Biol Trace Elem Res (2015) 163:184–192
DOI 10.1007/s12011-014-0141-2

Development of an Analytical Method for Assessment of Silver Nanoparticle Content in Biological Matrices by Inductively Coupled Plasma Mass Spectrometry

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Received: 25 September 2014 / Accepted: 29 September 2014 / Published online: 12 October 2014

Abstract Silver nanoparticles (AgNPs) are a broad class of synthetic nanoparticles that are utilized in a wide variety of consumer products as antimicrobial agents. Despite their widespread use, a detailed understanding of their toxicological characteristics and biological and environmental hazards is not available. To support research into the biodistribution and toxicology of AgNPs, it is necessary to develop a suitable method for the assessment of AgNP content in biological samples. Two methods were developed and validated to analyze citrate-coated AgNP content that utilize acid digestion of rodent feces and liver tissue samples, and a third method was developed for the dilution and direct analysis of rodent urine samples. Following sample preparation, the silver content of each sample was determined by inductively coupled plasma mass spectrometry (ICP-MS) to quantify the silver and AgNP levels present. Analysis of rat feces matrix yielded analytical recoveries ranging from 82 to 93 %. Liver tissue spiked with a formulation of AgNPs over a range of concentrations yielded analytical recoveries between 88 and 90 %, providing acceptable accuracy results. The analysis of silver in urine samples exhibited recovery values ranging from 80 to 85 % for AgNP formulations and 62–84 % for standard silver ion solutions. All determinations exhibited a high degree of analytical precision. The results obtained here suggest that matrix interference plays a minimal role in AgNP recovery in feces and liver tissue, while the urine matrix can exhibit a significant effect on the determination of silver content.

Keywords Silver nanoparticles · Bioanalytical · Tissue distribution · Validation · Plasma spectroscopy

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Introduction

Silver nanoparticles (AgNPs) are a class of synthetic material that are typically composed of a silver-containing core with a coating that can be made of a wide range of substances including polyvinylpyrrolidone (PVP) and citrate [1, 2]. The particles are produced in a range of sizes on the scale of 10 to 110 nm, which when combined with the chemical properties of the coating can affect their physicochemical characteristics in biological systems [2]. The chemical properties of the coating contribute to the surface charge of the particle, which is related to the biological properties and activity of the particles [3].

The primary application of AgNPs revolves around their use in commercial products as an antimicrobial agent, particularly in textiles, household supplies, and toiletries [4, 5]. AgNPs have been shown to be a broad-spectrum biocide that functions through a slow release of silver, which is toxic to cells when exposed over an extended period of time [6]. The mechanism of toxicity has been proposed to occur through a number of routes including the production of reactive oxygen species, binding to thiol groups in the cell, and binding intracellularly to DNA and RNA [7]. This antimicrobial effect has also led to the application of AgNPs in medical products such as topical creams and wound dressings [4]. Due to the expanding use of AgNPs in consumer products and medical devices, there is a growing concern over the risks of

exposure to high concentrations of AgNPs, both by wildlife and humans. The physical form and size of nanoparticles can be related to toxicological effects that differ significantly from the dissolved chemical forms of the metallic components dissolved in the same systems. It will be important to understand the toxicology of exposure to AgNPs if information is to be obtained on the safe use of AgNPs in consumer products. Some studies have shown reproductive and developmental effects on animals exposed to AgNPs [8–11].

One potential exposure route that is of great concern is oral exposure through ingestion, although exposure can also be a result of direct contact with the particles, or inhalation, due to the size of the particles [12]. Environmental exposure to AgNPs can lead to uptake by plants through the root system [13, 14]. In addition to exposure through consumption of contaminated plants, AgNPs are sometimes applied to animal feed, resulting in the possibility of uptake in livestock [15, 16]. Oral exposure to AgNPs and biodistribution after exposure can thus be of interest in both humans and animals. One type of animal dosing study can be performed by oral administration of formulations containing the chemical of interest and monitoring the mass balance of the nanoparticles as they are retained or excreted by the subjects. Previous tissue distribution studies have shown that animals dosed with AgNPs exhibit the most elevated amounts of Ag through routes of excretion (feces and urine) [17]. Due to the high excretion levels of AgNPs in the animal models, it is necessary to develop a suitable assay for the determination of AgNP content in all excreta. Nanoparticles that are not excreted are most commonly retained in the organs of the subject, making a robust assay for nanoparticles in biological tissues, particularly in organs involved in the removal of toxins, an essential tool in toxicology studies. One organ that has been found to be a strong indicator of silver exposure in organisms is the liver [17]. A previous study found that in rats exposed to AgNPs, the concentration of silver found in the liver peaked at 24 h after exposure [18].

In order to develop a reliable method for quantifying synthetic nanomaterials in biological media, it is necessary to use a sensitive analytical technique that can specifically determine the concentration of the metal present in the system. Previous methods have been developed for the analysis of AgNPs in water, soil, and plant tissue, but these methods made use of flame or graphite furnace atomic absorption spectroscopy, which exhibits lower sensitivity than some other spectroscopic techniques [17, 19–22]. Some methods rely on UV-Visible absorbance to measure the surface plasmon resonance of unagglomerated nanoparticles [23]. However, the high chloride content in biological samples, sulfhydryl groups contained in proteins, and other chemical factors can potentially result in agglomeration and precipitation of nanoparticles, making this method of quantification unreliable for total silver analysis [24, 25]. A reliable, robust, and sensitive method for the analysis of AgNPs in animal tissues and waste products has not yet been evaluated in detail. Some reports have described the digestion of biological samples for tissue distribution studies, but to our knowledge, very few reports have provided detailed validations of their digestion and analysis methods [12, 26–29]. Previous studies have used an acid dilution of urine samples for analysis by inductively coupled plasma mass spectrometry (ICP-MS) to determine the content of uranium, but there are potential difficulties related to the presence of chloride in urine and the possibility of precipitation of silver that require investigation [30, 31]. The development and validation of a method for the analysis of AgNPs in biological media taken from nonhuman species will not only provide a method for analysis of biological samples in toxicological studies, but can also serve as the basis for the adaptation of the method to use on biological samples obtained from humans.

ICP-MS provides specificity on an elemental basis, can account for interferences through the application of collision cell technology (for polyatomic interferences) or correction equations (for isobaric interferences), and exhibits limits of detection in the parts-per-trillion range [32]. These characteristics make ICP-MS well-suited to the quantification of AgNPs in biological tissues. Analysis by ICP-MS also requires the development of an appropriate sample digestion method that can break down the sample matrix and provide a homogenous solution capable of passing through a nebulizer, while still maintaining the elemental composition of the sample. Some previous studies have made use of ICP-MS as the analytical technique of choice, or in conjunction with other techniques such as transmission electron microscopy and field-flow fractionation to provide information regarding the physical characteristics of the nanoparticles in addition to chemical concentration [33, 34]. Inductively coupled plasma-optical emission spectroscopy (ICP-OES) has been employed in some previous studies of AgNPs, with limits of detection reported in the micrograms per liter range, orders of magnitude higher than those possible with ICP-MS analysis [35, 36].

In this study, three assays for determining the content of AgNPs in rat feces, liver tissue, and urine have been developed featuring digitally controlled hotblock and microwave digestions of the sample matrices in nitric acid. The assays in feces and liver were found to exhibit acceptable accuracy and precision, while the urine assay exhibited slightly low analytical recoveries. The methods developed here provide the basis for the performance of future toxicological studies of AgNPs with the potential for application to other synthetic nanoparticles and other similar biological matrices.

Method

Materials

All preparation of samples and solutions used 18 MΩ cm^{-1} deionized (DI) water. Sample digestion was performed with trace metal-grade concentrated nitric acid (SCP Science, Quebec). Stock silver standards for sample spiking were obtained from High Purity Standards (Charleston, SC) and used as received. Samples of male rat feces, urine, and liver were commercially purchased from Bioreclamation LLC (New York) and were used as received for sample preparation. AgNP stock formulations were prepared using 20-nm citrate-coated AgNPs produced by nanoComposix, Inc (San Diego, CA).

Instrumentation

Sample digestion was performed with a combination of a DigiPREP graphite hotblock (SCP Science, Quebec) and a Discovery SP-D microwave unit (CEM Corporation, Matthews, NC) in 35 mL quartz vessels. Analysis of prepared samples was performed on a Thermo X-Series 7 ICP-MS equipped with a Peltier-cooled spray chamber and an ASM-500X autosampler. All sample preparation and analysis steps were performed in low-light or yellow-light environments to minimize redox activity or precipitation of silver contained in samples.

Feces Sample Preparation

Feces samples were prepared by massing approximately 0.40 g, followed by spiking with either diluted elemental silver stock standards or AgNP formulation solutions. Samples were spiked with AgNP formulation solutions in triplicate at three concentrations from 6 to 90 ng/mL. Additional samples were prepared in triplicate at a concentration of 1000 μg/L for preparation of dilution verification samples using silver stock standard. Six replicate AgNP formulation-spiked samples were prepared at the low spiking concentration to challenge the limit of quantification. Silver stock standard-spiked samples were prepared in triplicate at concentrations of 5–75 ng/mL. Matrix blank samples were prepared by digestion of six replicates of control rat feces without spiking and analysis by the same method as the spiked samples. The spiked and unspiked samples were frozen and lyophilized for 72 h. After lyophilization, the sample mass was measured again to monitor loss of mass; mass loss for all feces samples ranged from a 16 % loss to a 51 % loss with the samples containing the higher volumes of spiking solution exhibiting the largest weight loss. Five milliliters of concentrated nitric acid was added to each 35 mL digestion vessel, which was then placed in a graphite hotblock for digestion at temperatures gradually increasing from 65 to 110 °C (5 °C increase/30 min). The vessels were then removed and allowed to cool to room temperature, followed by digestion in the SP-D microwave unit at a maximum temperature of 180 °C and pressure of 400 psi for 15 min. The resulting digest was then diluted to volume with DI water and stored in a refrigerator at approximately 8 °C until analysis.

Liver Sample Preparation

Liver samples were prepared by thawing, followed by homogenization using a Polytron® with a handheld tissue homogenizer (Kinomatica, Switzerland) followed by massing of approximately 2.0 g of the tissue homogenate. Prior to homogenization, the blades of the homogenizer were cleaned to minimize the risk of contamination by submersion in DI water and operation for 10 s, submersion in a 5 % HNO_3 solution and operation for 10 s, and then submersion in DI water and operation for 10 s. The tissue samples were spiked with AgNP formulation solution at six concentrations ranging from 30 to 96 ng/mL. Additional samples were prepared in triplicate at 1000 ng/mL for dilution verification purposes, at masses ranging from 2.2 to 5.3 g using silver stock standard. Matrix blank samples were prepared by digestion of six replicates of control rat liver tissue of approximately the same mass and analysis by the same method as the spiked samples. Five milliliters of concentrated nitric acid was added to each vial, which was then placed in a hotblock for digestion at temperatures gradually increasing from 65 to 110 °C (5 °C increase/30 min). The vessels were then removed and allowed to cool to room temperature, followed by digestion in the SP-D microwave unit at a maximum temperature of 180 °C and pressure of 400 psi for 15 min. The resulting digest was then diluted to volume with DI water and analyzed immediately.

Urine Sample Preparation

Urine samples were prepared by spiking a nominal volume of 0.500 mL of control urine with an AgNP formulation or an elemental silver stock standard, followed by a tenfold dilution of the sample with a 1 % nitric acid solution. Samples were spiked at three concentrations ranging from 6 to 75 μg/L. Additional samples were prepared at 3000 μg/L for dilution verification purposes using either AgNP formulation or silver stock standard. Six replicate AgNP formulation-spiked samples were prepared at the low spiking concentration for analysis. All other spiking levels of AgNP formulation were prepared at medium, high, and dilution verification levels. Stock standard-spiked samples were prepared at the same concentrations in quadruplicate. Matrix blank samples were prepared by dilution of six replicates of control urine and

analysis by the same method as the spiked samples. Samples were analyzed immediately after preparation.

ICP-MS Analysis of Samples

Digested samples were analyzed for total silver content against calibration standards prepared from stock standards over the concentration range of 0.5–100 ng/mL. Calibration standards were prepared daily prior to analysis to account for potential photodegradation. Silver signal was monitored at ^{107}Ag and ^{109}Ag, and data are reported for ^{107}Ag. Indium was added to calibration standards and samples as an internal standard to monitor and correct for instrument drift over time. Calibration curves were calculated by plotting the mass-uncorrected counts as a function of concentration of silver nanoparticle (in ng/mL) and performing linear least squares regression on the plot with no weighting. Quality control samples consisted of acid matrix-matched calibration verification standards that were analyzed immediately following calibration and again after a maximum of every 10 samples. Carryover was assessed by analysis of calibration blank samples after measurement of the calibration verification samples.

Results

Method Figures of Merit

Method limits of detection and quantification were determined for all matrices by analyzing the silver signal of six replicates of matrix blanks, taking the standard deviation of the detected silver concentration, and multiplying by the Student's T value at 99 % confidence interval for the appropriate degrees of freedom. Statistical outliers were excluded from the sample set by application of Dixon's q test at a 99 % confidence interval. The estimated limit of quantification was taken as the lowest concentration calibration standard with a back-calculated concentration within 25 % of its nominal concentration. The method limit of detection in feces for sample masses ranging between 0.27 g and 0.47 g was found to be 5.1 ng Ag/g feces; in urine for nominal volumes of 0.500 mL was found to be 0.415 ng Ag/mL urine; and in liver for sample masses ranging between 3.4 g and 7.3 g was found to be 0.64 ng Ag/g tissue. The lowest acceptable calibration standard was observed to be 0.50 ng/mL Ag in all cases. This makes the estimated limit of quantification in feces matrix 50 ng Ag/g feces; in urine matrix, 5 ng Ag/mL urine; and in liver matrix, 12.5 ng Ag/g tissue.

Analytical Recoveries—Accuracy and Precision

Analytical recovery for all samples was calculated using the following equation:

$$\%\text{Recovery} = \frac{[\text{Ag}]_{\text{measured}}}{[\text{Ag}]_{\text{nominal}}} \times 100\% \quad (1)$$

Recovery of spiked rat feces using a commercially purchased Ag solution in dilute HNO_3 and DI water was assessed (Table 1), with recoveries ranging from 89 to 93 % (Fig. 1). The relative standard deviation for triplicate preparations was 2.9 % at the low-concentration-spiked samples, 0.77 % in medium-concentration-spiked samples, and 0.90 % at the higher spiking concentrations, demonstrating a high degree of precision. High-level dilution verification sample analysis in feces matrix spiked with silver stock solution provided an average recovery of 88 % (0.82 % relative standard deviation (RSD)). Samples of rat feces that were spiked with varying concentrations of AgNP formulation exhibited analytical recoveries ranging from 82 to 92 %. The %RSD for replicate measurements for AgNP digests at all concentrations was between 2.4 and 2.6 %.

Six homogenized liver samples were spiked to yield final concentrations of 30, 48, 60, 72, 84, and 96 ng/mL of AgNP. Observed recoveries ranged from 88 to 90 % (Fig. 2). Recovery of spiked rat livers using a commercially purchased Ag solution in HNO_3 and DI water was also assessed, with recoveries of 72–90 %. High-level dilution verification sample analysis provided an average recovery of 96 %.

Recovery of rat urine spiked with a commercially purchased Ag solution in HNO_3 was also assessed, with much lower recoveries of 62–73 % (Table 1 and Fig. 3). High-level dilution verification sample analysis for urine spiked with stock silver solution provided an average recovery of 84.2 %. Quadruplicate analyses by two analysts at each

Table 1 Average analytical accuracy and precision for the analysis of rat feces, and urine for stock silver solutions and 20-nm citrate-coated AgNP

Matrix		Stock Ag		AgNP formulation	
		% Recovery	%RSD	% Recovery	%RSD
Feces	Low	90.7	2.9	92.2	2.6
	Med	92.5	0.77	83.9	2.4
	High	89.4	0.90	81.8	2.4
	Dilution	88.5	0.82	NA	NA
Urine	Low	62.2	8.4	79.5	4.4
	Med	73.2	8.1	84.7	1.2
	High	71.4	11	84.7	0.27
	Dilution	84.2	6.4	83.2	3.6

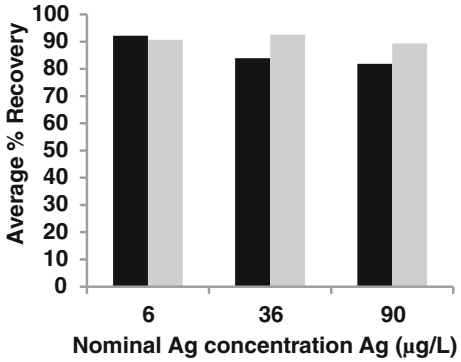

Fig. 1 Average percent recovery for replicate analyses of feces spiked with silver stock standards and AgNP formulation (n=6 for AgNP low concentration, n=3 for all others). Key: *black bar*, AgNP formulation spike; *gray bar*, Ag stock standard spike

concentration demonstrated 6.4–11 %RSD, exhibiting slightly decreased method precision compared to other preparation methods. Urine spiked with concentrations of 6, 36, 90, and 1000 ng/mL of AgNP exhibited analytical recoveries ranging from 80 to 85 %. Replicate analysis of AgNP formulation-spiked stock solutions demonstrated 0.27–4.4 %RSD, exhibiting strong precision for the method.

Relationship Between Feces Mass Loss and Sample Recovery

During method development, it is important to establish the relationship between the physical characteristics of samples and the ability to obtain consistent recoveries. Several methods of feces analysis involve the lyophilization of the sample prior to digestion [37]. The amount of moisture lost during lyophilization may be related to the physical and chemical characteristics of the sample, suggesting that there could potentially be a link between the amount of moisture lost during sample preparation and the ability to obtain consistent recoveries of AgNPs. During this investigation, observed feces mass lost during lyophilization varied widely, ranging from 19.7 to 51.0 %, while recoveries of AgNPs from those feces samples were found to range from 79.3 to 95.9 %. These observations suggest that there is a minimal correlation between moisture loss in feces samples and the recoveries of AgNPs spiked into the samples and that the digestion method

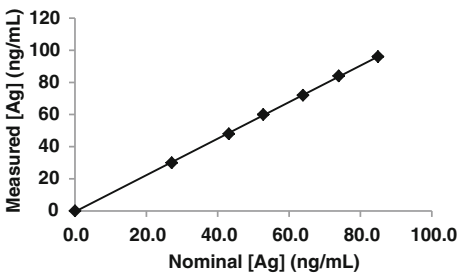

Fig. 2 Average analytical recovery for liver tissue samples spiked with AgNP formulation plotted against expected recoveries

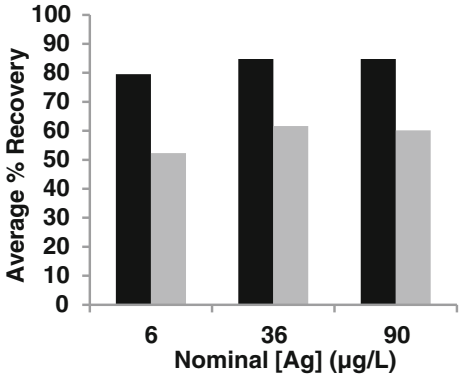

Fig. 3 Average percent recovery for replicate analyses of urine spiked with silver stock standards and AgNP formulation (n=6 for AgNP low concentration, n=3 for all others). Key: *black bar*, AgNP formulation spike; *gray bar*, Ag stock standard spike

used is robust for the determination of AgNPs in feces despite the variability of biological samples.

Discussion

Method Development Considerations

In order to adequately determine the biodistribution of AgNP in test subjects, a robust digestion method is necessary to account for all the differences throughout a single biological system. A high degree of robustness is also necessary to provide accurate analytical results in biologically variable samples such as feces. In previous method development work, the addition of nitric acid to fecal samples has resulted in exothermic expansion and frothing of the mixture during the hotblock digestion due to the mixture of nitric acid and water in the feces, which can result in sample loss in small-volume sample vessels like the 35 mL digestion vessels used here (personal observation). Lyophilization of samples prior to any digestion step helps to minimize this reaction and prevents the frothing from occurring. The lack of frothing results in higher precision and accuracy of spiked sample analysis. This sample preparation step is of vital importance because of the possibility of variability in sample moisture depending on the diet and health of individual specimens.

For ICP-MS measurements, the analyte signal was monitored for two isotopes, 107 and 109, and results are reported here for ^{107}Ag. Samples were analyzed in a standard mode or in the absence of a collision cell gas that could exclude polyatomic interferences. This method of analysis provides higher sensitivity for the analysis and allows measurement of lower concentrations of Ag than would be possible in the presence of a collision cell gas. Silver suffers from very few biologically relevant polyatomic interferences on either mass, primarily ^{67}Zn^{40}Ar on ^{107}Ag [38]. Since the isotopic

abundance of ^{67}Zn is relatively low (4.10 % natural abundance), the potential for interference by that polyatomic species is low despite the relatively high concentration of Zn in biological samples. The limits of detection and quantification that are reported for the method developed here were significantly lower than or comparable to those of a number of similar studies in the literature, which ranged from LOD= 0.26–700 ng/mL in fluid samples and 200 ng/g in a representative tissue sample [39, 29, 40]. Reported limits of quantification are lower than those reported here (0.08–0.67 ng/mL), but it is unclear whether their reported limits take into account sample preparation and dilution factors [39, 41]. Also, some of these reports use different matrices than those explored in this study.

Initial attempts were made to analyze silver stock-spiked solutions and AgNP formulation-spiked samples in whole liver organs. The method applied in these initial attempts did not include homogenization of the tissue prior to spiking and used a higher sample mass. The analysis of silver for silver stock-spiked samples and AgNP formulation-spiked samples provided low, inconsistent recoveries at concentrations above 36 ng/mL of Ag for both stock-spiked and formulation-spiked samples (data not shown). It was found that decreasing the mass of tissue coupled with homogenization allowed successful recovery of AgNP formulation spiked into tissue samples over a range of concentrations, making the tissue homogenization critical to successful application of the method to tissue studies.

The experiments described here were designed to support investigators exploring the biological distribution and fate of AgNPs administered under different dosing scenarios. One previous study of the biodistribution of silver nanocrystals in rats after intraperitoneal injection of the nanocrystal solution described analytical results for tissue concentrations of silver, but the method of digestion was not described and no evidence was provided for analytical accuracy of the method applied [26]. A recent review article discussed the advances in the area of analysis of engineered nanomaterials in biodistribution studies, which discusses some studies of silver nanoparticles in biological media [12], but many of the studies referenced therein utilize methods that either were not validated or used large amounts of sample due to their application of the method of standard addition [42]. By spiking a known concentration of AgNP into three common biomonitoring matrices, it is possible to assess the viability of the method for application to other biological samples that could possibly be obtained from such a study. Similarly, it is essential to determine the comparability of the analytical results obtained with free silver ions and AgNP formulations in the digestion and analysis of samples. The comparability of the results can be assessed either by (a) comparison of stock- and formulation-spiked samples (as will be addressed below) or (b) preparation and analysis of sample matrix calibration curves and acid matrix calibration curves against each other. A sample matrix calibration curve in the liver tissue was prepared and analyzed against a stock standard calibration curve, as will be discussed below, but in addition, a series of stock standard-prepared standards were analyzed against a matrix sample-digested calibration curve, and the stock standard-prepared standards were found to provide an average analytical recovery of 102 %. These results suggest that the digestion process results in the breakdown of the AgNPs in solution to free silver ions prior to analysis.

Analytical Recoveries

To facilitate suitable recoveries of AgNPs from the biological tissue and excreta, digestion methods were developed to provide optimal recoveries of silver content while avoiding the use of chloride anions, which could potentially result in the precipitation of silver as AgCl. Three unique sample preparation methods, one for each matrix, exhibited analytical recoveries between 72.3 and 95.9 %, generally with excellent method precision. The advantage of the methods described here are that they utilize less sample than those of previously described studies, making the methods applicable to toxicological studies where limited sample mass is available [42]. It may be possible to further decrease the mass of the biological samples used for digestion, although such a revision of the method will adversely affect the limits of detection and quantification reported here. Further, the methods described here provide analytical techniques that can be performed routinely using common analytical laboratory equipment, rather than those of previous studies that use specialized equipment such as single-particle ICP-MS for the characterization of nanoparticles in biological samples [43, 44].

It was expected that the prevention of AgCl precipitate would be most challenging in rat urine matrix due to a naturally high abundance of Cl ion. Results showed a 71–85 % recovery for AgNPs. Though the urine recoveries were the lowest of the three, the level of suppression observed in urine was lower than expected, with no linear relationship between AgNP concentration and analyte loss observed. It is possible that the primary cause of the better-than-expected recoveries of AgNPs in urine is a protective effect of the coating of the nanoparticle, which minimizes the reactivity of silver atoms with chloride in solution. The carbon containing shell likely forms a partially hydrophobic barrier between the Ag components of the nanoparticle and the bulk solution, preventing much of the free Cl^- from combining with any Ag. A previous study investigated the aggregation of 70-nm citrate-coated AgNPs in salt solutions and demonstrated that the critical coagulation concentration of chloride in a primarily sodium chloride solution was 47.6 mM [1]. There is a minimal probability that the stability of the citrate coating found on the particles studied here would be compromised by the dilute acid conditions (approximately 1 %) and lack of heat used for

digestion of the urine samples. However, it is difficult to account for the possibility of AgCl precipitate formation without studying the reaction in detail and developing appropriate correction equations. As both components would be introduced into the plasma simultaneously, any silver signal measured would correspond to a mixture of Ag^+ and AgNPs. One of the limitations of this technique is that the analysis of silver by ICP-MS provides results of total amounts of elements in a sample without regard to the speciation of the elements. Future work will be necessary to elucidate the relationship between total silver in digested samples and the speciation of the silver contained in samples. One previous report detailed a speciation method for silver nanoparticles in environmental samples that used a cloud point extraction-based system, and other physical methods can be applied to biological samples for speciation analysis, including TEM, SEM, field-flow fractionation, and UV-Visible spectroscopy [23, 26, 34, 45]. It is also possible that a digestion step involving the use of microwave technology or hotblock heating may allow a more accurate determination of silver in urine samples. Further, preliminary experiments have suggested that utilization of HCl in place of HNO_3 during digestion allows increased recovery of AgNP formulations in high-salt matrices such as cellular growth media (data not shown).

The results obtained in digested liver tissue samples exhibited a great degree of variability of observed percent recovery of nanoparticles. The physical size of livers ranged in experiments from approximately 1.3 to 2.0 g of homogenate, suggesting that the volume limitations of the digestion vessels used and the variability of biological tissue samples may have resulted in incomplete sample digestions. Homogenization of the tissue samples followed by digestion of a subsample of the organ can assist in minimizing the variability of the results due to "hot spots" or localized deposits of AgNP in the tissue samples. This method is especially important if the tissue sample is larger than 4 g, so that it may be separated into subsamples which can be summed for the entire tissue. The method reported here for tissue is sufficiently robust to provide acceptable analytical recoveries over a range of concentrations and can be applied to AgNPs with different coatings. Personal observations of tissue analysis experiments performed with PVP-coated AgNPs have demonstrated comparable spike recoveries in quality control samples, suggesting the applicability of the digestion and analysis method to a broader selection of materials, but this discussion is beyond the scope of this manuscript.

Conclusions

AgNPs are a growing concern in the environment due to their increased use in consumer products. Therefore, it is important to develop reliable methods for the determination of AgNPs in biological samples. Three biological matrices that could be targets for analysis in toxicological studies are urine, feces, and liver samples. The method developed here provides a validated, reliable determination of silver content in feces, urine, and liver tissue samples and will be applied in upcoming reports of biodistribution studies of AgNPs in rats. Further method development and studies will be necessary to understand the speciation of silver in digested samples and its relationship to the determined concentration in biological samples.

Acknowledgments This study was supported, in part, by the National Institute of Environmental Health Sciences (U19 ES019525) and National Institute of Diabetes and Digestive and Kidney Diseases (1U24DK097193-01). The authors would also like to acknowledge RTI International for providing internal funds for the study. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

Ethical Statement The samples analyzed in this study were collected from animals that were maintained under RTI IACUC guidelines, and all protocols were performed with IACUC review and approval.

Conflict of Interest The authors acknowledge that there are no financial conflicts of interest in this report.

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