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Quantitative Analysis of Gold Nanoparticles in Single Cells by Laser Ablation Inductively Coupled Plasma-Mass Spectrometry

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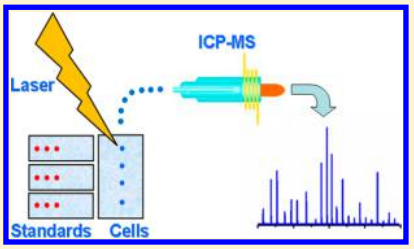
ABSTRACT: Single cell analysis has become an important field of research in recent years reflecting the heterogeneity of cellular responses in biological systems. Here, we demonstrate a new method, based on laser ablation inductively coupled plasma mass spectrometry (LA-ICPMS), which can quantify *in situ* gold nanoparticles (Au NPs) in single cells. Dried residues of picoliter droplets ejected by a commercial inkjet printer were used to simulate matrix-matched calibration standards. The gold mass in single cells exposed to 100 nM NIST Au NPs (Reference material 8012, 30 nm) for 4 h showed a log-normal distribution, ranging from 1.7 to 72 fg Au per cell, which approximately corresponds to 9 to 370 Au NPs per cell. The average result from 70 single cells (15 ± 13 fg Au per cell) was in good agreement with the result from an aqua regia digest solution of 1.2×10^6 cells (18 ± 1 fg Au per cell). The limit of quantification was 1.7 fg Au. This paper demonstrates the great potential of LA-ICPMS for single cell analysis and the beneficial study of biological responses to metal drugs or NPs at the single cell level.



Nanoparticles (NPs), due to their distinct chemical and physical properties, have been widely studied for various applications, for example, imaging, sensing, and drug delivery systems.[1] These applications require a profound understanding of the mechanisms of NPs entering and leaving cells. Elucidating the endocytosis and exocytosis of NPs is also essential in assessing their biological effects in organisms.[2,3] Therefore, study of the cellular uptake of NPs plays a crucial part in both biomedical applications and toxicological assay of NPs.

Conventional methods for estimating cellular uptake of NPs are based on acid digestion of large numbers of cells and their subsequent analysis by an elemental analysis technique, such as inductively coupled plasma-optical emission spectrometry.[4] These methods can only provide information on an imaginary average cell and cannot reveal cell-to-cell differences within the cellular population. It is well-known that individual cells, even those of identical genotype and phenotype, show clear heterogeneity after environmental stimuli.[5] Analysis of NPs in single cells is therefore crucial for elucidating cellular diversity and heterogeneity.[6]

Some methods, such as surface-enhanced Raman scattering (SERS) and fluorescent-based cytometry or microscopy, can be used to determine specific NPs in single cells. However, these methods often rely on plasmonic or fluorescent properties of the NPs and thus are hard to apply to NPs without observable optical properties. In addition, the physicochemical properties of NPs are highly susceptible to complex biological microenvironments. Possible aggregation or interaction with biological molecules makes it difficult to accurately quantify NPs in organisms by optical methods. For example, quantum dots can be oxidized in organisms and thus induce fluorescence quenching.[7] Therefore, a versatile, sensitive, and reliable method for quantification of NPs in single cells is urgently required.

Recently, inductively coupled plasma mass spectrometry (ICPMS) has emerged as a new option for single cell analysis. The ICP is a high temperature source (approximately 7000 K) where all the chemical bonds in the sample are broken and the elemental isotopes are efficiently ionized with a positive charge, giving ICPMS remarkable sensitivity for elemental detection.[8,9] Tsang et al. monitored metal contents in *H. pylori* at the single-cell level,[10] and Zheng et al. determined intracellular quantum dots in single cells.[11] By using element-labeled antibodies, mass cytometry, an adaptation of ICPMS, was successfully used to measure 34 parameters simultaneously in single cells.[12] However, all of the above applications were limited to analyzing single cells in suspension. This work develops a new method based on laser ablation ICPMS (LA-ICPMS) that can be used for *in situ* quantification in single cells.

LA-ICPMS is a technique used for the *in situ* analysis of trace elements in solid samples with high spatial resolution.[13] In LA-ICPMS, the surface of a solid sample is ablated by a high-powered laser and the resultant aerosol is introduced into the

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ICP for elemental analysis by mass spectrometry. Because of the impressive detection limits (typically better than parts-per-million for most elements), wide dynamic range (up to 9 orders of magnitude), and spatial resolution capacity in the micrometer range, LA-ICPMS has been widely used in many research fields, a relevant example being elemental bioimaging.[14] Unlike solution-based techniques (e.g., mass cytometry), LA-ICPMS enables *in situ* analysis of microscopic targets, offering new opportunities for single cell analysis.

Although LA-ICPMS has been described as a promising method for single cell analysis, there are only a few papers on this topic in the literature.[15,16] One of the challenges for LA-ICPMS is the lack of matrix-matched standards whose sizes are similar to single cells. Inkjet printing technology is expected to offer an ideal solution to this issue. Inkjet printing can accurately and reproducibly eject picoliter volumes of liquid from microsized apertures onto a substrate in a defined pattern.[17,18] Hoesl et al. described the use of an ink jet printer to deposit an overlay of an internal standard and quantification of blotted proteins.[19] Alternatively, individual droplets have been successfully applied as calibration standards for X-ray fluorescence analysis[20] and ion mobility spectrometry.[21] For the first time, this work describes the use of individual printed droplets as a calibration method for the quantification of gold nanoparticles (Au NPs) in single cells as a proof of concept.

EXPERIMENTAL SECTION

Chemicals and Reagents. Thirty nm Au NPs (NIST RM 8012) were purchased from the National Institute of Standards and Technology (NIST). DMEM/F12 media and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific (Rockford, USA). Dulbecco's Phosphate Buffered Saline (PBS) and trypsin-EDTA solution were obtained from the Beyotime Institute of Biotechnology (Beijing, China). High purity concentrated nitric acid, hydrochloric acid, and hydrogen peroxide were bought from Beijing Chemical Reagent Company (Beijing, China). Ultrapure water (18.2 MΩ cm^{-1}) from a Milli-Q water purification system (Millipore, MA, USA) was used throughout the experiments. Au standard solution was purchased from the National Research Center for Certified Reference Materials (Beijing, China). Gold chloride was obtained from Strem chemicals (Newburyport, MA, USA).

Cell Experiments. Raw 264.7 cells (a mouse leukemic monocyte macrophage cell line) were used in this study. Extra care was taken to avoid elemental contamination. Cells were grown in optimal cell growth conditions to ensure that they were not stressed prior to the experiments. Raw 264.7 cells were cultured at 37 °C in 5% CO_2 in DMEM/F12 medium containing 10% heat inactivated FBS, 2.0 g L^{-1} glucose, 100 μg mL^{-1} streptomycin, and 100 IU mL^{-1} penicillin.

The NIST Au NPs solution was sonicated to avoid aggregation. Cells were incubated with 5 mL of Au NPs (0.1 μM in DMEM/F12 media) for 4 h. In order to remove extracellular Au NPs, the cells were thoroughly washed with PBS. After the cells were counted using a hemocytometer, 50 μL of cell solution was added to a Nunc Lab-Tek II Chamber Slide System (Thermo Fisher Scientific, Waltham, USA) for LA-ICPMS analysis. The cells in the remaining solution were digested by aqua regia and determined by solution ICPMS.

ICPMS Measurement. Gold chloride was weighed and diluted with water to yield Au standards of 0.2, 2, and 20 μg g^{-1}. In order to visualize the ejected droplet residues and match the cellular matrix, 2% (wt) rhodamine B was added into the standard solution. A MicroFab Jetlab Printing Platform with a 20 μm orifice (MicroFab Technologies, Inc., Texas, U.S.A.), which can precisely dispense picoliter droplets, was used to print standard solution onto glass slides (bare silica slides, from Citoglas Labware Manufacturing Co., China). A known number of droplets from each Au standard (e.g., 10^6 droplets from the 2 μg g^{-1} Au standard) were dispensed into a weighing vessel, and the Au content was determined by solution ICPMS.

LA-ICPMS measurements were carried out using a NWR 213 laser ablation system (ESI, Fremont, USA) coupled to a NexION 300D ICPMS instrument (PerkinElmer, Norwalk, USA). Helium was used as the ablation gas, at a typical flow rate of 0.8 L min^{-1}, with argon introduced through a Y-piece after the ablation cell. The system was tuned for maximum ^{115}In signal intensity, while keeping the UO/U ratio at a low level during the ablation of NIST 611 glass. Only single cells were targeted, while cells present in clusters or closer than 40 μm apart were disregarded. For solution analysis, the ICPMS was tuned using a multielement standard solution (1 μg L^{-1} each of Be, Ce, Fe, In, Li, Mg, Pb, and U in 1% v/v nitric acid). Typical LA-ICPMS operating conditions are given in Table 1.

Table 1. LA-ICPMS Operating Parameters

ICP Parameters	
nebulizer gas flow/L min^{-1}	0.94
auxiliary gas flow/L min^{-1}	0.9
plasma gas flow/L min^{-1}	18
ICP RF power/W	1600
analog stage voltage/W	−1650
sample and skimmer cone	Pt
isotope monitored	^{197}Au
dwell time/ms	10
scan type	data only
Solution Analysis	
spray chamber	quartz cyclonic
nebulizer	concentric
LA Analysis	
laser wavelength	213 nm (Nd:YAG)
laser ablation method	single spot analysis
He carrier gas flow rate/L min^{-1}	0.8
laser warm up/s	25
spot size/μm	40
fluence/J cm^{-2}	6.0

RESULTS AND DISCUSSION

Au NPs, due to their unique characteristics, have become one of the most widely studied NPs for cell imaging, targeted drug delivery, cancer diagnostics, and therapeutic applications.[22] Although accurate quantification of Au NPs in single cells is urgently required to provide deep insight into cellular heterogeneity and the complex interaction between Au NPs and cells, it is still a challenge for currently available methods. Therefore, Au NPs were chosen as a proof-of-concept analyte in this study. RAW 264.7 macrophage cells are able to engulf and digest cellular debris, foreign particles, and microbes. Thus, the cells were chosen as good models to study the uptake of AuNPs.

One major obstacle to accurate quantification by LA-ICPMS is a lack of suitable single-cell standards. In this study, a commercial inkjet printer, MicroFab JetLab, was used to

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simulate matrix-matched standards for single cell analysis. Under controlled conditions, the droplets dispensed from the printer are almost identical with less than 1% mass variations.[23] The sizes of the droplets observed on glass slides however are dependent on the orifice diameter, substrate surface condition, and other environmental factors, and thus, even equal mass droplets may have different diameters on the substrate. Figure 1

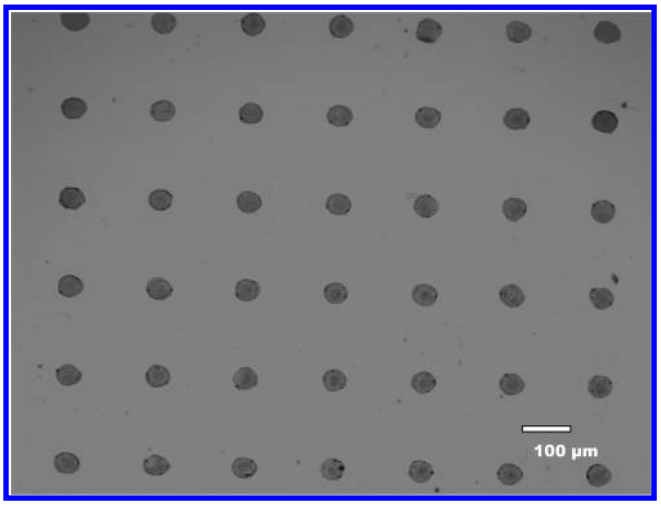

Figure 1. Images of droplet residues of 2 μg g^{-1}Au standard on a glass slide under a light microscope. The scale bar is 100 μm.

shows that the droplet residues of the Au standards (2 μg g^{-1}) appear uniform on glass slides with droplet diameters of 40 ± 3 μm (n = 30), suggesting that these droplets have similar masses. The average mass of the droplets was calculated by measuring the gold content in a weighing vessel which contained a known number of droplets ejected from a gold standard solution. The results indicated that there were no significant differences in the droplet masses between the three concentrations of Au standards used. The average mass of droplets ejected under these experimental conditions was calculated to be 18 ± 1 ng.

The droplet residues were used as calibration standards for LA-ICPMS. To guarantee complete ablation of each residue, a 40 μm spot size and 6.0 J cm^{-2} fluence was used. Figure 2 shows typical ^{197}Au signal intensities from the ablation of droplet residues from three concentrations of gold standard solutions on glass slides. No detectable Au signals were determined outside the droplet residues, and thus, good signal-to-noise ratio could be obtained. The droplet signals showed very good reproducibility with RSDs better than 8% for four parallel measurements of each Au standard. The correlation coefficient of the calibration curve was 0.999, ranging from 3.6 to 360 fg Au (Figure 2b). The limit of detection (LOD) and limit of quantification (LOQ), calculated from 3 and 10 times the standard deviation of the blank signals, were 0.5 and 1.7 fg Au, respectively. The above results demonstrate that such droplet residues can be used as calibration standards for LA-ICPMS analysis, thus demonstrating a novel calibration method for quantitative analysis by this technique. In LA-ICPMS, elemental response is dependent on the chemical composition and physical structure of the matrix, as a consequence of changes in the amount of ablated material and differences in the transport of the ablated material.[24] In this study, the individual droplets simulated matrix matched standards for the following reasons. First, the standards and samples were both dried residues from picoliter-sized droplets or single cells which were

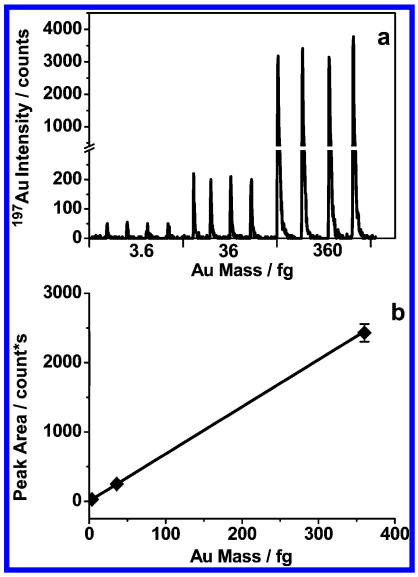

Figure 2. (a) Transient signals obtained by LA-ICPMS from droplet residues ejected from gold standards (0.2, 2, and 20 μg g^{-1}) onto a glass slide. (b) Calibration curve from three Au standards by LA-ICPMS (R^2 = 0.999). Limit of quantification: 1.7 fg Au.

fully ablated by the laser beam. Second, 2% (wt) rhodamine B was added into the Au standard solutions, and thus, the printed droplets had the same order of magnitude of carbon content as macrophage cells, which was estimated by taking a mean carbon-to-volume ratio in a cell (0.15 pg μm^{-3})[25] and the known volume of a macrophage cell (1.2 × 10^3 μm^{-3}).[26] The ablated aerosol from the standards and samples had similar major element composition, i.e., mainly carbon-containing aerosol from the samples or standards and silicon-containing aerosol from the glass slides. Third, studies have shown that laser-ablated particles under 800 nm in diameter behave like solutions in the ICP,[27] indicating that 30 nm Au NPs should be quantifiable using Au solution as the calibration standard. However, the signals from nanoparticles are known to produce discrete signal spikes so that, even if the mean signals from similar mass loadings (solution vs particles) are consistent, the nanoparticle signals occur under instantaneously high mass loading conditions. The microdroplet standards ablated here are designed to approximate these conditions. In summary, the standards described here simulate the ideal matrix-matched standards and offer a viable route for calibration.

Individual cells were readily observed by the laser microscope and individually ablated by the laser beam. Thus, Figure 3

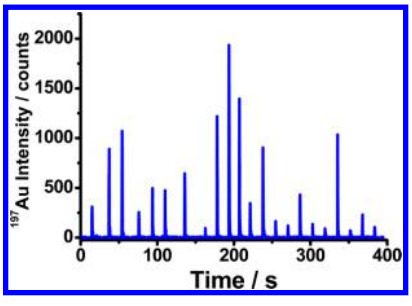

Figure 3. Transient Au signals of 22 individual cells determined by LA-ICPMS after incubation with 100 nM Au NPs for 4 h (note that uneven time spacing corresponds to moving to isolated single cell targets).

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shows Au signal intensities for the ablation of individual cells on chamber slides, where each integrated signal represents the total gold mass in a single cell. No detectable Au could be determined in control cells, and thus, the Au signals came from the Au NPs although LA-ICPMS cannot differentiate in-tact NPs from wholly or partially dissolved ones. From the certification report for NIST RM 8012, it can be calculated that each 30 nm Au NP contains approximately 5.9×10^5 Au atoms. Therefore, the number of Au NPs in a single cell can be estimated. In this experiment, 71 individual cells were analyzed by LA-ICPMS. Among the 71 cell signals, 70 signals were above the limit of quantification with a range from 1.7 to 72 fg Au per cell, which corresponds to 9 to 370 Au NPs per cell. The average mass loading from the 70 single cells, 15 ± 13 fg Au per cell or 77 ± 67 Au NPs per cell (shown as average ±1 SD), was in good agreement with the data from digestion/solution analysis of 1.2×10^6 cells, 18 ± 1 fg Au per cell, or 92 ± 5 Au NPs per cell (shown as average ±1 SD). The overall efficiency of the instrumental system used was estimated as $\sim 2 \times 10^{-6}$ given that 5×10^5 Au atoms yielded ~1 count by LA-ICPMS. The accuracy of the method, as shown by the similarity of the means, is good, but as expected, the variance of the laser method is much greater. This is to be expected given the larger number of factors affecting the laser experiment (namely, fg sample masses, variability in cell uptake, and laser power and transport) and the difficulty of accurately measuring transient signals.

Figure 4 shows that a log-normal distribution approximates the Au masses of the 70 individual cells. Previous studies have

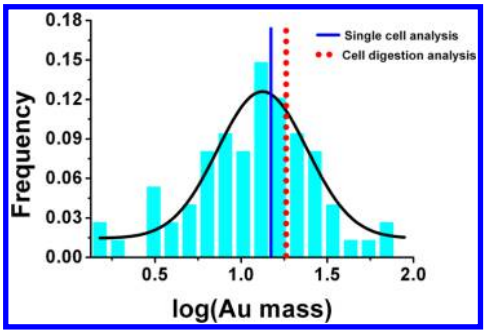

Figure 4. Histogram showing the log-normal distribution of Au mass for the ablation of 70 cells. The two averages from single cell analysis (the blue solid line) and cell digestion analysis (the red dot line) are 15 and 18 fg, respectively.

shown that population heterogeneity is an inherent condition of cell populations, possibly as a result of stochasticity in gene expression and protein synthesis.[6] The microenvironment, such as different sedimentation or aggregation states of Au NPs around an individual cell, could also affect the uptake of Au NPs of individual cells.[28] Finally, cell cycle phase also has a significant effect on the uptake of NPs.[29] For example, cell division can dilute the concentration of NPs in cells. Therefore, all these factors ultimately lead to a reasonably wide distribution of Au NPs in single cells, as shown in Figure 4. The data provide significant evidence that seemingly identical single cells respond differently to Au NPs and thus contain different Au masses after exposure. This method has the capability to quantitatively analyze Au NPs in single cells and will aid better understanding of cell-to-cell variation, giving deeper insights into basic biological processes.

Compared to other single cell analysis methods, LA-ICPMS has many advantages when applied to NP determinations. First, LA-ICPMS has the capability to absolutely and accurately quantify NPs in single cells via elemental analysis. The proof of this statement ultimately depends on the development of a suitable cell-based reference material. Second, LA-ICPMS can provide *in situ* information on cell−NP interactions within microenvironments. Third, as a multielement detector, LA-ICPMS is a versatile tool for the determination of other metal NPs in single cells, especially for those without any detectable physicochemical property as required by other methods.

■ CONCLUSION

We have demonstrated the principles of a new quantitative method for single cell analysis by LA-ICPMS. Dried residues of individual picoliter droplets generated by an inkjet printer have been used as suitable calibration standards for the quantitative analysis of Au NPs in single cells. The results show that individual cells, even those from a single type and identical in appearance, exhibit different uptake of Au NPs when exposed and, in turn, could have varied biological responses to Au NPs. We conclude that LA-ICPMS is a sensitive and versatile technique for single cell analysis and will offer great benefit to the study of biological responses to metal drugs or NPs at the single cell level.

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Notes

The authors declare no competing financial interest.

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