Title: Assessment of Total Silver and Silver Nanoparticle Extraction from Medical Devices

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Abstract (200 words max)

There is concern over the release of silver nanoparticles (AgNPs) from medical devices due to their potential toxicological consequences inside the body. Towards developing the exposure component of a risk assessment model, the purpose of this study was to determine the amount and physical form of silver released from medical devices. Scanning electron microscopy was used to confirm that three of five marketed medical devices contained nanosilver coatings (mean feature sizes 115 to 341 nm). Aqueous device extracts (water, saline and human plasma) were analyzed with inductively coupled plasma mass spectrometry, UV-visible spectroscopy, dynamic light scattering, transmission electron microscopy, and nanoparticle tracking analysis. The amount of silver extracted from the devices ranged from 1×10^{-1} to 1×10^6 ng/cm² (conditions ranged from 37 to 50 °C, over one hour to seven days). The results further indicated that one of the five devices (labeled MD1) released significantly more AgNPs than the other devices. This data

suggests that some but not all devices that are formulated with nanosilver may release detectable levels of AgNPs upon extraction. Further work is underway to quantitate the proportion of silver released as AgNPs and to incorporate this data into a risk assessment for AgNP exposure from medical devices.

Keywords (6 max): medical device; silver nanoparticle; extraction; exposure assessment

Abbreviations

AgNP silver nanoparticle

Ag silver

AU arbitrary units

DLS dynamic light scattering

EDS electron dispersion x-ray spectroscopy

ICP-MS inductively couple plasma mass spectrometry

MD medical device

nAg nanosilver

ND none detected

NTA nanoparticle tracking analysis

PDI polydispersity index

SEM scanning electron microscopy

TEM transmission electron microscopy

UV-VIS ultraviolet-visible

1. Introduction

Silver (Ag) is a broad-spectrum antimicrobial element with a record of historical as well as modern use in the healthcare industry (Maillard and Hartemann, 2012). As medical device infections continue to negatively impact health outcomes for patients (Busscher et al., 2012), silver is being added to devices to inhibit the colonization of their surfaces by microorganisms (Monteiro et al., 2009). Silver has been used or is being considered for a wide range of applications including wound care (Chaloupka et al., 2010; Cortivo et al., 2010; Gee Kee et al., n.d.; Innes et al., 2001; Ip et al., 2006; Leaper, 2006; Rigo et al., 2013), catheters (Arjun Srinivasan et al., 2006), orthopedic (Slane et al., 2015), dental (Chladek et al., 2012), and cardiovascular devices (Grunkemeier et al., 2006).

The formulation of medical devices with silver is based on the performance and manufacturing requirements of the device. A number of chemistries may be used to impart silver into medical devices, including ionic formulations and engineered nanosilver (nAg) surfaces (Chaloupka et al., 2010). nAg refers to bulk silver with features sizes of about 100 nm or less and may take the form of a continuous coating or discrete silver nanoparticles (AgNPs). Such nAg configurations are thought to be advantageous because they afford greater control over the rate of silver release; however, toxicological concern has been raised surrounding the issue of AgNPs that are released into the body (Garcia-Reyero et al., 2014; Johnston et al., 2010; Wijnhoven et al., 2009). Specifically, there is an extensive research effort to understand the potential toxicological impact of AgNPs exposure via various routes, including broken skin (George et al., 2013), inhalation (Haberl et al., 2013; Kaewamatawong et al., 2014), ingestion (Hadrup and Lam, 2014; Munger et al., 2014), and intravenous routes. It is known that AgNPs are transported (Lankveld et al., 2010; Lee et al., 2007), accumulated (Lee et al., 2013), and affect cellular processes (Bressan et al., 2013; Haase et al., 2014) differently than ionic forms of silver, and thus some speculate that increased scrutiny over their use is warranted.

In developing a risk assessment of AgNP exposure from medical devices, the potential nature of the hazard and exposure levels are considered. While some investigators have addressed the issue of AgNP release from consumer products (Benn et al., 2010; Benn and Westerhoff, 2008; Echegoyen and Nerín, 2013;

Monteiro et al., 2012), there are still a limited number of reports addressing medical devices specifically (Holbrook et al., 2014; Rigo et al., 2012). Further, additional methodological development is needed in the area of characterization of nanoparticles using common laboratory equipment that may be found in a research and development setting. The present study is an early step in determining the potential AgNP exposure from medical devices by performing extraction studies. Extractions were carried out in various aqueous media at different times and temperatures, followed by analysis of the amount of silver released and investigation for evidence of AgNP release. The results suggest the presence of nanoparticles in some extracts, but also highlight the difficulty in interpreting data obtained using typical medical device extraction protocols to identify nanoparticle release. Ultimately, this research is intended to promote patient health through a better understanding of AgNP release from medical devices.

2. Materials and Methods

2.1 Test sample preparation

Five types of medical devices containing silver were obtained by purchasing through third party vendors and consisted of three wound dressing products and two catheter products (manufacturer and product names withheld, randomized in **Table 1**). These devices were chosen based on the characteristics of silver within the device. Two of the wound dressings and one of the catheters were known to contain nAg through public disclosure by the device manufacturer (labeled “nano” in Table 1), while the other wound dressing and catheter served as controls as they were understood to contain ionic silver only (labeled “ionic” in Table 1).

Device Characteristics		
Device	Silver	Measured Ag Coverage [$\mu\text{g}/\text{cm}^2$]
MD1	Nano	295 ± 15
MD2	Ionic	201 ± 14
MD3	Nano	271 ± 11
MD4	Ionic	2.32 ± 0.35
MD5	Nano	150 ± 8

Table 1. Medical devices studied in the present report. Three types of wound dressings and two types of catheters were studied (randomized and labeled MD1 through MD5). All medical devices contained silver, and they are designated as either “nano” or “ionic” to reflect whether the manufacturer used a process known to create nanosilver in the production of the device.

Devices were removed from their sterile packaging and carefully cut into smaller test samples as follows: wound dressings were cut into discs measuring 10 mm in diameter using sterile biopsy punches (Acuderm, Fort Lauderdale, FL) and catheters were cut into 2.45 cm length segments using clean laboratory scissors. About 20 individual devices of each product were used to complete the study. In initial studies (data not shown). We observed that sample-to-sample variance introduced through sample preparation was the greatest source of error in the study. Additionally, we observed that devices were manufactured quite consistently and were unable to distinguish any device-to-device variability.

2.2 Extraction Protocol

Samples were separately extracted, at various time points in water, saline and citrated human plasma. Water was obtained from a Thermo Scientific (Waltham, MA) Barnstead Nanopure water deionizer and measured $>18.2 \text{ M}\Omega$ resistance. Saline was prepared from deionized water by adding sodium chloride (Sigma Life Sciences, St. Louis, MO) to obtain a 0.9% wt/vol solution, which was filtered using a $0.2 \mu\text{m}$

Stericup filter unit (EMD Millipore, Billerica, MA). Citrated human plasma was purchased from Valley Biomedical (Winchester, VA).

Extractions were performed in 25 mL scintillation vials with polyethylene cone lid inserts (Wheaton, Millville, NJ). The extracts were prepared for four time/temperature endpoints (1 hour at 37°C, 24 hours at 37°C, 72 hours at 50°C, and 7 days at 37°C) in each of the three media. Separate extractions were carried out for each time point (n=3). Ten (10) mL of each medium was added to vials containing test samples using sterile 10 mL pipettes. For the plasma extractions, the scintillation vials were briefly irradiated with UV light to reduce the likelihood of microbial contamination. Once filled, all vials were placed into a MaxQ4450 (Thermo Scientific, Waltham, MA) incubator-shaker at a shaking rate of 60 rpm at the specified temperature for the duration of the extraction. At the conclusion of each extraction period, the vials were removed from the shaker and the 10 mL of solution from each vial was transferred into labeled 15 mL BD Falcon (Franklin Lakes, NJ) polystyrene centrifuge tubes in order to separate the sample from the extraction media.

Positive and negative control extractions were conducted in conjunction with all time/temperature endpoints. For negative controls, extractions were carried out in the absence of a test material. For positive controls, 10 µg/mL and 10 mg/mL Ag⁺ from silver nitrate (AgNO₃) was spiked into media prior to exposing to extraction conditions. Control extracts were analyzed in parallel to test sample extracts.

2.3 Scanning Electron Microscopy (SEM)

A Hitachi (Troy, MI) SU-70 Schottky field emission gun scanning electron microscope was used to image carbon-sputtered medical device samples. The secondary electron images were acquired under 7.5 kV accelerating voltage at 200-250,000 times magnification. Feature sizes were quantified using the measure tool of ImageJ Software (National Institutes of Health, Bethesda, MD) by manually locating features, assuming an elliptical shape, and drawing a line across the major axis (longest dimension of each feature). The length of each line was considered the feature size.

2.4 Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

In order to determine the concentration of silver in device extracts, analysis using an XSeries2 (Thermo Fisher) inductively coupled plasma mass spectrometer was carried out. To prepare the samples for ICP-MS, 171.4 μL of Fisher Scientific Optima 70% HNO_3 was added to 6 mL aliquots of extract resulting in a 2% HNO_3 solution. From the 2% minimum dilution, each extract was further diluted using 2% nitric acid (HNO_3) to achieve final solutions within the quantifiable range of the ICP-MS (0.1 to 100 ppb) based on calibration curves. The final dilutions of the extracts ranged from 1:1 to 1:1000. All of the samples were run in parallel with Pure Plus (Perkin Elmer, Waltham, MA) internal standard containing Bi, Ge, In, Li⁶, Sc, Tb, and Y diluted to 1 ppb.

2.5 Dynamic Light Scattering (DLS)

Dynamic light scattering was used to determine average particle size in water and saline extracts. 1 mL of each extract was transferred into a Sarstedt (Nümbrecht, Germany) polystyrene cuvette and DLS analysis was carried out using a Malvern (Worcestershire, UK) Zetasizer Nano ZS according to the manufacturer's instructions. Measurements of each extract solution were replicated three times (n=9 total). In certain instances samples could not be analyzed due to low count rates, and thus these samples were not included in the final calculation (reported as lower n in Table 3).

2.6 Transmission Electron Microscopy (TEM)

To prepare samples for TEM, 5 μL of the undiluted extract was dropped onto a 300 Mesh Cu TEM grid (Pelco, Inc.) and was left to dry for about 2 minutes prior to imaging using a JEOL (Peabody, MA) JEM-1400 TEM. Each sample was dropped on three separate grids for a total of 9 grids per extract condition. Manual searching was employed in order to identify nanoparticles in the extract by viewing all of the sections of each grid. Once particles were visually located, electron dispersive x-ray spectroscopy (EDS) was used to confirm the composition of the particles as containing silver. High resolution images of these silver-positive particles were recorded. Particle size was measured using ImageJ Software.

2.7 Ultraviolet-Visible Spectroscopy (UV-VIS)

For UV-VIS, 300 μ L of each extract was pipetted into wells of a Greiner 96 well UV-Star UV-transparent microplate (Greiner, Monroe, NC). Full spectra absorption data was collected using a SpectraMax 190 plate reader spectrophotometer (Molecular Devices, Sunnyvale, CA). Using 50 nm citrate-coated Biopure AgNPs as controls (Nanocomposix, San Diego, CA), the limit of detection of AgNPs in water was determined to be 200 ng/mL. To roughly correlate absorbance intensity of extracts to nanoparticle concentration, the purchased nanoparticles were also used to construct a five-point calibration curve with R^2 value of 0.99 and linear equation of $y=1.79x+0.95$.

2.8 Nanoparticle Tracking Analysis (NTA)

NTA was used to characterize the size and concentration of nanosilver in each extract. When the flow of the extract through the instrument was visualized on the screen, the abundance of particles, or lack thereof, was observed. The cuvette containing the extract was placed into the sample holder of the Nanosight (Amesbury, UK) NS500. The receiving tube was placed in the cuvette and the program was run to analyze the sample. Once the sample was taken into the instrument, the screen produced a live video feed of the sample running through the instrument. The feed was recorded and used in analysis.

2.9 Statistics

All extractions were replicated 3 times ($n=3$), and each analytical method was carried out with three technical replicates. The study was repeated once and the data reported herein is from the second round, which closely followed the findings in the first. All means are reported $\pm$ the standard deviation. Statistical comparisons are made by a two-tailed Student's t-test assuming equal variances of the means using Microsoft Excel (Seattle, WA).

3. Results

3.1 Characterization of Devices

3.1.1 SEM

SEM images of the medical devices examined in this study are shown in **Figure 1A**. These representative images illustrate the segment of the device containing the greatest amount of silver, which was confirmed by EDS and quantified by calculating the ratio of the intensity of the silver peak in the EDS spectra to the intensity of the adjacent background signal. For all devices except MD4, these silver-rich areas were from the outer surface of the device, while for MD4 silver was found only in the cross-section of the device. The SEM images demonstrate that the silver-rich regions of each device had vastly different appearances, which is attributable to the different manufacturing processes and material compositions of each device. For the three devices containing nAg (MD1, MD3, and MD5), the silver-rich regions were greater in number and more apparent compared to the ionic silver-containing devices, in which the silver-rich regions were small and had corresponding low intensities in the EDS spectra.

The results of quantifying silver feature size in the SEM images by measuring the major diameter are shown in **Figure 1B**. The mean diameters for the three nAg devices were measured as 115 ± 35 , 205 ± 147 and 341 ± 267 nm for MD1, MD3, and MD5, respectively. The shapes and densities of these nanoscale features varied significantly. For MD1, a dense layer of nAg was observed to completely cover the device surface with relatively uniform size (relative standard deviation 34%). For MD3, the nAg layer consisted of larger particles in a bed of silver with smaller feature sizes. Finally, for MD5, the largest individual particles were observed (some exceeding 1000 nm), but overall the population was mixed, leading to the largest mean feature diameter and relative standard deviation (78%).

Figure 1. A) Scanning electron microscopy (SEM) images of the test articles illustrate the nano or ionic nature of the silver contained in each medical device. The magnification shown is the greatest that could be obtained for each device while still showing a detailed picture and minimizing image artifacts due to charging. A red “X” in each image indicates the location of maximal silver signal (~ 3 eV) in energy dispersive x-ray spectroscopy (EDS) analysis that was performed on each sample. MD1, MD3, and MD5 contain nanoscale silver characterized by large deposits of silver confirmed with EDS while MD2 and MD4 contain ionic silver, characterized by small difficult-to-find deposits of silver with low EDS peak intensities. **B)** Results of diameter analysis of nanosilver features in SEM images for the three nanosilver devices.

3.2 Analysis of Extracts

Changes to test samples and media over the extraction period are summarized in **Table 2**. MD1 turned from a metallic blue/silver color to brownish. MD2, a hydrogel, swelled in the aqueous environment, and MD3, which contained a fibrous inner structure, condensed upon being wetted. These observations became more apparent with longer extraction times. In general, extracts did not change from their original appearance over the extraction periods; however, a couple notable exceptions did occur. For MD1, water and saline extracts turned a faint yellow color and for MD3, the device material released numerous small fibers, which was attributed to cutting the device during sample preparation.

Device	Changes to Device	Changes to Extracts
WD1	Changed from blue/silver to brown color	Water and saline extracts turned yellowish color
WD2	Swelled upon being wetted	ND
WD3	Condensed upon being wetted	Small fibers released
Cat1	ND	ND
Cat2	ND	ND

Table 2. Summary of visual changes to medical device samples and extract solutions that occurred over the course of the extraction process. ND indicates none detected.

3.2.1 ICP-MS

ICP-MS analysis of the amount of silver in device extracts revealed that extraction medium, time, and temperature all influenced the silver extraction rate (**Figure 2**). Additionally, the devices were each unique in the amount of silver released and which extraction medium led to the greatest release from a particular device. Silver release across all of the devices and extraction conditions ranged from about 1×10^{-1} (the limit of detection) to 1×10^6 ng/cm² (seven orders of magnitude). The extract media was observed to have a significant effect on the measured rate of silver extraction from devices. In particular, silver extraction into water was highly variable between devices, silver extraction into saline was lower, and silver extraction into plasma most similar when comparing devices.

The most consistent silver release was observed between MD1, MD2 and MD3 in plasma, which all released between 1×10^3 to 1×10^5 ng/cm² of silver across all time points. Relatively low silver release from MD4 and MD5 (1×10^{-2} to 1×10^2 ng/cm²) was observed throughout the various extraction conditions. On a per-sample basis, silver extraction from MD1 was the greatest among the tested devices (ranged from 1×10^3 to 1×10^6 ng/cm² depending on the media). Measured silver in extracts was greatest into water (up to 1×10^6 ng/cm², **Figure 2A**) and much less so into saline and plasma (up to 5×10^4 ng/cm², **Figures 2B and 2C**). For MD2 and MD3, silver extraction kinetics were largely similar to MD1, except that the magnitude

of silver released into water was significantly reduced, by approximately two to three orders of magnitude, respectively. For saline, silver extraction was reduced by approximately one order of magnitude.

Release of silver from MD4 was the lowest among all of the devices tested and below $5 \times 10^1 \text{ ng/cm}^2$ for all extract conditions. For some experiments, the amount of silver was below the limit of detection and so no values are reported on the figure. Release of silver from MD5 exhibited a monotonic increase with each subsequent time point after the initial amount released at 1 hour. The amount released ranged from 0.5×10^0 to $1 \times 10^2 \text{ ng/cm}^2$. The pattern of gradual increase in release was consistent for all extracts.

Figure 2. Effect of extraction conditions on silver release from medical devices. Each device was extracted in three types of media [water (A), saline (B), plasma (C)] to four different time/temperature endpoints (1 hour, 24 hours, and 7 days at 37 °C and 72 hours at 50 °C). * indicates that the concentration of silver was below the limit of detection.

3.2.2 UV-VIS

UV-visible spectroscopy was carried out on water and saline extracts in order to determine if the absorbance of the media changed following extraction (**Figure 3**). The plasma extract samples were not analyzed using UV-VIS due to the strong absorbance properties native to plasma, which masked any absorbance contributions of potential AgNPs themselves. Particular attention was paid to the 350 to 450 nm band as AgNPs are known to exhibit absorbance characteristics in this region due to the surface plasmon resonance effect. Among the devices tested, only extracts from MD1 exhibited an increase in absorbance in this band compared to the background at 700 nm, which was observed to be greater for water extracts (range 1.9 to 2.8) than saline extracts (range 1.0 to 1.7). Using a calibration curve constructed using commercially available 50 nm AgNPs in water, the observed absorbance values corresponded to AgNP concentrations of approximately 200 to 1100 ng/mL in extracts. The only other absorbance shift noted for the medical device extracts was a peak at ~280 nm for MD4. This UV peak was outside the absorbance range for silver, but instead was attributable to some other unidentified, extractable material in the device. AgNO₃ controls showed no changes in absorbance upon exposure to extraction conditions compared to silver-free controls.

Figure 3. UV-visible spectroscopy indicated that MD1 was the only medical device for which extracts exhibited significant absorbance in the 400 nm range, the region where AgNPs are expected to exhibit absorbance due to the surface plasmon resonance effect. **A)** Example absorbance spectra of seven-day water extracts of medical devices. The yellow band indicates the region of interest. **B and C)** Quantitation of absorbance intensity at 400 nm (normalized to the intensity at 700 nm) for water and saline extracts,

respectively. * indicates $p < 0.05$ in comparison with the blank control. AgNO_3 controls were tested at 10×10^6 ng/mL.

3.2.3 TEM

TEM of air-dried extracts was carried out in order to visually evaluate the morphology and abundance of AgNPs in extracts (Figure 4), if present. Only water and saline extracts were analyzed by TEM, as the protein content of plasma interfered with visualization of AgNPs. In order to compare the relative differences in abundance of AgNPs between extracts, qualitative abundance scores were assigned to each device/extract combination based on the observations of the non-blinded TEM operator (same person evaluated all samples). This score ranged from “0” indicating no AgNPs identified to “4” indicating the greatest relative abundance of AgNPs. These scores are shown in **Figure 4** along with representative images of positively-identified AgNP and corresponding EDS spectra. No AgNPs were detected in the extract-only or AgNO_3 controls (data not shown).

In general, the abundance of AgNPs increased with extraction time and temperature. No abundance value greater than zero was assigned to the one hour or 24 hour extracts for any devices. Nonzero values were assigned, however to the longer extractions, with the greatest AgNP abundance observed in the 7 day water extracts. AgNP abundance in water extracts was generally greater than that for saline extracts. Samples from saline extracts also contained a greater number of features absent of silver, which were attributed to dried salt deposits. The greatest abundance of AgNPs identified using TEM was found in 7-day water extracts of MD1, which was the only extract, assigned a value of 4. The shape of AgNPs in these samples was generally irregular. There were only enough particles identified from MD1 in order to determine a meaningful average 20 ± 9 nm particle size (average of measurements from 15,000, 25,000, and 50,000 x magnification images).

Figure 4. Transmission electron microscopy of water and saline extracts of medical devices. Table indicates the abundance with which EDS-confirmed AgNPs were found in extract samples using a qualitative scale in which “4” indicates highly abundant and “0” indicates none found. Included for each device are representative TEM images illustrating the appearance of AgNPs at high magnification. The red “X” indicates the spot at which the EDS scan was conducted and corresponding spectra are shown on the right.

3.2.4 DLS

Results of DLS analysis of medical device extracts are shown in **Table 3**. The greatest evidence for AgNPs in extracts was found for MD1, for which the average particle size was <1000 nm for five of eight extracts analyzed. The three remaining extracts with average z-average >1000 nm had very high standard deviations (greater than 2 times the mean) due to outlier values. For MD2 and MD3, DLS data was inconsistent, with z-average values generally >1000 nm. For MD4 and MD5, only a few extracts produced reportable DLS

results. No particles were detected in the extract-only and AgNO₃ control samples (the analytical runs of these samples timed out due to low count rates and thus no numerical data is reported). The PDI values reported varied widely and were between 0.215 and 0.937, indicating very heterogeneous samples for all extracts.

Z-Avg Size [nm] (n, mean PDI) in Water Extracts				
	1 h	24 h	72 h	7 d
MD1	468 ± 177 (8, 0.514)	522 ± 123 (9, 0.572)	4,250 ± 5,206 (9, 0.717)	80 ± 17 (9, 0.254)
MD2	6,406 ± 10,733 (8, 0.324)	15,766 ± 9,720 (9, 0.937)	9,856 ± 5,322 (9, 0.929)	10,709 ± 7,563 (8, 0.488)
MD3	1,092 ± 461 (9, 0.776)	7,705 ± 6,832 (9, 0.919)	21,972 ± 16,809 (9, 0.793)	9,133 ± 6,644 (8, 0.838)
MD4	***	3,665 ± % (3, 0.408)	299 ± 201 (9, 0.431)	211 ± 11 (9, 0.310)
MD5	***	682 ± % (1, 0.694)	***	702 ± 357 (7, 0.499)

Z-Avg Size [nm] (n, mean PDI) in Saline Extracts				
	1 h	24 h	72 h	7 d
MD1	198 ± 79 (9, 0.313)	4,250 ± 5,206 (9, 0.717)	99 ± 23 (9, 0.215)	7,325 ± 12,479 (9, 0.308)
MD2	71 ± 30 (9, 0.493)	9,856 ± 5,322 (9, 0.929)	33 ± 10 (9, 0.310)	2,356 ± 1,027 (9, 0.896)
MD3	22,799 ± 7,906 (9, 0.819)	21,972 ± 16,809 (9, 0.793)	789 ± 561 (9, 0.626)	236 ± % (3, 0.265)
MD4	***	152 ± 39 (9, 0.284)	225 ± 139 (9, 0.290)	***
MD5	***	769 ± 666 (6, 0.779)	***	***

Table 3. Results of dynamic light scattering (DLS) analysis of medical device extracts. Values are displayed as mean z-average value ± standard deviation [n number of reportable data points out of 3 technical repeats of 3 samples (9 total), mean PDI value] for each extract. *** indicates that the sample did not produce any data output due to low count rates. % indicates that there were too-few data points to compute the standard deviation.

3.2.5 NTA

The results of NTA of medical device extracts are shown in **Figure 5**. Only MD1 provided robust enough results to enable the determination of an average particle size in extracts (110 ± 14 nm and 111 ± 59 nm for

water and saline extracts, respectively). For the other medical devices, due to low count rates, it was not possible to obtain replicable data in order to compute an average particle size (fewer than 50% of runs produced a number result compared to 100% for MD1). No particles were detected in the extract-only and AgNO₃ control samples (the minimum number of tracks could not be satisfied to permit quantification of these samples). Using 50 nm citrate-coated Biopure AgNPs as controls (Nanocomposix, San Diego, CA), the limit of quantitation of AgNPs in water was determined to be 1 µg/mL (not shown).

Figure 5. Nanoparticle tracking analysis (NTA) of medical device extracts (7 days @ 37 °C) indicated that only extracts of MD1 contained quantifiable nanoparticulates within the size and detection limits of the instrument. * indicates that it was not possible to obtain replicable results for the device. Only data from the 7 days @ 37 °C extraction condition are shown as the other conditions led to less-robust results.

4. Discussion

The present study was carried out to improve our understanding of the release profile and physical form of silver extracted from medical devices. Three of these medical devices (MD1, MD3, and MD5) are known to contain nAg as part of their device construction, while MD2 and MD4 were included as “ionic controls,” meaning the silver present in those devices is formulated as a non-physical ionic compound. These

characterizations, which we determined by reviewing publicly-available documentation and advertising, were confirmed by SEM (Figure 1). While we noted that silver in the ionic controls could be pinpointed to small regions of the device, we observed that the intensity of silver-associated peaks at the sites of these inclusions in the EDS spectra were low, and the physical form of these inclusions, while of very small scale, did not resemble solid physical structures or nanoparticles. In clear contrast, SEM indicated that MD1, MD3, and MD5 all contained nanoscale physical depositions of silver. Therefore, the SEM results provided us with confidence in our initial designations of the silver in these devices as “nano” and “ionic.”

The amount of silver that could be extracted from the devices was highly dependent on the extraction conditions. The finding reinforces previous studies and highlights the importance for careful extract medium choice depending on the physiological process being modeled. The extract media (water, physiological saline, citrated human plasma), time and temperature points were chosen in order to elucidate the kinetics of silver release from the devices under conditions commonly employed in medical device testing. Specifically, these conditions are outlined in International Organization for Standardization (ISO) Biological evaluation of medical devices 10993 Part 12: Sample preparation and reference materials [ISO 10993-12:2007(E)], however, they were modified to allow a time course examination in the present study. Except for MD4, sample preparation was expected to have minimal effect on the silver extraction rate. For MD4, the release rate was likely enhanced by exposing the cross-section during sample preparation, but this device exhibited the lowest silver extraction rates nevertheless.

Strikingly different patterns of silver release were observed using ICP-MS. Of particular note, while some MDs appeared to release very similar amounts of silver on a per surface area basis in plasma, there was a large amount of variation from these values in water and saline. In agreement with previous work (Rigo et al., 2012), these data suggest that the choice of extract medium can have greater than order-of-magnitude impacts on the silver extraction rate. While plasma extraction might be most relevant for estimating silver release into blood, it appears that saline extractions suppress the silver release rate and water extracts are less predictable. Saline may be a poor extract medium because chloride ions in saline might react with Ag^+

ions from the devices (Rigo et al., 2012), leading to precipitate of AgCl, although no precipitates were observed in this study.

In this study, four analytical methodologies were used to identify the possible presence of nanoparticles in extract solutions (UV-VIS, TEM, DLS and NTA). These were chosen because they are all commonly available techniques in characterization laboratories that are commercially available and require minimal end-user customization. While each method individually does not guarantee detection of the presence of AgNPs in a sample, agreement among multiple orthogonal techniques does create a preponderance of evidence to that effect. Analyses of these data revealed that extracts from only one of the five devices (MD1) contained evidence of AgNPs in each assay. For extracts of MD1, a characteristic absorbance peak at 400 nm was observed, there was direct visual evidence of silver-positive nanoparticles in TEM (mean diameter 20 ± 9 nm), and sub-1000 nm particles were measured in DLS and NTA. For the other devices, no such consensus was obtained, but this does not indicate a lack of presence of AgNPs, but merely that the techniques employed are not sensitive enough to detect them, if present.

AgNPs exhibit characteristic light absorbance at around 400 nm, which is due to the surface plasmon resonance effect (Mahmoud et al., 2012). Others have demonstrated the usefulness of UV-VIS in differentiating silver ions and AgNPs (Zook et al., 2011). The precise wavelength maximum and intensity of this peak is affected by the physical and chemical properties and the absolute concentration of the AgNP solution. The absence of an absorbance peak suggests that AgNPs, if present, are below the limit of detection of the experiment (200 ng/mL here) or that the particle diameter is larger which would cause a broadening of the shape and reduction of the intensity of the peak. Therefore, the results here (**Figure 3**) suggest that only extracts from MD1 contained AgNPs with a concentration greater than ~ 200 ng/mL. While we were able to estimate the concentration of AgNPs in MD1 extracts as 200 to 1100 ng/mL by using commercial 50 nm AgNPs to construct a calibration curve, these values only serve as an approximation since the extinction coefficient of the AgNPs in extracts was unknown.

AgNP toxicity is known to vary with particle size (Park et al., 2011), and thus multiple techniques were used to characterize the size of AgNPs in extracts. TEM in combination with EDS is one such technique for providing visual and chemical evidence for the presence of AgNPs in a sample. However, samples must be dried prior to analysis, which could introduce physical artifacts not natively present in the wet sample. Cryogenic TEM and environmental SEM, which were not available for this study, are two techniques that can be used to avoid sample drying issues. Further complicating TEM analysis is the difficulty in analyzing dilute samples of particles. Typically, we analyze nanoparticle solutions at 1 mg/mL for TEM analysis using the method described above. As solutions become more dilute, it becomes noticeably more difficult to locate AgNPs on the TEM grid. In the present study, it was considerably easier for the microscopist to locate and positively-identify (using EDS) AgNPs in extracts of MD1 (**Figure 4**). Further, it was noted that the abundance of AgNPs was further increased with samples from longer extraction time points. While there were some bona fide AgNPs identified in extracts from other devices, the abundance was noted to be quite low. The relative difficulty in finding these features makes the determination ambiguous regarding whether these few AgNPs are representative of the sample or simply chance pieces of debris/drying artifacts. Thus, TEM analysis here provides substantial evidence for the presence of AgNPs only from MD1.

DLS and NTA are both techniques rooted in determining the size of NPs by measuring their rate of Brownian motion in a fluid. However, the measurements are carried out quite differently in that DLS interrogates the sample with laser light and uses the autocorrelation function to determine size, while NTA tracks nanoparticles optically and directly measures Brownian motion through analysis of video. As such, DLS has a lower size and concentration limit of detection than NTA, but NTA is more useful for characterizing heterogeneous mixtures of NPs of sufficient concentration. In the present study, particles were detected in DLS more often than NTA (**Figures 4 and 5**), suggesting the presence of low levels of particles or aggregates. Regarding DLS, given the high (> 0.2) PDI values, the z-average reported is difficult to interpret and thus cannot be relied upon to accurately describe the particle size. Nevertheless, the signal detected indicates the potential presence of particles, which was supported by finding low-abundance particles using TEM, but does not definitively demonstrate the presence of an AgNP population

in the native sample. We suggest exploration of other techniques such as single particle ICP-MS to quantify potential low levels of AgNPs (Liu et al., 2014). Additionally, as the samples were not filtered prior to DLS analysis, we are unable to rule out the role of large particle/fibers interfering with DLS measurement, which is being explored in ongoing studies.

The methods used in this study were not designed for determining the relative contributions of silver ions and NPs to the levels of total silver in the extracts. It is thought that silver ions are primarily responsible for the antimicrobial effect (Chernousova and Epple, 2013), whether they originate from the devices as ions or are shed from AgNPs. While the relative proportions of silver ions and AgNPs can be estimated through the use of calibration curves for the UV-VIS and NTA combined with ICP-MS, ideally time-resolved ICP-MS or separation techniques (e.g. centrifugation, chromatography, or field flow fractionation) would be employed to separate the ion and particle populations (Mitrano et al., 2012; Ramos et al., 2014; Sekine et al., 2014). Additionally, ongoing studies are being designed to consider the possibility of transformation of silver ions and particles, once exposed to extract media (Reidy et al., 2013). While we did attempt to control for the spontaneous reduction of Ag^+ to AgNPs by including AgNO_3 controls, other transformations could have taken place during extraction, including dissolution, aggregation, and adsorption to the extraction vessel (Stebounova et al., 2010).

Taken together, the greatest evidence for AgNP extraction came from one of the five medical devices (MD1), which was one of the devices known to have a surface coated with AgNPs. Other studies have investigated AgNP release from medical devices and other substrates (Benn and Westerhoff, 2008; Dair et al., 2010). In one recent study, the authors observed substantially more AgNP release from one of two wound dressings, which was attributed to differences in the manufacturing technique of the dressings (Holbrook et al., 2014). In another study, release of silver from four wound dressings into media similar to the present study (water, saline, and human serum substitute) was investigated (Rigo et al., 2012). In agreement with our findings, medical devices released silver at a rate dependent on their construction and the extract medium. Separately, we are pursuing studies to characterize the potential hazard of AgNPs with various physical-chemical properties that may be released from medical devices. Ultimately, this type of

testing is meant to protect patient health by contributing to a risk assessment for patient exposure to AgNPs from medical devices.

5. Conclusion

In this study, only one of the five medical devices tested showed observable levels of extractable AgNPs in all assays, suggesting that devices which contain AgNPs do not necessarily release them in observable amounts upon exposure to aqueous environments. Further work is under way to quantify these exposure levels and understand the mechanism of release. This research is contributing to a risk assessment for AgNP exposure from medical devices.

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7. Disclaimer

The mention of commercial products, their sources, or their use in connection with material reported herein is not to be construed as either an actual or implied endorsement of such products by the Department of Health and Human Services. The statements in this report should not be construed as representing official agency policies.

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