.0%)	4/10 (40.0%)
P-value	0.085	0.005**	0.002**	0.043*
Severity	–	6 0 0 0	7 0 0 0	4 0 0 0
Nose				
Incidence	0/10 (0.0%)	2/10 (20.0%)	3/10 (30.0%)	4/10 (40.0%)
P-value	0.023*	0.237	0.105	0.043*
Severity	–	2 0 0 0	3 0 0 0	4 0 0 0

(continued)

TABLE 5. Continued

Female Rats		Vehicle Control	AgNP-10 nm 36 mg/kg bw	AgNP-75 nm 36 mg/kg bw	AgNP-110 nm 36 mg/kg bw
Eye					
	Incidence	0/10 (0.0%)	9/10 (90.0%)	1/10 (10.0%)	1/10 (10.0%)
	P- value	0.018 N*	<.001***	0.5	0.5
	Severity	–	9 0 0 0	1 0 0 0	1 0 0 0
Kidney					
	Incidence	0/10 (0.0%)	10/10 (100.0%)	10/10 (100.0%)	10/10 (100.0%)
	P-value	<.001***	<.001***	<.001***	<.001***
	Severity	–	9 1 0 0	10 0 0 0	10 0 0 0
Male Rats		Vehicle control	AgNP-10 nm 36 mg/kg bw	AgNP-75 nm 36 mg/kg bw	AgNP-110 nm 36 mg/kg bw
Intestine Large, Cecum:					
	Incidence	0/9 (0.0%)	9/10 (90.0%)	5/10 (50.0%)	2/10 (20.0%)
	P-value ^a	0.215 N	<.001***	0.022*	0.263
	Severity	–	9 0 0 0	5 0 0 0	2 0 0 0
Intestine Large, Colon					
	Incidence	0/9 (0.0%)	9/10 (90.0%)	3/10 (30.0%)	2/10 (20.0%)
	P-value	0.122 N	<.001***	0.124	0.263
	Severity	–	9 0 0 0	3 0 0 0	2 0 0 0
Intestine Small, Duodenum					
	Incidence	0/9 (0.0%)	10/10 (100.0%)	7/10 (70.0%)	3/10 (30.0%)
	P-value	0.392 N	<.001***	0.002**	0.124
	Severity	–	10 0 0 0	7 0 0 0	3 0 0 0
Intestine Small, Ileum					
	Incidence	0/9 (0.0%)	7/10 (70.0%)	3/10 (30.0%)	1/10 (10.0%)
	P-value	0.135 N	0.002**	0.124	0.526
	Severity	–	7 0 0 0	3 0 0 0	1 0 0 0
Intestine Small, Jejunum					
	Incidence	0/9 (0.0%)	10/10 (100.0%)	3/10 (30.0%)	1/10 (10.0%)
	P-value	0.028 N*	<.001***	0.124	0.526
	Severity	–	10 0 0 0	3 0 0 0	1 0 0 0
Liver					
	Incidence	0/9 (0.0%)	4/10 (40.0%)	0/10 (0.0%)	0/10 (0.0%)
	P-value	0.048 N*	0.054	–	–
	Severity	–	4 0 0 0	–	–
Pancreas					
	Incidence	0/9 (0.0%)	4/10 (40.0%)	2/10 (20.0%)	1/10 (10.0%)
	P-value	0.332 N	0.054	0.263	0.526
	Severity	–	4 0 0 0	2 0 0 0	1 0 0 0
Stomach, Glandular					
	Incidence	0/9 (0.0%)	10/10 (100.0%)	7/10 (70.0%)	10/10 (100.0%)
	P-value	0.002**	<.001***	0.002**	<.001***
	Severity	–	10 0 0 0	7 0 0 0	10 0 0 0
Lymph Node, Mandibular					
	Incidence	0/9 (0.0%)	6/10 (60.0%)	4/10 (40.0%)	1/10 (10.0%)
	P-value	0.258 N	0.008**	0.054	0.526
	Severity	–	6 0 0 0	4 0 0 0	1 0 0 0
Lymph Node, Mesenteric					
	Incidence	0/9 (0.0%)	10/10 (100.0%)	7/10 (70.0%)	6/10 (60.0%)
	P-value	0.163	<.001***	0.002**	0.008**
	Severity	–	10 0 0 0	7 0 0 0	6 0 0 0
Spleen:					
	Incidence	0/9 (0.0%)	8/10 (80%)	8/10 (80.0%)	10/10 (100.0%)
	P-value	<.001***	<.001***	<.001***	<.001***
	Severity	–	6 2 0 0	6 2 0 0	10 0 0 0
Brain, Brain Stem					
	Incidence	0/9 (0.0%)	6/10 (60.0%)	4/10 (40.0%)	0/10
	P-value	0.131 N	0.008**	0.054	–
	Severity	–	6 0 0 0	4 0 0 0	–
Nose					
	Incidence	0/9 (0.0%)	5/10 (50.0%)	1/10 (10.0%)	7/10 (70.0%)
	P-value	0.040*	0.022*	0.526	0.002**
	Severity	–	5 0 0 0	1 0 0 0	7 0 0 0

(continued)

TABLE 5. Continued

Male Rats	Vehicle control	AgNP-10 nm 36 mg/kg bw	AgNP-75 nm 36 mg/kg bw	AgNP-110 nm 36 mg/kg bw
Kidney				
Incidence	0/9 (0.0%)	7/10 (70.0%)	1/10 (10.0%)	1/10 (10.0%)
P-value	0.049 N*	0.002**	0.526	0.526
Severity	–	7 0 0 0	1 0 0 0	1 0 0 0
Female Rats	Water control	AgOAc 100 mg/kg bw	AgOAc 200 mg/kg bw	AgOAc 400 mg/kg bw
Intestine Large, Cecum				
Incidence	0/10 (0.0%)	10/10 (100.0%)	10/10 (100.0%)	10/10 (100.0%)
P-value	<.001***	<.001***	<.001***	<.001***
Severity	–	1 6 3 0	0 2 6 2	1 4 5 0
Intestine Large, Colon				
Incidence	0/10 (0.0%)	10/10 (100.0%)	10/10 (100.0%)	8/10 (80.0%)
P-value	<.001***	<.001***	<.001***	<.001***
Severity	–	1 5 4 0	0 2 8 0	0 3 4 1
Intestine Large, Rectum				
Incidence	0/10 (0.0%)	10/10 (100.0%)	10/10 (100.0%)	8/10 (80.0%)
P-value	<.001***	<.001***	<.001***	<.001***
Severity	–	10 0 0 0	10 0 0 0	7 1 0 0
Intestine Small, Duodenum				
Incidence	0/10 (0.0%)	10/10 (100.0%)	10/10 (100.0%)	9/10 (90.0%)
P-value	<.001***	<.001***	<.001***	<.001***
Severity	–	1 1 8 0	0 1 6 3	1 0 1 7
Intestine Small, Ileum				
Incidence	0/10 (0.0%)	10/10 (100.0%)	10/10 (100.0%)	8/10 (80.0%)
P-value	0.001**	<.001***	<.001***	<.001***
Severity	–	1 7 2 0	3 7 0 0	1 6 1 0
Intestine Small, Jejunum				
Incidence	0/10 (0.0%)	10/10 (100.0%)	10/10 (100.0%)	9/10 (90.0%)
P-value	<.001***	<.001***	<.001***	<.001***
Severity	–	1 9 0 0	0 9 1 0	1 2 6 0
Liver				
Incidence	0/10 (0.0%)	9/10 (90.0%)	10/10 (100.0%)	10/10 (100.0%)
P-value	<.001***	<.001***	<.001***	<.001***
Severity	–	2 7 0 0	1 9 0 0	2 5 3 0
Pancreas				
Incidence	0/10 (0.0%)	9/10 (90.0%)	10/10 (100.0%)	8/10 (80.0%)
P-value	<.001***	<.001***	<.001***	<.001***
Severity	–	8 1 0 0	10 0 0 0	6 2 0 0
Salivary Glands				
Incidence	0/10 (0.0%)	9/10 (90.0%)	10/10 (100.0%)	6/10 (60.0%)
P-value	0.026*	<.001***	<.001***	0.005**
Severity	–	9 0 0 0	10 0 0 0	6 0 0 0
Stomach, Glandular				
Incidence	0/10 (0.0%)	9/10 (90.0%)	10/10 (100.0%)	8/10 (80.0%)
P-value	<.001***	<.001***	<.001***	<.001***
Severity	–	9 0 0 0	10 0 0 0	8 0 0 0
Heart				
Incidence	0/10 (0.0%)	9/10 (90.0%)	9/10 (90.0%)	7/10 (70.0%)
P-value	0.009**	<.001***	<.001***	0.002**
Severity	–	9 0 0 0	9 0 0 0	7 0 0 0
Adrenal Cortex				
Incidence	0/10 (0.0%)	0/10 (0.0%)	3/10 (30.0%)	7/10 (70.0%)
P-value	<.001***	–	0.105	0.002**
Severity	–	–	3 0 0 0	5 2 0 0
Adrenal Medulla				
Incidence	0/10 (0.0%)	8/10 (80.0%)	8/10 (80.0%)	7/10 (70.0%)
P-value	<.008**	<.001***	<.001***	0.002**
Severity	–	8 0 0 0	8 0 0 0	6 1 0 0
Parathyroid Gland				
Incidence	0/10 (0.0%)	9/10 (90.0%)	9/9 (100.0%)	5/9 (55.6%)
P-value	0.042*	<.001***	<.001***	0.011*
Severity	–	9 0 0 0	9 0 0 0	5 0 0 0

(continued)

TABLE 5. Continued

Female Rats	Water control	AgOAc 100 mg/kg bw	AgOAc 200 mg/kg bw	AgOAc 400 mg/kg bw
Pituitary Gland				
Incidence	0/10 (0.0%)	5/10 (50.0%)	9/10 (90.0%)	8/10 (80.0%)
P-value	<.001***	0.016*	<.001***	<.001***
Severity	–	5 0 0 0	9 0 0 0	8 0 0 0
Thyroid Gland				
Incidence	0/10 (0.0%)	7/10 (70.0%)	9/10 (90.0%)	7/10 (70.0%)
P-value	0.004**	0.002**	<.001***	0.002**
Severity	–	7 0 0 0	9 0 0 0	7 0 0 0
Clitoral Gland				
Incidence	0/10 (0.0%)	4/10 (40.0%)	6/10 (60.0%)	3/10 (30.0%)
P-value	0.146	0.043*	0.005**	0.105
Severity	–	4 0 0 0	6 0 0 0	3 0 0 0
Ovary				
Incidence	0/10 (0.0%)	9/10 (90.0%)	10/10 (100.0%)	8/10 (80.0%)
P-value	<.001***	<.001***	<.001***	<.001***
Severity	–	6 3 0 0	6 4 0 0	2 4 2 0
Uterus				
Incidence	0/10 (0.0%)	0/10 (0.0%)	6/10 (60.0%)	8/10 (80.0%)
P-value	<.001***		0.005**	<.001***
Severity	–	–	6 0 0 0	8 0 0 0
Bone Marrow				
Incidence	0/10 (0.0%)	5/10 (50.0%)	10/10 (100.0%)	9/10 (90.0%)
P-value	<.001***	0.016*	<.001***	<.001***
Severity	–	5 0 0 0	4 6 0 0	3 5 1 0
Lymph Node, Mandibular				
Incidence	0/10 (0.0%)	8/10 (80.0%)	10/10 (100.0%)	10/10 (100.0%)
P-value	<.001***	<.001***	<.001***	<.001***
Severity	–	8 0 0 0	10 0 0 0	10 0 0 0
Lymph Node, Mesenteric				
Incidence	0/10 (0.0%)	10/10 (100.0%)	10/10 (100.0%)	10/10 (100.0%)
P-value	<.001***	<.001***	<.001***	<.001***
Severity	–	1 6 3 1	0 0 1 9	1 1 1 7
Spleen				
Incidence	7/10 (70.0%)	8/10 (80.0%)	10/10 (100.0%)	8/10 (80.0%)
P-value	0.332	0.5	0.105	0.5
Severity	4 3 0 0	4 4 0 0	4 6 0 0	3 4 1 0
Mammary Gland				
Incidence	0/10 (0.0%)	2/10 (20.0%)	0/10 (0.0%)	0/10 (0.0%)
P-value	0.375 N	0.237		
Severity	–	2 0 0 0	–	–
Brain, Brain Stem				
Incidence	0/10 (0.0%)	6/10 (60.0%)	3/10 (30.0%)	3/10 (30.0%)
P-value	0.282	0.005**	0.105	0.105
Severity	–	6 0 0 0	3 0 0 0	3 0 0 0
Brain, Cerebellum				
Incidence	0/10 (0.0%)	0/10 (0.0%)	5/10 (50.0%)	1/10 (10.0%)
P-value	0.19		0.016*	0.5
Severity	–	–	5 0 0 0	1 0 0 0
Brain, Cerebrum				
Incidence	0/10 (0.0%)	0/10 (0.0%)	2/10 (20.0%)	0/10 (0.0%)
P-value	0.505		0.237	
Severity	–	–	2 0 0 0	–
Nose				
Incidence	0/10 (0.0%)	0/10 (0.0%)	5/10 (50.0%)	3/10 (30.0%)
P-value	0.025*		0.016*	0.105
Severity	–	–	5 0 0 0	3 0 0 0
Eye				
Incidence	0/10 (0.0%)	8/10 (80.0%)	10/10 (100.0%)	7/10 (70.0%)
P-value	0.002**	<.001***	<.001***	0.002**
Severity	–	8 0 0 0	10 0 0 0	7 0 0 0
Harderian Gland				
Incidence	0/10 (0.0%)	9/10 (90.0%)	10/10 (100.0%)	7/10 (70.0%)
P-value	0.006**	<.001***	<.001***	0.002**
Severity	–	8 1 0 0	9 1 0 0	7 0 0 0

(continued)

TABLE 5. Continued

Female Rats		Water control	AgOAc 100 mg/kg bw	AgOAc 200 mg/kg bw	AgOAc 400 mg/kg bw
Kidney					
Incidence		0/10 (0.0%)	9/10 (90.0%)	10/10 (100.0%)	9/10 (90.0%)
P-value		<.001***	<.001***	<.001***	<.001***
Severity		–	0 1 5 3	0 0 7 3	1 0 7 1
Male Rats		Water control	AgOAc 100 mg/kg bw	AgOAc 200 mg/kg bw	AgOAc 400 mg/kg bw
Intestine Large, Cecum					
Incidence		0/10 (0.0%)	10/10 (100.0%)	10/10 (100.0%)	8/10 (80.0%)
P-value		0.001**	<.001***	<.001***	<.001***
Severity		–	0 7 3 0	0 6 4 0	5 0 3 0
Intestine Large, Colon					
Incidence		0/10 (0.0%)	10/10 (100.0%)	10/10 (100.0%)	8/10 (80.0%)
P-value		0.001**	<.001***	<.001***	<.001***
Severity		–	0 8 2 0	0 7 3 0	5 3 0 0
Intestine Large, Rectum					
Incidence		0/10 (0.0%)	9/10 (90.0%)	10/10 (100.0%)	6/10 (60.0%)
P-value		0.028*	<.001***	<.001***	0.005**
Severity		–	8 1 0 0	10 0 0 0	6 0 0 0
Intestine Small, Duodenum					
Incidence		0/10 (0.0%)	10/10 (100.0%)	10/10 (100.0%)	9/10 (90.0%)
P-value		<.001***	<.001***	<.001***	<.001***
Severity		–	0 0 7 3	0 0 0 10	4 1 1 3
Intestine Small, Ileum					
Incidence		0/10 (0.0%)	10/10 (100.0%)	10/10 (100.0%)	5/10 (50.0%)
P-value		0.106	<.001***	<.001***	0.016*
Severity		–	3 3 4 0	1 8 1 0	2 1 2 0
Intestine Small, Jejunum					
Incidence		0/10 (0.0%)	10/10 (100.0%)	9/9 (100.0%)	9/10 (90.0%)
P-value		<.001***	<.001***	<.001***	<.001***
Severity		–	0 7 3 0	0 5 4 0	4 2 2 1
Liver					
Incidence		0/10 (0.0%)	10/10 (100.0%)	10/10 (100.0%)	9/10 (90.0%)
P-value		<.001***	<.001***	<.001***	<.001***
Severity		–	1 9 0 0	3 7 0 0	6 3 0 0
Pancreas					
Incidence		0/10 (0.0%)	10/10 (100.0%)	10/10 (100.0%)	2/10 (20.0%)
P-value		0.497 N	<.001***	<.001***	0.237
Severity		–	10 0 0 0	9 1 0 0	0 2 0 0
Salivary Glands					
Incidence		0/10 (0.0%)	10/10 (100.0%)	10/10 (100.0%)	1/10 (10.0%)
P-value		0.318 N	<.001***	<.001***	0.5
Severity		–	10 0 0 0	10 0 0 0	1 0 0 0
Stomach, Glandular					
Incidence		0/10 (0.0%)	9/10 (90.0%)	10/10 (100.0%)	7/10 (70.0%)
P-value		0.007**	<.001***	<.001***	0.002**
Severity		–	8 1 0 0	10 0 0 0	7 0 0 0
Heart					
Incidence		0/10 (0.0%)	4/10 (40.0%)	8/10 (80.0%)	1/10 (10.0%)
P-value		0.439	0.043*	<.001***	0.5
Severity		–	4 0 0 0	8 0 0 0	1 0 0 0
Adrenal Cortex					
Incidence		0/10 (0.0%)	0/10 (0.0%)	2/10 (20.0%)	3/10 (30.0%)
P-value		0.018*	–	0.237	0.105
Severity		–	–	2 0 0 0	3 0 0 0
Adrenal Medulla					
Incidence		0/10 (0.0%)	8/10 (80.0%)	8/10 (80.0%)	3/10 (30.0%)
P-value		0.323	<.001***	<.001***	0.105
Severity		–	8 0 0 0	8 0 0 0	3 0 0 0
Parathyroid Gland					
Incidence		0/10 (0.0%)	8/9 (88.9%)	9/10 (90.0%)	1/10 (10.0%)
P-value		0.382 N	<.001***	<.001***	0.5
Severity		–	8 0 0 0	9 0 0 0	1 0 0 0

(continued)

TABLE 5. Continued

Male Rats	Water control	AgOAc 100 mg/kg bw	AgOAc 200 mg/kg bw	AgOAc 400 mg/kg bw
Pituitary Gland				
Incidence	0/10 (0.0%)	9/10 (90.0%)	8/10 (80.0%)	3/10 (30.0%)
P-value	0.372	<.001***	<.001***	0.105
Severity	–	9 0 0 0	8 0 0 0	3 0 0 0
Thyroid Gland				
Incidence	0/10 (0.0%)	9/10 (90.0%)	7/10 (70.0%)	1/10 (10.0%)
P-value	0.317 N	<.001***	0.002**	0.5
Severity	–	9 0 0 0	7 0 0 0	1 0 0 0
Preputial Gland				
Incidence	0/10 (0.0%)	3/10 (30.0%)	5/10 (50.0%)	0/10 (0.0%)
P-value	0.459 N	0.105	0.016*	–
Severity	–	3 0 0 0	5 0 0 0	–
Bone Marrow				
Incidence	0/10 (0.0%)	3/10 (30.0%)	10/10 (100.0%)	5/10 (50.0%)
P-value	0.009**	0.105	<.001***	0.016*
Severity	–	3 0 0 0	3 7 0 0	3 2 0 0
Lymph Node, Mandibular				
Incidence	0/10 (0.0%)	7/10 (70.0%)	10/10 (100.0%)	5/10 (50.0%)
P-value	0.041*	0.002**	<.001***	0.016*
Severity	–	7 0 0 0	10 0 0 0	5 0 0 0
Lymph Node, Mesenteric				
Incidence	0/10 (0.0%)	10/10 (100.0%)	10/10 (100.0%)	10/10 (100.0%)
P-value	<.001***	<.001***	<.001***	<.001***
Severity	–	1 6 2 1	0 0 2 8	4 2 1 3
Spleen				
Incidence	1/10 (10.0%)	4/10 (40%)	8/10 (80.0%)	3/10 (30.0%)
P-value	0.228	0.152	0.003**	0.291
Severity	1 0 0 0	3 1 0 0	7 1 0 0	1 2 0 0
Brain, Brain Stem				
Incidence	0/10 (0.0%)	7/10 (70.0%)	7/10 (70.0%)	2/10 (20.0%)
P-value	0.456	0.002**	0.002**	0.237
Brain, Brain Stem				
Incidence	0/10 (0.0%)	7/10 (70.0%)	7/10 (70.0%)	2/10 (20.0%)
P-value	0.456	0.002**	0.002**	0.237
Severity	–	7 0 0 0	–	–
Nose				
Incidence	0/10 (0.0%)	0/10 (0.0%)	7/10 (70.0%)	0/10 (0.0%)
P-value	0.358	–	0.002**	–
Severity	–	–	7 0 0 0	–
Eye				
Incidence	0/10 (0.0%)	9/10 (90.0%)	8/10 (80.0%)	1/10 (10.0%)
P-value	0.341 N	<.001***	<.001***	0.5
Severity	–	9 0 0 0	8 0 0 0	1 0 0 0
Harderian Gland				
Incidence	0/10 (0.0%)	10/10 (100.0%)	10/10 (100.0%)	0/10 (0.0%)
P-value	0.167 N	<.001***	<.001***	–
Severity	–	10 0 0 0	9 1 0 0	–
Kidney				
Incidence	0/10 (0.0%)	10/10 (100.0%)	10/10 (100.0%)	3/10 (30.0%)
P-value	0.397	<.001***	<.001***	0.105
Severity	–	1 5 4 0	0 2 7 1	0 0 3 0

^aIncidence is reported as the number of animals with pigmentation/number of animals examined microscopically. Significantly increased incidence of pigmentation is indicated with probability (p) values denoted by $p < .05^*$, $p < .01^{**}$, and $p < .001^{***}$. Significantly decreased incidence of pigmentation is indicated with P values appended with "N".

^bPigmentation was graded for severity as minimal, mild, moderate, or marked. For each tissue type, the vertical bar symbol is used to separate the 4 severity grades of pigmentation, and the integers indicate the number of rats in a treatment group that were detected with pigmentation within each severity grade.

Statistically significant sex differences in the distribution and accumulation of silver were noted for a number of tissues and organs in this study. In general, silver accumulations were significantly higher in female rats administered AgNP when compared with male rats, especially for the kidney, liver,

jejunum, and colon, and a similar profile in silver distribution was observed for rats exposed to AgOAc (100 mg kg bw). Sex difference were noted in the pattern of silver distribution in the kidney, with female rats showing higher levels of silver than that in the spleen, and male rats showing higher levels in

the spleen than that in the kidney. In addition, a 10-fold higher concentration of silver was noted in the kidney of female rats exposed to AgNP-10 nm when compared with male rats. Sex-related differences in the accumulation of AgNP have been reported by others. In 28-day oral toxicity studies, Kim *et al.* (2008) reported a 2-fold higher accumulation of silver across all dose groups in the kidneys of female rats when compared with male rats. In a follow-up study, histological evaluations showed that female rats exposed to AgNP had higher accumulations of silver compared with male rats in all regions of the kidney, but in particular the glomerulus in the cortex of the kidneys (Kim *et al.*, 2009). One potential explanation for the sex differences in the distribution of silver may be hormonal regulation in the rat kidney. However, sex differences in silver accumulation were observed in nearly every tissue in this study. Estrogens appear to exert an effect on calcium transport, and exogenous estrogens have been shown to improve calcium absorption from the intestine and enhance transport of calcium across epithelia (Khanal and Nemere, 2008). At present, the effect of hormones on the clearance and accumulation of AgNP remains unclear but may be an important regulator for the sex differences observed in this study.

This study also revealed differences in the localization patterns between 10-nm AgNP and AgOAc. Deposits of both forms of silver were found within the same tissues; however, the TEM micrographs revealed that, as confirmed by ICP-MS results, the absolute amount of silver accumulated in the tissues was visually greater for AgOAc than for 10 nm AgNP.

TEM micrographs also revealed differences in the distributional patterns between the 10-nm AgNP and AgOAc. The AgNP appeared to be located predominantly within cells (intracellular), in particular epithelial cells; whereas, AgOAc appeared to have an affinity for the basement membranes (extracellular) of tissues. These findings provide additional support that the AgNP were absorbed as intact particles. It is well established that the oral administration silver nitrate results in the deposition of silver within the matrix of basement membranes, as well as in loose connective tissue associated with the basement membranes and phagocytic cells, and not in epithelial cells or in elastic fibers (Cooper *et al.*, 1956). Basement membranes usually consist of a homogeneous matrix that contains a rich source of negatively charged sulfhydryl and/or disulfide groups, and silver ions are known to have a high affinity for these groups and would be expected to precipitate in their presence (Walker, 1971).

EDS was used in this study to confirm the presence of silver in the observed granules within rat tissues and organs; however, there were no qualitative differences between the elemental composition of particles or granules in any of the examined tissues of rats exposed to either AgNP or AgOAc. Strong energy signals for silver were detected in the tissues of rats exposed to AgNP and AgOAc alike. In addition to silver, signals corresponding to selenium, sulfur, and chlorine were detected. The presence of sulfur and selenium in association with the AgNP and AgOAc suggests protein-binding of the granules, either at the site of absorption or at the site of accumulation, due to the high affinity of silver for sulfur and selenium. Body fluids of mammals are rich with chloride and sulfide ions and the Ag^+ produced from the silver metal form insoluble salts that lend protection against silver toxicity (Zhang *et al.*, 2013). The observation of sulfur, selenium, and chlorine in association with silver granules derived from an ionic silver form was anticipated, due to the high affinity of silver ions for these minerals; however, the similarity in elemental composition between AgOAc

and AgNP is difficult to explain. Loeschner *et al.* (2011) reported similar EDS findings in ileum lysosomes of rats that were exposed to PVP-coated 14 nm AgNP or AgOAc. The authors suggested 2 scenarios to explain the phenomena: either the AgNP were dissolved, the ions bound to proteins, and the silver-protein complex deposited as granules; or intact AgNP were absorbed, bound to proteins, and deposited in the tissue.

In the AgNP-exposed rats, mostly round, albeit agglomerated, smooth-surfaced granules were observed by TEM. The aspect ratios of the granules indicated that the granules were mostly spherical-to-oval in shape and the diameters of the individual granules were similar to the administered AgNP. Commonly used AgNP synthesis methods usually produce negatively charged AgNP (Tolaymat *et al.*, 2010). Studies have shown that the surfaces of AgNP adsorb Ag^+ , and this may account for the oval shape (Liu and Hurt, 2010). In addition, AgNP tend to aggregate in the presence of electrolytes, in particular the chloride ion, and the matrix in the intestinal tract may induce the aggregation and/or agglomeration of AgNP in the lumen. TEM micrographs showed that the surfaces of the granules were smooth and continuous, and the observed granules retained similar size and shape characteristics as the administered nanoparticles at most sites examined. These findings support the scenario that the 10-nm AgNP were absorbed intact.

In contrast, TEM micrographs of granules deposited in tissues of rats exposed to AgOAc showed the presence of irregular, convoluted shapes, and variable size distributions. Such findings support the scenario of dissolved silver ions absorbed unimpeded through the gastrointestinal barrier, the ions then binding to proteins, and the silver-protein complexes deposited as granules. Several of these silver-protein complexes may bind together to form granules with the irregular, convoluted shapes of granules observed in the tissues of rats exposed to AgOAc. The results presented in this study demonstrate that, at least for 10-nm AgNP, AgNP are absorbed and translocate to organs and tissues as mostly intact particles. This is the first direct comparison of the morphological differences between the accumulation of AgNP and soluble silver in organs and tissues of rats following daily oral administration (7 days/week) of AgNP and AgOAc for a period of 13 weeks.

Microscopic examination found no treatment-related histopathological findings in rats administered AgNP. Pigmentation, assumed silver accumulation, was observed in a number of tissues, with the highest levels of pigmentation detected in the small and large intestine, kidney, liver, and mesenteric lymph nodes; however, the presence of pigmentation in most tissues was minimal in both sexes or rats exposed to AgNP, and the presence of pigmentation did not induce lesions that could be detected by light microscopy.

In the future, studies are needed to understand the mechanism for sex differences in silver accumulation and pattern of distribution.

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SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

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