Using Single Particle ICP-MS as a Tool for Understanding Metallic Nanoparticle Transformation during Nanotoxicity Assays

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ABSTRACT

The advent of engineered nanoparticles (ENPs) and their increased use has remarkably improved the performance of basic materials and consumer products, but has also increased the potential for ENP release into the environment. Technical procedures, more accurate and specific than those for routine chemical analysis, are needed for characterization and quantification of ENPs at environmentally relevant concentrations in nanotoxicology studies. Single particle inductively coupled plasma mass spectrometry (sp-ICP-MS) has emerged as a technique potentially capable of measuring ENPs in aqueous media at the pg L^{-1} range. The work presented here establishes a method for employing sp-ICP-MS toward the characterization of ENPs following their exposure to a model organism, *Caenorhabditis elegans*. Sample preparation, ICP-MS analysis, data processing, particulate transport efficiency evaluation, and critical calculations are discussed.

Keywords: Single particle ICP-MS, *C. elegans*, nanomaterial characterization, gold nanoparticles

1 INTRODUCTION

With the bourgeoning nanotechnology industry, engineered nanoparticles (ENPs) are being incorporated into a number of industrial and consumer products, including cosmetics and food.[1] Usage of ENPs in these products will inevitably lead to ENP transfer to air, water, and soils during product disposal.[2] Questions related to the safety and deleterious effects of nanomaterials have sparked the need to develop analytical methods that are sensitive, accurate, and reliable for assessing the fate of ENPs in biological samples. Plasma source-mass spectrometry has been shown to have the potential to be the basis of an ideal ENP characterization procedure due to its ability to not only measure total metal content, but also provide ENP number density, as well as size distribution. Over the past decade, single particle inductively coupled plasma mass spectrometry (sp-ICP-MS) has emerged as a technique capable of measuring ENPs in aqueous media below the μg L^{-1} range.[3] Although reports of single-particle analysis by plasma spectrometry first appeared in 1988,[4] the potential for application toward the characterization of ENPs was developed by Degueldre and co-workers in a series of papers dating from 2003.[5,6,7,8] For sp-ICP-MS measurements, the arrival of a particle in the plasma generates a "flash" of ions that produces a unique transient event at the mass spectrometer detector. The frequency of the flashes is correlated with the number density of particles and the amplitude reflects particle diameter. The methodology was applied toward the characterization of a variety of ENPs including alumina, gold, uranium, titania, thoria, and zirconia. In recent years, there have been advances in sp-ICP-MS detection of ENPs, such as the differentiation of dissolved metal ions from suspended ENPs within a liquid sample,[9] the identification of critical/key parameters for method enhancement,[10] and the utilization of sp-ICP-MS to investigate the toxicity and uptake of silver nanowires in *Daphnia magna*.[11]

Caenorhabditis elegans is a simple organism that has been widely used as a model organism in genetics[12] and toxicology.[12,13] Easily cultured on agar, with an average lifespan of two to three weeks at 20 °C, *C. elegans* provide researchers with a tractable platform for quickly testing chemical effects on a simple multicellular organism.[14] Cultivating worms in adverse environments allows the observation of physiological and behavior effects, such as changes in feeding, locomotion, lifespan, reproduction, and morphology.[15] *C. elegans* has recently been used as a model organism for toxicological studies of ENPs including nano-ZnO,[16,17] nano-TiO_2,[16] nano-CuO,[17] nano-Ag,[6,18] and nanodiamonds,[11] revealing significant toxicity in many cases.

In this study, we employ sp-ICP-MS to investigate the uptake of gold (Au) ENPs in *C. elegans* following 96 h exposure. A sp-ICP-MS method was initially optimized for sizing Au ENPs, and then implemented for the characterization of internalized Au ENPs. Liberation of Au ENPs was accomplished through alkaline digestion of *C. elegans* tissue using tetramethylammonium hydroxide (TMAH).

2 MATERIALS AND METHODS

2.1 Materials[1]

Monodispersed citrate-stabilized spherical Au ENPs were used. Particles analyzed included commercially available nanoparticle suspensions purchased from

nanoComposix (nominal diameter of 30, 60, and 80 nm), NIST Reference Material (RM) 8011 (Gold nanoparticles, Nominal 10 nm Diameter), NIST RM 8012 (Gold nanoparticles, Nominal 30 nm Diameter) and NIST RM 8013 (Gold Nanoparticles, Nominal 60 nm Diameter), The NIST 30 nm and 60 nm reference materials were certified to contain 48.17 ± 0.33 μg g^{-1} and 51.86 ± 0.64 μg g^{-1} gold (mass fraction), respectively.[19,20,21] The nanoComposix gold nanoparticles were supplied at 49, 53, and 49 μg/g, as reported by the vendor (San Diego, CA). A soluble gold standard of 9.89 mg g^{-1} Au in 10 % (v/v) HCl (NIST SRM 3121) was used to prepare all soluble gold standards for instrument calibration.

2.2 Single Particle-ICP-MS

Single particle-ICP-MS measurements of aqueous samples were performed on an X-SERIES 2 ICP-MS (Thermo, Waltham, MA, USA) with PFA-ST nebulizer (Elemental Scientific, Omaha, NE, USA) and impact bead spray chamber. The sample uptake rate (0.18 mL min^{-1}) was measured in triplicate by weighing a vial containing deionized (DI) water before and after 4 min of aspiration. Au ENP suspensions were prepared by diluting the stock suspensions with DI water to particle concentrations ranging from 18,000 to 25,000 particles mL^{-1}. Instrument calibration was achieved using a blank and five soluble standards containing 0 ng g^{-1} to 5 ng g^{-1} of Au, prepared in a thiourea solution [2.4 % (v/v) HCl, 0.5 % (v/v) HNO_3, and 0.20 % (w/w) thiourea]. Biological extracts were analyzed with an X-SERIES 7 ICP-MS (Thermo) with Quartz C-Type nebulizer (Elemental Scientific) and impact bead spray chamber. Daily tuning of these instruments was accomplished using a multielement tuning solution [2 μg L^{-1} ^{7}Li, ^{9}Be, ^{59}Co, ^{115}In, ^{137}Ba, ^{140}Ce, and ^{238}U in 2 % (v/v) HNO_3] for maximum sensitivity and minimum oxide level (<2 %). The dwell time for all sp-ICP-MS experiments was 10 ms, and each sample was surveyed five times for 60 s each, totaling approximately 300,000 readings. Particle distributions were determined by following a modified data processing protocol.[9,20] For comparison, Figure 1 displays a typical time scan plot of a calibration blank, a standard, and an ENP suspension analyzed in time resolved mode. All data points (signal intensities are represented in Au counts per second) were exported to Microsoft Excel®, converted to counts per particle event and plotted against a time scale. The transport efficiency was determined using the particle size method and particle sizes were binned to generate size distribution histograms.[9,20]

2.3 Au ENP Uptake Experimental Design

***C. elegans* maintenance.** *Caenorhabditis elegans* (wild type, Bristol strain N2) was obtained from the *Caenorhabditis* Genetics Center (funded by the NIH National Center for Research Resource, Twin Cities, MN, USA) and maintained at 20 °C on nematode growth medium (NGM) plates seeded with *Escherichia coli*, strain OP50, as a food source. Larger quantities of nematodes were cultured in liquid medium.[21]

Nanoparticle exposure. Prior to ENP exposure, nematodes were separated from debris, residual bacteria, and dead worms, counted under a light microscope, and resuspended in fresh culture medium (complete S-Basal: contains NaCl, KH_2PO_4, cholesterol, trace metals, $MgSO_4$, $CaCl_2$, penicillin streptomycin, and amphotericin b). ENP exposures (an estimated 30,000 worms per exposure, n = 6, 96 h exposure period) were carried out in complete S-Basal spiked with Au ENPs to a concentration of 500 μg L^{-1}. All samples were placed on a shaker at 100 RPM and kept in the dark at 20 °C. After exposure, samples were placed in ice to settle the worms. Samples were transferred to individual centrifuge tubes, centrifuged at 1.2 x10^{3} *g* for 3 min, and excess exposure media was removed by vacuum filtration. Residual Au was removed by repeated washes with DI water, subsequent centrifugation, and vacuum filtration. After the final wash, the samples were transferred to cryovials and lyophilized for 24 h.

Total Au uptake via ICP-MS. Total Au concentrations were determined in control and exposed worms by weighing lyophilized samples into Teflon vessels with the addition of a concentrated HCl/HNO_3 mixture (3:1, v/v) and indium as an internal standard [NIST SRM 3124a Indium (In) Standard Solution] to evaluate matrix effects within the digestion process. Samples were evaporated on a hotplate at 120 °C and diluted to 25 mL with a 2 % (v/v) HNO_3 solution containing rhodium as an internal standard [NIST SRM 3144 Rhodium (Rh) Standard Solution], to evaluate instrumental drift. Calibration standards and sample digestions were analyzed by ICP-MS under the following conditions: 90 s sample uptake; 150 s sample rinse; 50 replicate measurements at *m/z* 103, 194, 195, and 197; and 0.7-0.8 mL min^{-1} sample uptake rate.

sp-ICP-MS measurements of Au ENP-exposed *C. elegans*. Liberation of Au ENPs in *C. elegans* samples was accomplished with the addition of 3 mL 7 % (w/v) tetramethylammonium hydroxide (TMAH).[22] Control and AuNP-exposed *C. elegans* samples (n = 6) were treated with TMAH, sonicated for 1 h, incubated overnight, and

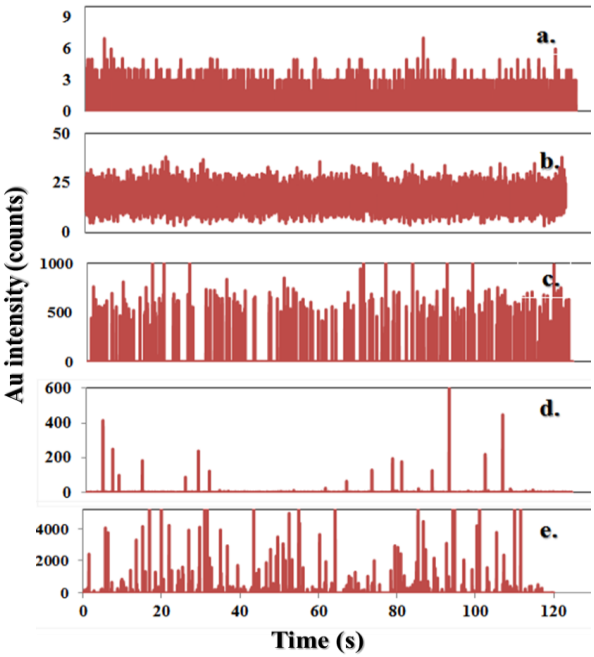

Figure 1. sp-ICP-MS analysis of gold in nanoparticle solutions (a, b, c) and *C. elegans* (d, e). (a) sp-ICP-MS time-resolved scans of ^{197}Au in a thiourea solution, (b) 0.04 ng g^{-1} soluble Au in thiourea solution, and (c) 0.032 ng g^{-1} (1.8 × 10^4 particle mL^{-1}) 60 nm NIST AuNPs in DI water. *C. elegans* from control (d) and after Au ENP exposure (e) were treated with 7 % (w/v) TMAH (dilution: 100-fold in DI water) and then analyzed by sp-ICP-MS. Dwell time: 10 ms, 120 s of data shown.

diluted 100-fold with DI water for sp-ICP-MS measurements.

3 RESULTS AND DISCUSSION

3.1 Validation of sp-ICP-MS for Au ENP

A sp-ICP-MS method was established for sizing metallic nanoparticles. Table 1 displays the calculated diameters resulting from sp-ICP-MS measurements of 30 and 60 nm Au ENPs from NIST and a commercial manufacturer (nanoComposix). The size distributions are in agreement with TEM values supplied by NIST and the manufacturer. Figure 2 shows the corresponding size distribution histograms from analysis of these four nanoparticles.

3.2 Au ENP uptake in C. elegans

Preliminary analysis of total Au uptake in *C. elegans*, shown in Figure 3, indicates that Au ENPs accumulate in *C. elegans*. Figure 1d and 1e display the sp-ICP-MS time resolved scans for *C. elegans* (control and 60 nm Au ENP-exposed) following treatment with 7 % (w/v) TMAH. Following preliminary sp-ICP-MS measurement, Au ENP-

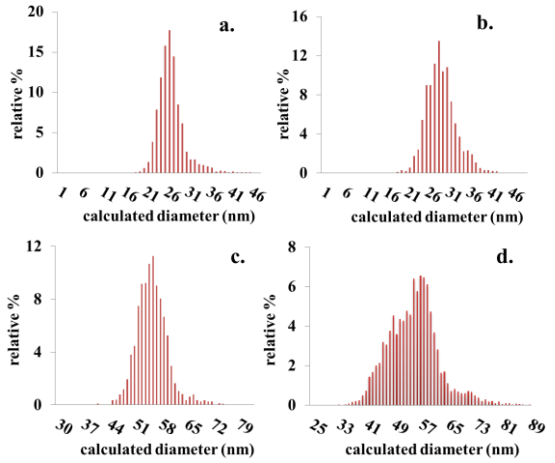

Figure 2. Size distribution histograms for 30 nm Au ENPs from NIST (a) and nanoComposix (b), respectively, and 60 nm AuNPs from NIST (c) and nanoComposix (d) in DI water. 3 replicate samples; 360 s measurement time; dwell time: 10 ms.

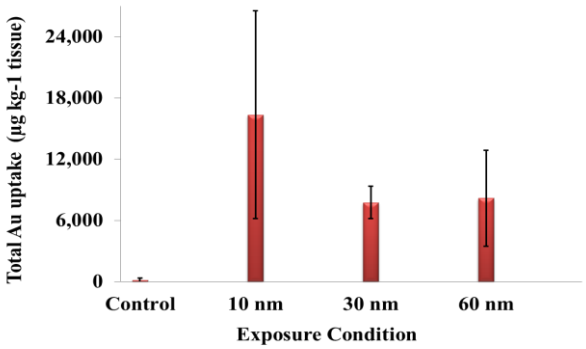

Figure 3. Comparison of total Au uptake in *C. elegans* after exposure to 10 nm, 30 nm, and 60 nm AuNPs (NIST). (n = 3; error bars: 95 % confidence interval).

exposed worm samples were diluted 100-fold to reduce particle number concentrations. Worms in the control experiments exhibited transient signals consistent with background concentrations of Au, whereas Au ENP-exposed worms exhibited signals consistent with uptake or epidermal adsorption of AuNPs. While data from total Au experiments, Figure 3, and the time resolved scan in Figure 1d indicate the presence of gold, this is attributed to an artifact of the system noise within the measurement technique and not actual Au ENPs liberated from the worms in the control experiment. Further investigation is needed to determine the source of the residual gold detected in the control worms. The large variability in uptake within each experimental condition can be attributed to the uncertainties common to biological

Table 1. sp-ICP-MS measurements of 30 and 60 nm Au ENPs.

Nanomaterial	Observed particle events (number/60 s)	Literature Particle Diameter (nm)[a]	Calculated Particle Diameter (nm)[b]
30 nm AuNP, NIST RM 8012	244 ± 13	27.6 ± 3.5	27.6 ± 2.1
30 nm AuNP, nanoComposix	215 ± 14	30.3 ± 2.7	29.7 ± 3.8
60 nm AuNP, NIST RM 8013	361 ± 32	56.0 ± 0.5	56.0 ± 4.5
60 nm AuNP, nanoComposix	344 ± 17	57.5 ± 7.2	55.1 ± 5.6

[a] Particle diameter used for NIST RM 8012 and 8013 were generated from TEM analysis at NIST. Particle diameter for 30 and 60 nm nanoComposix AuNPs are generated from TEM analysis performed by manufacturer.
[b] Generated from sp-ICP-MS analysis of Au ENPs. The average diameter and uncertainty (one standard deviation) are calculated from the size distribution measured in 3 replicate samples by using a dwell time of 10 ms, 3 surveys with an acquisition time of 360 s each.

studies. Alternative techniques will be employed to identify and size individual particles internalized by *C. elegans*, as well as to investigate the agglomeration and biotransformation of Au ENPs once ingested by *C. elegans*. The effect of TMAH digestion on Au ENPs will be studied in a parallel experiment to ensure that the alkaline digestion did not impact the Au ENP number concentration or size distribution measurements. However, with time-sensitive sample preparation, we do expect that 7 % (w/v) TMAH treatment will significantly affect measured size distributions.[22]

While total Au and sp-ICP-MS analysis will confirm the presence of Au ENPs, light microscopy techniques will allow visualization of localization of particles. Further studies include depuration and maternal transfer analyses to investigate transgenerational transfer of Au ENPs.

4 SUMMARY

This study focuses on applying sp-ICP-MS as a universal technique for the characterization of ENPs in environmental studies, using *C. elegans* as a model organism for ENP exposure. This work combines sp-ICP-MS techniques with tissue digestion to characterize ENPs in biological exposures. A sp-ICP-MS method was validated for sizing Au ENPs using NIST RMs (30 nm, NIST RM 8012 and 60 nm, NIST RM 8013) and then implemented for the size characterization of nanoComposix Au ENPs of the following sizes: 30, 60, 80 nm. The Au ENP uptake by *C. elegans* was established, and preliminary sp-ICP-MS analysis of the uptake of Au ENP was made. An experimental design was created for future implementation of this sp-ICP-MS methodology to analyze complex biological samples after exposure to metallic nanoparticles following alkaline digestion of biological tissues.

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