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Short communication

Determination of quantum dots in single cells by inductively coupled plasma mass spectrometry

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ABSTRACT

In order to assess cytotoxicity of quantum dots (QDs), new reliable analytical techniques that can provide comparative information at a single-cell level are required. In this study, a single cell ICP–MS (SC-ICP–MS) method was established to determine intracellular QDs in single cells after exposure. Uptake kinetics of QDs into cells was studied using the established method. The results were compared and validated by flow cytometry and cell digestion methods. In contrast to other methods, SC-ICP–MS can directly detect QDs and their degradation products via elements, and thus is a promising complement to available methods for single cell analysis and is expected to be a critical tool in the future.

1. Introduction

Quantum dots (QDs) are semiconductor nanocrystals of a few nanometers in diameter with unique optical and electrical properties, and thus have been widely applied in research and industry, such as in vivo biomedical imaging and electronics industries [1,2]. Meanwhile, research has raised potential risks of QDs to organisms and the environment under certain circumstances [3], because of their particulate nature and composition. Structurally, QDs are composed of a crystalline core and a shell that shields the core and gives QDs bioavailability. QD cores are typically made of chalcogenides of metals (such as CdSe). In order to improve water solubility and obtain a desired bioactivity, QDs usually need to be further coated by functional groups, such as peptides or proteins. Chemical composition of QD cores is considered as an important factor of QD cytotoxicity. Research shows that toxic elements (for example Cd) can be gradually released from the QDs after internalization in cells and produce many harmful effects including DNA and protein damage, cell functional impairment, and cell apoptosis [4]. In addition, the structure of the outer capping layer is also a key factor affecting QD cytotoxicity. For example, QDs with shells, e.g. CdSe/ZnS, are less toxic than "bare" QDs, e.g. CdTe [5]. In order to assess the QD cytotoxicity, it is prerequisite to quantitatively analyze intracellular QDs after exposure.

Up to now, intracellular QDs have been successfully determined by many analytical methods [3,6]. Some methods, however, can only provide the ensemble average from thousands of cells [7]. It is well known that cell heterogeneity is a widespread phenomenon among an isogenic cell population. Therefore, the ensemble average from a large amount of cells obtained by traditional methods usually masks the stochastic diversity of individual cellular response. For example, Liu et al. have shown the average results of gene expression from 50 cells were not representative of any one individual cell [8]. Other methods, such as some fluorescence techniques, may not reflect the actual concentration of QDs in single cells, because intracellular QDs can be partly oxidized and induce fluorescence quenching [9]. Another challenge in analysis of QDs in cells is correlating dosage across different studies. Intracellular QD concentrations reported in the literature vary in their unit of measurement due to different analytical methods used. It is usually difficult to compare results from different laboratories [3]. Therefore, characterization and quantification of intracellular QDs in cells are still challenging and new reliable techniques that can provide easily comparative information at a single cell level are urgently required as a complement to available methods in studies of QD cytotoxicity.

Inductively coupled plasma–mass spectrometry (ICP–MS), the most advanced and sensitive means for ultra-trace element analysis, has been widely used in many research fields, such as

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metallomics [10], quantitative proteomics [11,12], and nanoscience [13]. Unlike many other soft ionization sources in mass spectrometry, ICP is a high temperature source (approximately 7000 K) where all chemical bonds in the sample are easily broken and isotopes are effectively ionized into ions with a positive charge. Therefore, ICP–MS has minimal matrix effects and unmatched sensitivity for element detection. Moreover, because the response of an element in ICP–MS is independent of molecular environment of the element, semi-quantitative or quantitative analysis of samples in diverse matrices can be accomplished by using commercially available standards [14]. This characteristic makes data from ICP–MS easily comparative.

Recently, ICP–MS has been introduced to single particle and single cell analysis. In such analysis, ICP–MS operates in a time-resolved mode so that the frequency of signals is directly related to the number concentration of nanoparticles or cells and the intensity of signals is related to nanoparticle size or intracellular amount of elements. Degueldre et al. developed the theory of single particle ICP–MS (SP-ICP–MS) for nanoparticle analysis and showed that the concentration and size of particles can be derived from the time-resolved signals [15]. In combination with labeling techniques, such as nanoparticle labeling on antibodies or DNA probes, SP-ICP–MS has been successfully applied to detect proteins [16] and DNA [17].

On the other hand, single cell ICP–MS (SC-ICP–MS, usually using a quadrupole analyzer) or mass cytometry [18,19] (usually using time of flight analyzer) can offer the capability of single cell analysis, expected to become a useful tool for study of QD cytotoxicity. SC-ICP–MS was used to determine intrinsic magnesium [20], uranium [21], and bismuth-based drugs [22] in single cells. Mass cytometry succeeded in measuring 34 parameters simultaneously in single cells of healthy human bone marrow [23]. Mass cytometry has a similar workflow to conventional flow cytometry. After staining with target-specific antibodies labeled with element isotopes, rather than fluorescence dyes in normal flow cytometry, the cells are introduced and detected by mass cytometry. This 'post-fluorescence' single cell technology is theoretically capable of simultaneously measuring 70–100 parameters of cells and will probably play an important role in dissecting the complex interactions of cells [24]. In order to further improve the detection limits of mass cytometry, QDs or other nanoparticles, because of increasing the number of atoms bound per antibody, may also serve as reporters in mass cytometry [24]. Therefore, it is also envisaged that analysis of QDs by SC-ICP–MS may have wider application beyond addressing QD toxicological concerns.

In summary, ICP–MS is a promising complement to available methods for analysis of intracellular QDs at a single cell level. However, few studies on this topic can be found in the literature. In this study, a SC-ICP–MS method was established to determine intracellular QDs in single cells after different exposure times (2, 4, 8, and 12 h). The results were also compared with flow cytometry and cell digestion methods. Due to its unique capability for single cell analysis, SC-ICP–MS is expected to be crucial for study of QD cytotoxicity and biomedical applications in the future.

2. Material and methods

2.1. Chemicals

Quantum dots (Carboxyl CdSeS, 7 nm) were purchased from URA Quantum Dots Technology Development Co. Ltd (Tianjin, China). RPMI-1640 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 Beyotime Institute of Biotechnology (Beijing, China). The guaranteed grade concentrated nitric 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 experiment. Cadmium standard solution was purchased from National Research Center for Certified Reference Materials (Beijing, China).

2.2. Cell culture conditions and treatments

Raw 264.7 cells (a mouse leukemic monocyte macrophage cell line) were used in this study. Extra care was taken to avoid contamination. Cells were grown in optimal cell growth conditions to ensure that the cells were not stressed prior to experiments. Raw 264.7 cells were cultured (37 °C, 5% CO_2) in high-glucose RPMI-1640 medium (glucose 2.0 g L^{-1}). Both media also contained 10% FBS, streptomycin (100 μg mL^{-1}) and penicillin (100 IU mL^{-1}). After 24 h of plating, the cells were washed three times with PBS.

QD solution was sonicated for 2 min to avoid QD aggregation and diluted to 30 nM with RPMI-1640 media. Then 5 mL QD solution was added into each cell. After incubation with QDs for 2, 4, 8, 12 h, the cells were collected in 1.5 mL Eppendorf tubes and centrifuged at 1300*g* for 3 min.

For preparation of a single cell solution, cells were washed with PBS to remove excess QDs. After collection and dispersal in PBS, the cells were ready for ICP–MS or flow cytometer analysis (CFlow C6, Accuri Cytometers Inc.).

2.3. Confocal laser scanning microscopy

In order to image single cells, cells were grown on glass bottom dishes designed for confocal laser scanning microscopy. After exposure to QDs and washing with PBS as in the above treatments, cells were imaged by confocal microscopy (Ultra VIEW VOX, PerkinElmer Co.) with a filter (448 nm/514 nm). No background fluorescence of cells was detected under the settings used. Figures were created using Adobe Photoshop software.

2.4. ICP–MS measurement

A Thermo X7 ICP–MS (Thermo Electron Corp., USA) equipped with a micro-concentric nebulizer (MicroMist, Glass Expansion, Australia), a hexapole collision cell and Xi interfaces was used throughout this experiment. The ICP–MS instrumental and operational parameters are given in Table 1. Before analysis, the ICP–MS was tuned using multi-element standard solution (10 μg L^{-1} each of Be, Co, In, and U in 2% v/v nitric acid). The time-resolved analysis (TRA) mode was used and thus intensities were collected as a function of time (counts s^{-1}). The acquisition time of each run was 20 s with a data acquisition rate, or dwell time, of 5 ms. Raw data of this experiment were recorded using the PlasmaLab software (Thermo

Table 1
ICP–MS instrumental and operational parameters.

Forward power (W)	1200
Nebulizer gas (L min^{-1})	0.75
Cool gas (L min^{-1})	13.5
Auxiliary gas (L min^{-1})	0.69
Spray chamber	Quartz impact beads
Interface	Xi cone
Micro nebulizer	Concentric
Data acquisition mode	Time-resolved analysis (TRA)
Isotope	^{111}Cd
Uptake rate (mL min^{-1})	0.25
Dwell time (ms)	5
Acquisition time (s)	20

Electron Corp., USA) and translated into Microsoft Excel format for data handling. A syringe pump controlled the flow rate of the cell suspension. Under the optimal conditions, cells were introduced into the ICP one by one, which meant that statistically only one cell existed in the ICP at any instant, enabling QDs in a single cell to be analyzed by SC-ICP–MS.

For comparison with the results of single cell analysis, the average quantity of QDs in Raw 264.7 cells was also determined by ICP–MS after cell digestion. The washed cells (1 mL, approximately 1.0×10^5) were digested for 1 h with 3 mL concentrated nitric acid and 1 mL hydrogen peroxide in a microwave oven (Microwave MARS5, CEM Corporation, NC, USA). Then the digested solution was appropriately diluted and determined by calibration with Cd standard solution.

3. Results and discussion

Phagocytic cells such as macrophages are able to engulf and remove large particles from the blood that are recognized by the immune system. Therefore, RAW 264.7 cells are good models to study the uptake of QDs and were thus chosen in this study. As for QDs, carboxyl CdSeS was used as a model in this study for a variety of reasons, such as commercial availability, stability and uniform size. The QD dose used in this work (30 nM) was expected to induce no evident cytotoxicity, as shown in previous work [3]. No significant cell loss was detectable in comparison with untreated cells under the same conditions.

3.1. The confocal image of cells exposed by QDs

In this experiment, the cells exposed to QDs were washed with PBS three times in order to remove extracellular QDs (e.g. QDs adhered to cell surfaces) before single cells analysis by SC-ICP–MS, because it is important to accurately determine intracellular QDs for toxicological research. QD localization in cells was determined using laser confocal scanning microscopy. From the cell images shown in Fig. 1, we can draw the following conclusions. First, the QDs were found to enter the cells under our experimental conditions. Second, extracellular QDs can be removed completely by the wash method because there was no detectable fluorescence from the outer part of the cell membrane or culture dishes. Third, the fluorescence intensity of intracellular QDs increases with increasing incubation time. However, this result from the confocal image is merely qualitative.

3.2. Single cell analysis by time-resolved ICP–MS

The theory of single particle/cell analysis by ICP–MS has been discussed in detail [25]. Briefly, cells are sprayed in sequence into a high temperature plasma where each cell is desolvated and its constituents atomized and ionized. The resulting ions are then detected by mass spectrometry. In the mass spectra, the intensity of each transient signal corresponds to atomic constituents in a single cell and the frequency of transient signals is directly related to the number of cells. The number of cell detected by ICP–MS (F_{cd}) during acquisition time (t) can be calculated by the

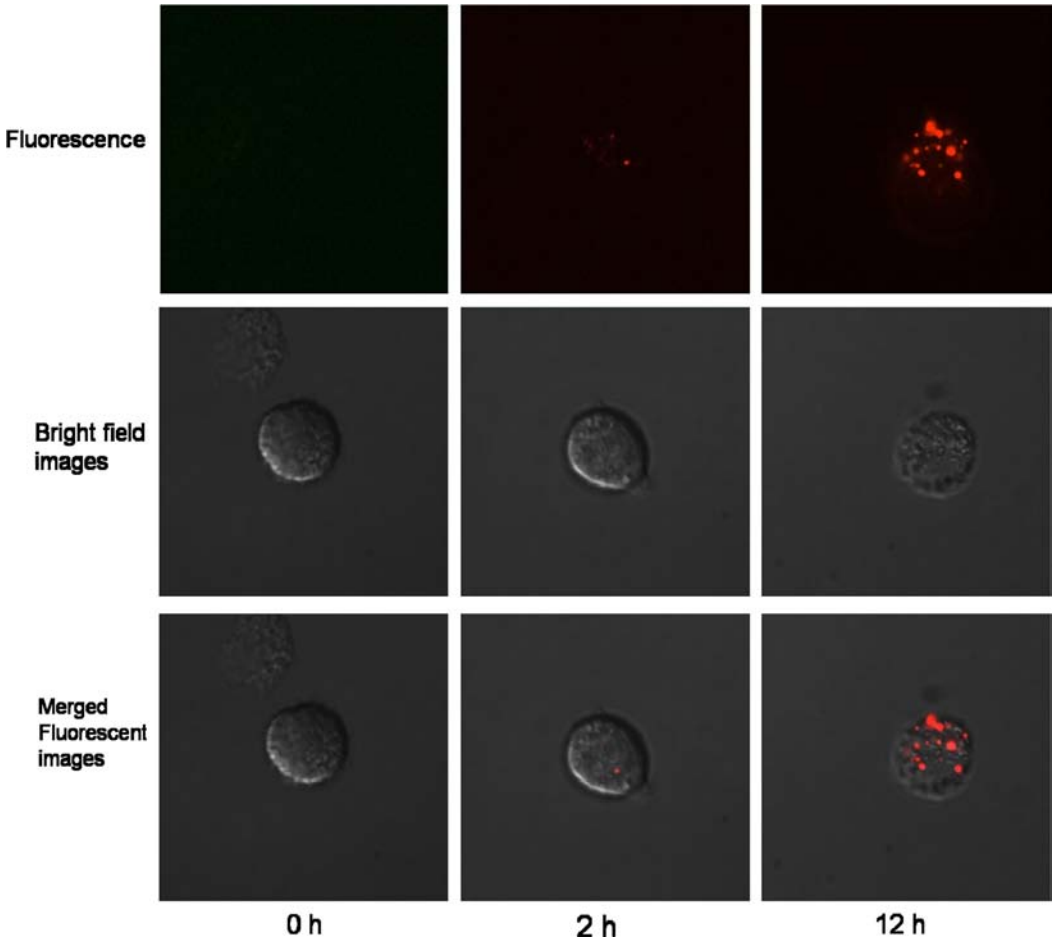

Fig. 1. Confocal microscopic images of Raw 264.7 incubated with QDs for 2 h and 12 h.

following equation:

$$F_{cd} = \varepsilon\, Q_{sam} N_{cd} t \quad (1)$$

where ε is the transport efficiency, Q_{sam} is the sample uptake rate (mL s^{-1}), N_{cd} is the cell number density (mL^{-1}), t is the acquisition time (s), and F_{cd} is the number of cells detected.

It is necessary for SC-ICP–MS analysis that only one cell enters the plasma at a time so that each transient signal in the mass spectrum corresponds to a single cell. In our experiment, dwell time, sample uptake rate, and cell number density were carefully chosen to meet this target. In traditional ICP–MS analysis, the dwell time is normally set at more than 100 ms in order to minimize signal variation due to ion statistics. In SC-ICP–MS or SP-ICP–MS analysis, shorter dwell times are utilized to improve the comparison between background and transient signals from single particles or cells, because the particle signal does not increase with increasing dwell time while the intensity of the background does. On the other hand, too short dwell time can also lead to deteriorating data because of a large proportion of incompletely measured particle events. Considering that each cell or nanoparticle can produce a plume of ions that enter the mass spectrometer in a period of usually less than 0.5 ms [26,27], the dwell time of 5 ms was chosen in our experiments. A low-flow nebulizer with a sample uptake rate of about 0.2 mL min^{-1} was used because its characteristics, such as higher transport efficiency and smaller droplet size than traditional nebulizers, are more suitable for SC-ICP–MS. The number of cells entering the ICP–MS per second must be limited so that the probability of overlapping signals from two cells or more can be very low. Taking Formula (1) into account, the cell number density should be in the range of 10^6 mL^{-1} if ε is estimated to be 1% like the nebulization efficiency in aqueous solution [28]. Under optimized conditions (i.e. t_{dwell}, N_{cd}, Q_{sam}), the real transport efficiency of SC-ICP–MS was determined by analysis of QD exposed cells, the number of which was accurately counted in advance by flow cytometry. The transport efficiency ε was calculated at 2.9%. Under these conditions, approximately three cells per second can be detected by ICP–MS, and thus overlapping of more than one cell in ICP within 5 ms integration time was insignificant.

Fig. 2 shows typical mass spectra of Raw 264.7 cells prior to and after QD exposure. The experimental conditions (see above) were carefully chosen to avoid overlapping of two cells within the dwell time. No visible ^{111}Cd transient signals can be detected for cells prior to QD exposure (Fig. 2a). This is because cadmium is usually considered as a non-essential element and cannot be detectable in normal cells. In contrast, a large number of ^{111}Cd transient signals above the baseline are observed for the cells exposed by QDs in Fig. 2c. Because there is no intrinsic Cd in the cultured cells, Cd transient signals in the mass spectra are solely from QDs. As shown in the confocal images, all extracellular QDs have been removed during the washing process; therefore all Cd transient signals in the mass spectra are from intracellular QDs. Moreover, according to the theory of SC-ICP–MS analysis, the intensity of each transient signal and the frequency of transient signals are directly related to the amount of intracellular QDs and the number concentration of cells exposed to QDs, respectively.

The signal distribution for the cells prior to QD exposure (cell blank) shows a Poisson distribution (Fig. 2b), whereas the signal distribution for the cells exposed to QDs shows an approximately lognormal distribution (Fig. 2d). These different distributions reflect the fact that cells are only present in a small fraction of droplets sprayed from the nebulizer whereas droplets from solution are homogenously distributed. These results are similar to those from single particle analysis by ICP–MS [27]. In the following sections, we will discuss how to determine cellular uptake of QDs by SC-ICP–MS and to develop a calibration method for SC-ICP–MS.

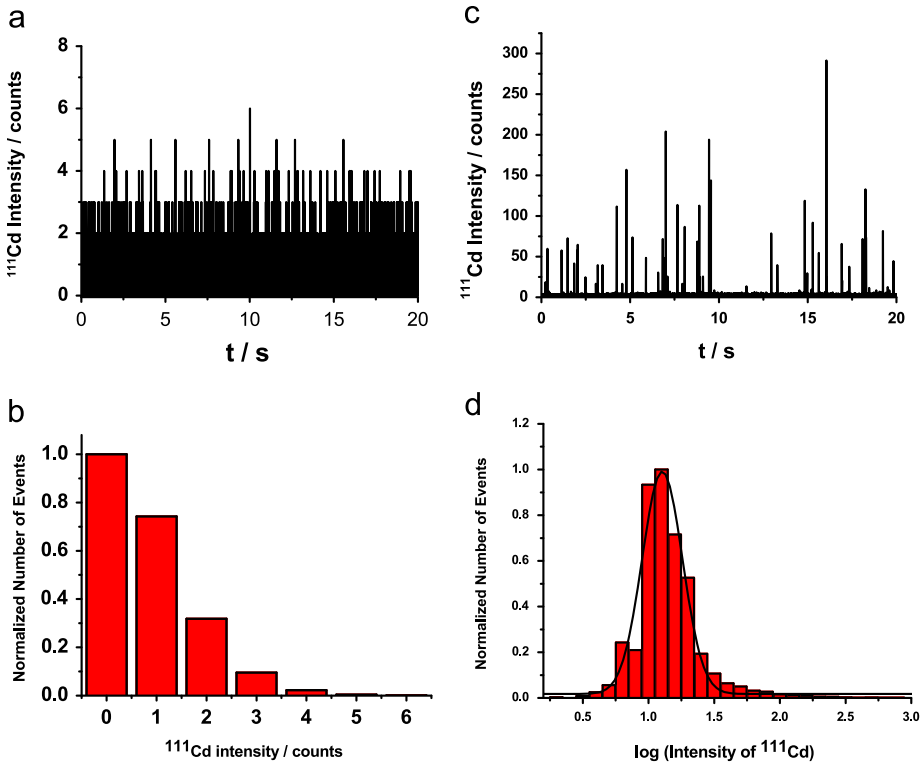

Fig. 2. Single cell analysis by ICP–MS: (a) mass spectra of Raw 264.7 cells without QD incubation and (b) frequency distribution histograms for Raw 264.7 cells without incubation of the QDs. Histograms are fitted to Poisson distribution (solid line). (c) Mass spectra of Raw 264.7 cells incubated with QDs for 8 h and (d) frequency distribution histograms for Raw 264.7 cells incubated with QDs for 8 h. Histograms are fitted to lognormal distribution (solid line).

3.3. The uptake kinetics of QDs into single cells

It is important for both biomedical application and toxicological concern of QDs to study uptake kinetics of QDs into cells. The uptake is highly variable and difficult to predict because of the wide variation in QD physicochemical features, culture conditions, and cell heterogeneity. In this study, the uptake kinetics of QDs was first determined by SC-ICP–MS and the results were further compared and confirmed by two other methods: flow cytometry and cell digestion. Flow cytometry is a well-established and widely used method in QD uptake research. Individual intracellular fluorescence of QDs can be measured and uptake kinetics of QDs can be derived using flow cytometry. The cell digestion method used was based on acid digestion of a mass of cells and determination of average element concentration by ICP–MS. Using such a cell digestion method, the uptake kinetics of QDs can also be obtained from average results of thousands of cells.

Fig. 3 shows uptake kinetics of QDs into single cells determined by SC-ICP–MS (Fig. 3a), cell digestion method (Fig. 3b), and flow cytometry (Fig. 3c). In order to compare the level of intracellular QDs determined by different methods, data in each figure were normalized to fluorescent or mass spectrometric intensity at 12 h incubation. From Fig. 3, we can see a similar trend in all methods that the uptake of QDs gradually increased for the first 8 h and reached a plateau at 8–12 h. The data from the cell digestion method have better precision, but cannot reflect cell heterogeneity, due to this bulk measurement being based on thousands of cells (Fig. 3b). In comparison, the data obtained from SC-ICP–MS and flow cytometry are based on single cell analysis, and thus have worse precision and can show cell individuality.

Also, the normalized intensity at each incubation time obtained from SC-ICP–MS is in good agreement with the corresponding intensity obtained from the cell digestion method. This agreement indicates that the established SC-ICP–MS method is reliable for study of uptake kinetics of QDs. However, an obvious difference can be found between data from flow cytometry (Fig. 3c) and data from both ICP–MS based methods (Fig. 3a and b). The normalized intensities from ICP–MS vary more significantly than those from flow cytometry. For example, the normalized fluorescence intensity at 12 h is only 1.4 times higher than the intensity at 2 h in Fig. 3c, whereas the normalized Cd intensity at 12 h is about 4 times higher than the intensity at 2 h. The low fluorescence intensity at 12 h may be due to QD aggregation and fluorescence quenching during cell culture, which are found in other literature [29]. Another possibility is that QDs have been shown to degrade under photolytic and oxidative conditions and their fluorescence intensity gradually decreases [3]. The toxic elements released from QDs are considered to be one of the causes of QD toxicity. The above discussion suggests that false information may result if only a fluorescence based method is used for study of uptake kinetics of QDs into cells. Qu et al. also found that the imaging method based upon QD optical fluorescence may not reflect the actual distribution of QDs in organisms due to QD aggregation or degradation when studying uptake of QDs into *C. elegans* [9]. In comparison, SC-ICP–MS can directly detect intracellular QDs and their degradation products via elements and thus can be a necessary complement in uptake kinetics of QDs. Moreover, SC-ICP–MS can also be used to analyze other intracellular nanoparticles that have not optical fluorophores and thus cannot be detected using fluorescence analysis, enabling SC-ICP–MS to be a versatile method to study nanoparticle cytotoxicity.

3.4. Semi-quantitative analysis of QDs in single cells

In this section, intracellular QDs in single cells were determined, thus making it possible to estimate the number of QDs in single cells

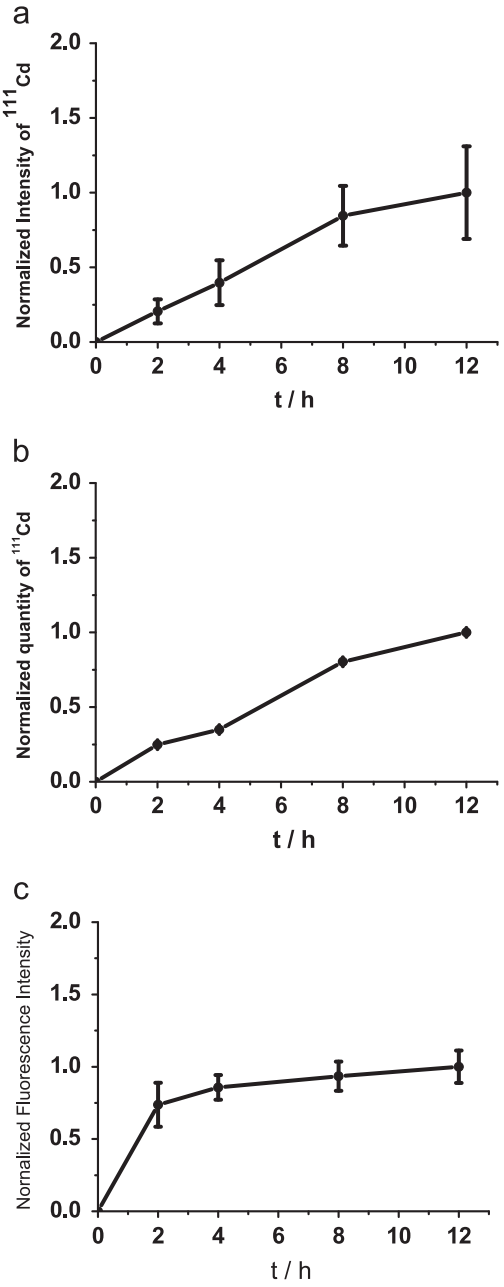

Fig. 3. Uptake kinetics of QDs into Raw 264.7 cells: (a) uptake kinetics determined by SC-ICP–MS. At each incubation time, 50 cells were analyzed. (b) Uptake kinetics determined by cell digestion method. At each incubation time, 1.0×10^5 cells were digested and analyzed. (c) Uptake kinetics determined by flow cytometry. At each incubation time, 50 cells were analyzed.

at each exposure time. Different approaches could be used to calibrate the relationship between the Cd signal and the number of QDs in a single cell. Abdelrahman et al. reported the synthesis of metal-containing polystyrene beads with and used for the calibration and internal standard in SC-ICP–MS [30]. A suspension of monodisperse QDs can also be used for calibration, but the monodisperse standard is often not available. In addition to dispersity, QDs used for calibration need to be of suitable size (neither too big nor too small), which guarantees complete atomization of particles in the ICP and unambiguous signals above background in mass spectra. In this experiment, a single QD 7 nm in diameter cannot be detected by our ICP–MS due to its small size. Therefore, another semi-quantitative approach, based on continuous introduction of standard solution, is chosen here. This approach assumes that ions from

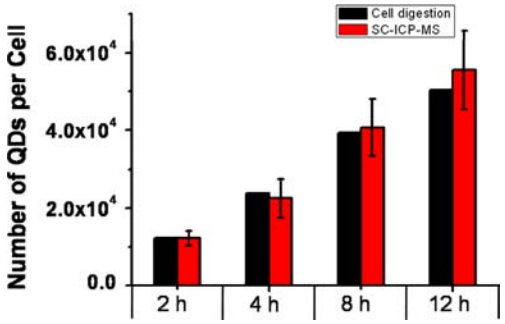

Fig. 4. The estimated number of QDs in a single Raw 264.7 cell. At each incubation time, 50 cells were analyzed by SC-ICP–MS and 1.0×10^5 cells were analyzed by cell digestion method.

intracellular QDs and ions from Cd standard solution will behave alike in the plasma, meaning that signal intensity in mass spectra is only dependent on the elemental mass transported into the plasma. This assumption is generally valid as much research has shown that the plasma ionizes particles with a similar efficiency to the corresponding dissolved species [31].

Cadmium standards (0.5, 1, 5, 10 ng mL^{-1}) were analyzed respectively in the same conditions as SC-ICP–MS analysis (i.e. solution uptake rate 0.25 mL min^{-1}, dwell time 5 ms). The transport efficiency of standards can be determined as 3.0% by the waste collection method, in which the waste stream is collected from the spray chamber and the total analyte into the plasma is calculated by comparing the waste volume to the sample uptake volume [32]. The number of Cd atoms corresponding to a count of ^{111}Cd in SC-ICP–MS can be calculated. Our results show that introduction of about 5.1×10^4 Cd atoms will produce one count of signal in the SC-ICP–MS. Assuming that the QDs have a spherical geometry and the same density as the corresponding bulk materials (5.816 g cm^{-3}), the number of Cd atoms in a single QD 7 nm in diameter can be estimated as 3.2×10^3. Based on the above data, the number of QDs in single cells at each exposure time can be estimated, as shown in Fig. 4. The estimation is of the same order of magnitude as the average number of QDs per cell by the cell digestion method. It should be noted that the cell digestion method can only provide the average based on thousands of cells while SC-ICP–MS can provide single cell information. Because the number of QDs entering a single cell can be determined by using commercially available standard solutions, data from ICP–MS can be easily compared.

4. Conclusions

We show that SC-ICP–MS is capable of measuring intracellular QDs in single cells and studying uptake kinetics of QDs. The established single cell analysis is validated by flow cytometry and cell digestion methods. In contrast to fluorescence based methods, SC-ICP–MS can directly detect QDs and their degradation products via elements. SC-ICP–MS is a promising complement to available methods for single cell analysis and is expected to be a critical tool in cell biology.

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