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RESEARCH PAPER

Use of alkaline or enzymatic sample pretreatment prior to characterization of gold nanoparticles in animal tissue by single-particle ICPMS

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Abstract Inductively coupled plasma mass spectrometry in single-particle mode (spICPMS) is a promising method for the detection of metal-containing nanoparticles (NPs) and the quantification of their size and number concentration. Whereas existing studies mainly focus on NPs suspended in aqueous matrices, not much is known about the applicability of spICPMS for determination of NPs in complex matrices such as biological tissues. In the present study, alkaline and enzymatic treatments were applied to solubilize spleen samples from rats, which had been administered 60-nm gold nanoparticles (AuNPs) intravenously. The results showed that similar size distributions of AuNPs were obtained independent of the sample preparation method used. Furthermore, the quantitative results for AuNP mass concentration obtained with spICPMS following alkaline sample pretreatment coincided with results for total gold concentration obtained by conventional ICPMS analysis of acid-digested tissue. The recovery of AuNPs from enzymatically digested tissue, however, was approximately four times lower. Spiking experiments of blank spleen samples with AuNPs showed that the lower recovery was caused by an inferior transport efficiency of AuNPs in the presence of enzymatically digested tissue residues.

Keywords Gold nanoparticles · Single-particle ICPMS · Biological tissue · Enzymatic digestion · TMAH solubilization

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Introduction

Inductively coupled plasma mass spectrometry in single-particle mode (spICPMS) is being increasingly used for determining the size distribution of metal-containing nanoparticles. The main advantage of the method is its ability to analyze a relatively large number of particles in a relatively short period of time (typically in the order of 1,000 particles per minute). It is furthermore one of the few methods that provide a size distribution of nanoparticles (NPs) based on particle number. Other methods for determination of particle number include microscopy techniques like transmission and scanning electron microscopy, atomic force microscopy, and particle tracking analysis [1]. These methods are, however, either time consuming or inaccurate when applied to complex matrices. A limitation of spICPMS is its limit of detection regarding particle size, which depends on the sensitivity of the ICPMS being used and the element of interest. Furthermore, the necessary high degree of dilution of the samples or sample extracts prior to analysis may potentially alter NP properties like the agglomeration state in comparison with the same particles in the original sample.

A detailed description of the spICPMS method was recently described by Pace et al. [2]. Briefly, a NP-containing liquid sample is diluted to a concentration in the nanogram-per-liter range (approximately 10^7 to 10^8 particles/L), and the signal of the isotope of interest is recorded using a short dwell time (in the range of a few milliseconds). This approach ensures that the plume of the ionized element originated from a single particle reaches the detector during one dwell time. The signal corresponding to one particle can subsequently be converted into particle mass based on a calibration curve, which is obtained by measuring the signal intensity corresponding to the relevant ionic standards or to NPs with known sizes and of the same composition as the analyte particle. The particle

diameter can be calculated from the determined mass if a spherical particle shape and a solid particle is assumed and the composition and density are known. The sum of masses of all particles divided by the measured volume of sample gives the mass concentration.

So far, the use of spICPMS has been mainly reported for the determination of NPs in aqueous suspensions including, for example, silver nanoparticles (AgNPs) in wastewater samples [3] and AgNPs migrating from food storage containers [4]. In order to apply the methodology to more complex matrices like biological samples, it is necessary to suspend or solubilize the sample matrix in such a way that the NPs are released into suspension, and clogging of the tubings or the nebulizer (inner diameters <200 μm) of the ICPMS instrument by matrix constituents are avoided. Until now, only one example has been reported, in which spICPMS was applied in an attempt to detect nanoparticles in tissue samples. AgNPs with average diameters of 12 and 18 nm were determined in the gut content and in tissues of rats following oral administration [5]. The samples were prepared by enzymatic digestion followed by dilution. Due to the size limit of detection of about 20 nm only, some larger NPs of the size distribution could be detected, but no quantitative analysis with respect to particle size distributions or particle concentrations in the sample could be made.

Tetramethylammonium hydroxide (TMAH) is a water-soluble strong base that has been used for alkaline solubilization of biological materials prior to determination of trace elements [6–8] by atomic spectrometric techniques. In comparison, proteinase K is a stable and highly active proteinase, which degrades proteins into amino acids. Proteinase K has been widely used in molecular biology for digesting proteins during the purification of DNA or RNA from microorganisms, cells, and plants. Recently, it was used also to liberate NPs from biological samples [5, 9, 10]. In the present study, we applied and compared the two sample preparation strategies, alkaline and enzymatic digestion, for liberation of gold nanoparticles (AuNPs) from biological tissues from animals that were intravenously dosed with 60-nm AuNPs and determined the number-based size distribution and mass concentration of AuNPs by spICPMS analysis.

Materials and methods

Materials

Ultrapure water (18.2 mΩ/cm) was obtained from a Millipore Element apparatus (Millipore, Milford, MA, USA) and used throughout the work. Single element PlasmaCAL standard of Au at 1,000 μg/mL was obtained from SCP Science (Quebec, Canada). ReagentPlus sodium dodecyl sulfate (SDS) with ≥98.5 % purity, bovine serum albumin (BSA) containing ≈98 % protein monomer, ReagentPlus calcium acetate, calcium acetate, and proteinase K-buffered aqueous glycerol solution (≥500 U/mL) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Tris(hydroxymethyl) aminomethane (Tris) analytical grade was obtained from Merck (Darmstadt, Germany). A 25 % (*v*/*v*) aqueous solution of electronic grade TMAH was obtained from Alfa Aesar (Ward Hill, MA, USA).

A reference AuNP suspension at 60 nm in nominal diameter (RM8013) was obtained from the National Institute for Science, Technology, NIST (Gaithersburg, MD, USA). The total gold concentration of the suspension was 51.86± 0.64 mg Au/L. The NIST Report of Investigation provides the average NP diameters determined by seven different sizing techniques. By the spICPMS technique, particle diameter is determined by converting mass to diameter assuming spherical particle shape. As none of the techniques in the report have measured particle diameter based on the same principle, the weighted average value of 55.4 nm was used for calculations.

AuNPs with a nominal diameter of 60 nm used for the rat experiments were obtained from British Biocell International (BBI, Cardiff, UK). The mean diameter reported by the supplier was 58.7 nm with a coefficient of variation of <8 %. The Au concentration in the suspensions was 44.5±1.1 μg/mL based on conventional ICPMS analysis following *aqua regia* digestion. The measured hydrodynamic diameter based on asymmetric flow field flow fractionation coupled on-line to dynamic light scattering was 62±0.6 nm [11].

Spleen samples were obtained from a previously conducted animal study [11]. Briefly, female Wistar rats with an age of 4 weeks were administered AuNPs or a control medium by intravenous injection of a volume of 1 mL in the tail vein. In this way, the rats were dosed 41.3 μg of 60-nm AuNPs in isotonic phosphate buffered saline (PBS). To avoid agglomeration of AuNPs suspended in the PBS solution, BSA was added at a final concentration of 1.3 mg/mL to sterically stabilize the AuNPs. Without addition of BSA, the AuNPs immediately aggregated in PBS which was visible by a change of the suspension color from red to blue–gray. The rats in the control group were dosed with a solution of BSA in PBS at the same concentration. After 24 h, the rats were sacrificed. The animal study was performed under conditions approved by the Danish Agency for Protection of Experimental Animals and by the in-house Animal Welfare Committee.

Instrumentation

For mixing of homogenized spleen sample with TMAH, an MS2 minishaker (IKA Works, Inc., Wilmington, NC, USA) was used. Sonication of samples was performed using a Branson 5510 sonication bath (VWR International, Leicester, UK) at a frequency of 42 kHz and an input power of 185 W. The pH value was measured with a PHM 240 pH/ION meter (Radiometer, Copenhagen, Denmark).

A Thermo Scientific iCAP Q ICPMS instrument (Thermo Fisher Scientific GmbH, Bremen, Germany) was used for all spICPMS experiments. Instrument tuning was performed prior to analysis by using a tuning solution according to the manufacturer's recommendation. Instrument settings are given in Table 1.

Methods

Unless stated otherwise, results based on repeated measure ments are given as mean±1 SD. The number of repetitions *N* is stated in parentheses.

Determination of the Au concentration by conventional ICPMS

The details of the analysis are described in [11]. Briefly, spleens were dissected and weighted, and subsequently homogenized by a blender with addition of water (spleen to water ratio=1+ 9 m/m). The homogenates were stored at −80 °C until time of analysis. The content of Au in the homogenates was determined following microwave-assisted digestion of 2 mL of tissue homogenate by aqua regia (3 mL of HCl and 1 mL of HNO_3). The Au content was quantified by ICPMS against an external calibration curve using Rh as internal standard.

Sample preparation for spICPMS

Before further treatment, all thawed rat spleen homogenates were sonicated for 1 h. Then, TMAH was added to 200 μL of homogenized spleen to a final TMAH concentration of 5 % (*v*/*v*). Immediately thereafter, BSA solution was added corresponding to approximately 300 BSA molecules per AuNP (assuming a diameter of 60 nm), which would theoretically allow the formation of a BSA monolayer [12]. The samples (total volume 2 mL) were then sonicated for 1 h and rotated mechanically at room temperature overnight.

For enzymatic treatment, a digestion buffer (10 mM Tris, 0.5 % SDS, and 1 mM calcium acetate) was prepared according to the enzyme supplier's recommendation. Proteinase K is proteolytically active in a broad pH range from 7.5 to 12. The pH was adjusted to 8 because at this value maximum stability during storage can be expected (according to the supplier). A volume of 1,880 μL of digestion buffer was added to 100 μL of homogenized spleen. The sample was vortexed for 10 s, and 20 μL of the diluted enzyme solution (100 U/mL) was added. The samples (total volume 2 mL) were sonicated for 1 h and rotated mechanically at room temperature overnight. At room temperature, the activity is at least 80 % of the maximum activity, which is achieved at 37 °C.

To allow double determinations, each homogenized spleen sample was divided into two subvolumes before alkaline or enzymatic treatment. The NIST RM8013 AuNPs were spiked to one of the control group samples each after alkaline and enzyme treatment.

NP characterization by spICPMS

Prior to spICPMS analysis, all prepared samples were sonicated for 15 min. For each sample, the ^{197}Au signal intensity was recorded. Dwell times of 1 to 10 ms and suspension concentrations of 25 to 500 ng/L (corresponding to 1.2×10^7 to 2.3×10^8 particles/L) were tested using RM8013 AuNPs to find optimum settings. The number of collected data points (or dwells) was 60,000 in each case. Following the analysis of each sample, ultrapure water was analyzed to control if carryover from the previous measurement could be detected. For all analyses, raw signal intensity data were plotted versus number of events to create a signal distribution histogram using a spreadsheet routine (Microsoft Excel). Very low signal intensities (less than 10 counts per 3 ms) were considered to be instrument background. Slightly higher signal intensities were considered as incomplete events, i.e., partial gold ion plumes that were detected during two consecutive dwell times. Instrument calibration was achieved by analysis of ultrapure water as blank (mass zero) and RM8013 (average mass per particle=1.7 fg) spiked to the relevant control sample as calibrant. The average intensity for the blank was calculated. For the RM8013 AuNPs, the mean peak intensity corresponding to particle events was determined from the frequency distribution. Both intensity values were plotted against mass. The slope was used to convert the measured intensities of AuNPs in the samples into particle masses. By assuming a spherical particle shape and a particle density of 19.3 g/cm^3 (density of gold), the masses were finally converted into particle diameters. The transport

Table 1 spICPMS settings

Parameter (unit)	Value
Plasma power (W)	1,550
Plasma gas flow rate (L/min)	14
Nebulizer flow rate (L/min)	0.96–0.99
Axillary gas flow rate (L/min)	0.8
Sample uptake flow rate (mL/min)	0.41–0.45[a]
Monitored isotope (*m*/*z*)	^{197}Au
Dwell time (ms)	1–10 (3[b])
Analysis time (s)	60–600 (180[b])
Wash time (s)	120
Spray chamber type	Quartz cyclonic type, Peltier-cooled
Nebulizer type	MicroFlow PFA nebulizer

[a] Determined on a daily basis (corresponding peristaltic pump speed was 40 rounds/min)

[b] Optimum value for 60-nm AuNPs

efficiency, which was necessary to determine the particle concentration in the samples, was determined according to the "particle frequency" method [2] by measuring RM8013 again spiked to the relevant matrix. The transport efficiency, which was determined daily, was calculated as number of particles detected by spICPMS in percentage of the theoretical (calculated) particle number in the aspirated AuNP suspension. The sample flow rate was accurately determined daily by weighing the amount of water that was delivered by the peristaltic pump of the sample introduction system during 1 min.

Results and discussion

Optimization of the spICPMS analysis

The gold nanoparticle material RM8013 was analyzed at a concentration of 100 ng/L (approximately 4.6×10^7 particles/L) using a range of dwell times (1, 3, 5, 10 ms). The analysis time was adjusted so that for each dwell time the same number of data points (60,000) was recorded. The resulting frequency plots are presented in Fig. 1. For the shortest dwell time, the signal corresponding to particles overlaps with the background signal due to a large fraction of incomplete particle events (the ion plume from one particle is only partially detected within one dwell time), which makes the determination of a threshold in signal intensity for separation of background and partial events from particle events difficult. With increasing dwell time, the probability for detecting two or more NPs simultaneously during one dwell time increased. For the given particle size, these "multiple particle events" started to occur for a dwell time of 10 ms. Dwell times of 3 and 5 ms gave similar results. The necessary analysis time to record 60,000 data points was shorter for a dwell time of 3 ms (180 s) than for 5 ms (300 s). Therefore, it was decided to use a dwell time of 3 ms for all further analyses.

A similar experiment was performed for optimization of the sample concentration at a fixed dwell time of 3 ms. With higher concentrations of the same AuNP suspension, the probability of two or more NPs entering the plasma at the same time increases, and two NPs are erroneously detected as one single particle. Multiple events started to occur at a concentration of 260 ng/L or 1.2×10^8 particles/L (Fig. 2). Thus, a concentration of 100 ng/L was chosen for further analyses.

The presented frequency distribution (Fig. 3) shows that a clear separation of (instrumental) background signal and particle-related signal was not possible. Ionic background can be excluded for the studied NP because the ionic Au concentration was below 0.07 μg/g (<0.13 % of the Au mass concentration of AuNPs) based on the 3 σ detection limit (information provided in NIST's Report of Investigation). An iterative algorithm was tested where particle events were distinguished as outliers from the background/dissolved ion signal if the measured intensity was more than n times the standard deviation of the whole data set as described in [3]. With $n=5$ (threshold two counts), too many false-positive events were included whereas $n=6$ (threshold 1,164 counts) overestimated the threshold.

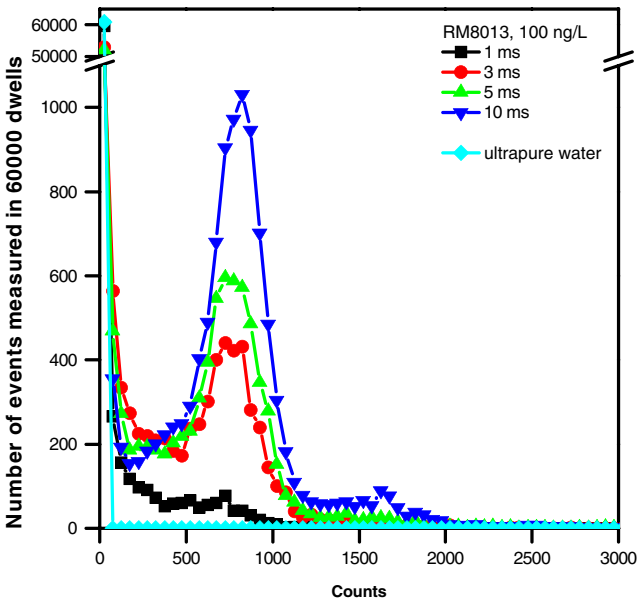

Fig. 1 Influence of dwell time on the frequency distribution of the obtained signal for RM8013 (100 ng/L). For the sake of clarity, the histograms (bin width 50 counts/dwell time) are presented as *curves* with each *point* representing a bin center

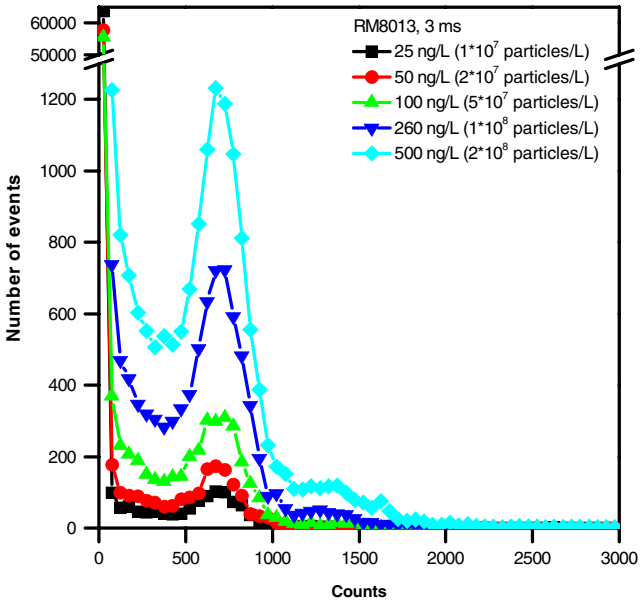

Fig. 2 Influence of sample concentration on the frequency distribution of the obtained signal for RM8013 (dwell time 3 ms). For the sake of clarity, histograms (bin width 50 counts/dwell time) are presented as *curves* with each *point* representing a bin center

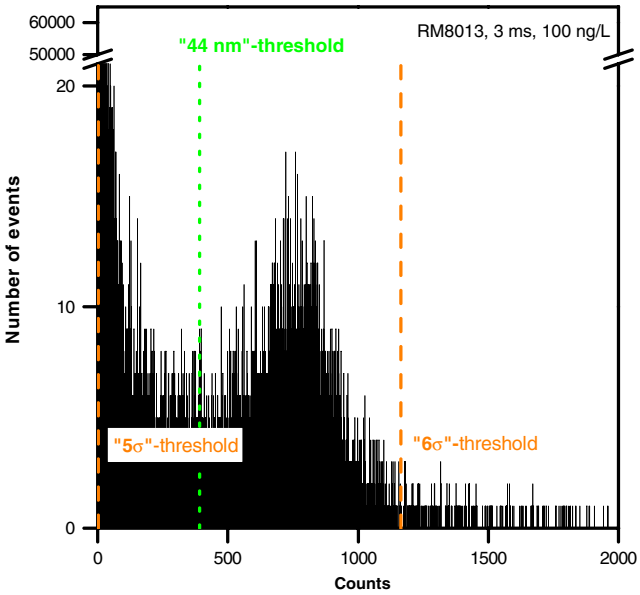

Fig. 3 Frequency distribution for RM8013 at 100 ng/L (dwell time 3 ms); the *vertical orange lines* present the thresholds determined by an iterative algorithm and the *green line* the chosen threshold value which corresponded to a lower boundary of NP sizes of 44 nm in the size distribution

Determination of the transport efficiency based on the "frequency method" [2] is based on calculating the ratio between detected particles and the theoretical number of particles. Setting a proper threshold is important because it determines which events are regarded as particle events and which are considered partial events or (ionic) background signal. To allow repeatable conditions, the threshold intensity of RM8013 was always set to a value, which yielded a particle size distribution with a minimum particle diameter of >44 nm (green line in Fig. 3). This minimum particle diameter was chosen based on the TEM-based size distribution provided by NIST. For the AuNPs used in the animal study, which had a mean diameter close to that of RM8013, the same threshold value was applied because no quantitative data from TEM analysis was available.

Further work is required to find a more suitable method for determination of the threshold value to allow a correct determination of the number-based particle size distribution. However, for a comparison between samples and sample preparation methods, our approach which used a fixed value of 44 nm as threshold was sufficient. This approach ensured that a constant fraction of nanomaterials (i.e., above a certain size limit) was considered in the data analysis. The influence of sample treatment, etc., was evaluated based on this fixed fraction.

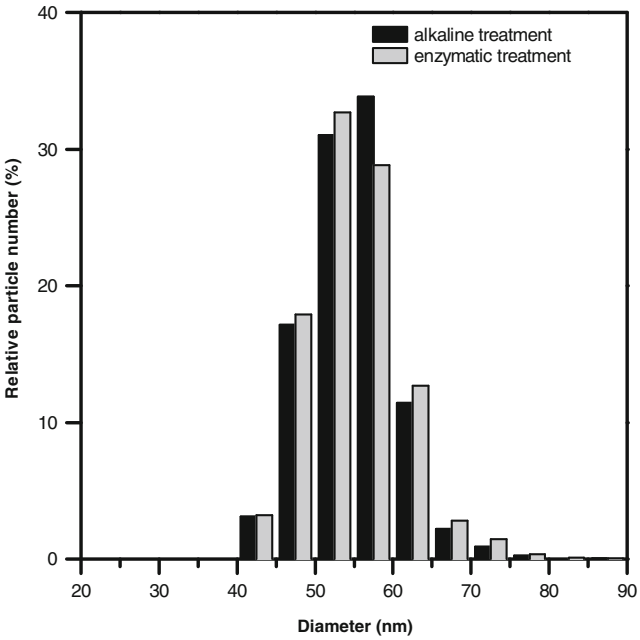

Fig. 4 Particle size distributions of AuNPs (bin size 5 nm) obtained by spICPMS for the spleen tissue from animal 1 after alkaline and enzymatic treatment

Table 2 Comparison of the particle size distribution characteristics after alkaline and enzymatic treatment based on the analysis of spleen tissue from three animals (mean±1 standard deviation, $N=3$)

	2.5 % percentile	50 % median	97.5 % percentile
Alkaline	44.9±0.0	54.6±0.6	65.7±0.4
Enzymatic	44.8±0.1	53.6±0.7	68.4±0.7

Size determination of AuNPs in tissues

Figure 4 presents the obtained particle size distribution (PSD) for spleen tissue from one animal after alkaline or enzymatic treatment, respectively. The PSDs were similar, which showed that the two sample preparation methods did not affect the

Table 3 Comparison of Au concentrations (in nanograms Au per gram spleen tissue) obtained by spICPMS after alkaline and enzymatic treatment as well as by conventional ICPMS after digestion with aqua regia. The given values are based on double determinations. The relative standard deviation (RSD) of repeatability was determined as the pooled RSD from the three double determinations for each treatment

Animal	Alkaline (spICPMS)	Enzymatic (spICPMS)	Aqua regia (ICPMS)
1	3,368	1,794	4,970
2	2,875	633	3,170
3	2,144	1,433	3,922
Mean	2,795	1,287	4,021
Relative standard error of the mean (%)	15.6	32.7	15.9
Repeatability RSD (%)	13.0	19.6	17.2

determination of the particle sizes differently. The mode of the particle size distribution was for both sample preparation techniques between 50 and 60 nm. This was in agreement with the weighed mean diameter of 58.7 nm. In our previous work, TEM investigations of liver tissue treated similarly with TMAH have shown that AuNPs did not agglomerate and occurred individually with the same size as observed for the stock suspension of AuNPs [11].

To allow quantitative comparison of the PSDs, the 2.5, 50, and 97.5 % percentiles of the size distributions were calculated (Table 2). There was no significant difference (t test) for the 2.5 and 50 % percentile values between alkaline and enzymatic treatment. For the 97.5 % percentile value, however, significantly higher values ($p<0.01$) were observed when using enzymatic treatment. The lower number of large particles obtained after alkaline solubilization could be explained with a stronger disaggregation of NP agglomerates by this treatment. The conversion of NP mass into diameter assumes a spherical particle shape. Therefore, the possible existence of NP agglomerates translates into large NPs in the PSD.

Determination of mass concentration of AuNPs in tissues

In samples from the control group, only one to two NPs were detected per analysis, which was probably due to carryover from previous measurements (results not included). The measured Au concentrations in spleen tissues of exposed animals after alkaline or enzymatic treatment are presented in Table 3. The values were compared with Au concentrations determined by conventional ICPMS after aqua regia digestion. The total mass concentration of Au (corresponding to AuNPs) detected by spICPMS was 2,795±616 and 1,287±594 ng Au/g spleen tissue (mean±1 standard deviation, $N=3$) after alkaline or enzymatic treatment, respectively. The total concentration of Au (as ions after aqua regia digestion) determined by conventional ICPMS was 4,021±904 ng Au/g spleen tissue (mean±1 standard deviation, $N=3$). There was no significant difference (t test) between the Au concentration determined after alkaline treatment (spICPMS) and aqua regia digestion (conventional ICPMS). The Au concentrations determined after enzymatic treatment were much lower in comparison to the alkaline treatment (approximately 60 % lower). The calculation of the AuNP concentrations already included a correction for the transport efficiency based on spiking of the RM8013 AuNPs to each of the relevant matrices. The determined transport efficiencies were 1.15 % for the enzyme matrix and 3.6 % for the alkaline matrix. The AuNP concentration was calculated as

$$\text{particle mass concentration} = \frac{\text{sum of all particle masses}}{\text{transport efficiency} \times \text{flow rate} \times \text{analysis time}}$$

Despite the applied corrections for transport efficiency, still lower concentration values were obtained after enzymatic treatment.

To further study the observed differences between alkaline and enzymatically treated samples, the RM8013 AuNP sample was analyzed at the same concentration (100 ng/L) in ultrapure water as well as spiked to alkaline or enzymatically treated spleen tissue, and the number of detected AuNPs was determined. There was no significant difference (t test) between the number of detected particles in ultrapure water (2,997±32, $N=2$) and that in alkaline-treated tissue (2,866±30, $N=2$). In contrast, the number of detected particles in enzymatically digested tissue was much lower (1,009±287, $N=2$). A possible explanation of this result could be that organic molecules from the enzymatic digestate adhere to surfaces in tubings and spray chamber and act as a "trap" for the AuNPs. Theoretically, 10^8 enzyme molecules per nanoparticle were present in the samples. This large excess of molecules could explain the suggested mechanism of inferior transport efficiency.

Conclusion

The potential of spICPMS for quantification of size and number concentration of 60-nm AuNPs in tissue samples has been demonstrated. Alkaline solubilization and release of the AuNPs from the sample tissue is a promising sample preparation technique, which led to high recoveries of AuNPs. It is necessary to mention that application of this sample preparation method was successful for AuNPs in animal tissue and is probably restricted to NPs that do not degrade and can be stabilized under such alkaline conditions. The use of enzymatic digestion gave a similar number-based size distribution as that determined for the alkaline-treated samples. The presence of hydrolyzed sample residues, however, strongly reduced the number of detected NPs by spICPMS. Further studies are necessary to identify the mechanism behind this result and which part of the analytical system causes this effect.

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References

1. Linsinger T, Roebben G, Gilliland D, Calzolai L, Rossi F, Gibson P, Klein C (2012) JRC reference report EUR 25404 EN
2. Pace HE, Rogers NJ, Jarolimek C, Coleman VA, Higgins CP, Ranville JF (2011) Anal Chem 83:9361–9369
3. Tuoriniemi J, Cornelis G, Hassellöv M (2012) Anal Chem 84:3965–3972
4. Goetz Nv, Fabricius L, Glaus R, Weitbrecht V, Guenther D, Hungerbuehler K (2013) Food Addit Contam: Part A Chem Anal Control Expo Risk Assess 30:612–620

5. van der Zande M, Vandebriel RJ, Van Doren E, Kramer E, Herrera Rivera Z, Serrano-Rojero CS, Gremmer ER, Mast J, Peters RJB, Hollman PCH, Hendriksen PJM, Marvin HJP, Peijnenburg AACM, Bouwmeester H (2012) ACS Nano 6:7427–7442
6. Tao G, Willie N (1998) Analyst 123:1215–1218
7. da Silva JBB, Borges DLG, da Veiga MAMS, Curtius AJ, Welz B (2003) Talanta 60:977–982
8. Batista BL, Grotto D, Rodrigues JL, de Oliveira Souza VC, Barbosa F Jr (2009) Anal Chim Acta 646:23–29
9. Deering C, Tadjiki S, Assemi S, Miller J, Yost G, Veranth J (2008) Particle and Fibre Toxicol 5:18
10. Loeschner K, Navratilova J, Købler C, Mølhave K, Wagner S, Kammer F, Larsen E (2013) Anal Bioanal Chem doi:10.1007/s00216-013-7228-z
11. Schmidt B, Loeschner K, Hadrup N, Mortensen A, Sloth JJ, der Koch C, Larsen EH (2011) Anal Chem 83:2461–2468
12. Dominguez-Medina S, McDonough S, Swanglap P, Landes CF, Link S (2012) Langmuir 28:9131–9139