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Development of Desolvation System for Single-cell Analysis Using Droplet Injection Inductively Coupled Plasma Atomic Emission Spectroscopy

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With a view to enhance the sensitivity of analytical instruments used in the measurement of trace elements contained in a single cell, we have now equipped the previously reported micro-droplet injection system (M-DIS) with a desolvation system. This modified M-DIS was coupled to inductively coupled plasma atomic emission spectroscopy (ICP-AES) and evaluated for its ability to measure trace elements. A flow rate of 100 mL/min for the additional gas and a measurement point –7.5 mm above the load coil (ALC) have been determined to be the optimal parameters for recording the emission intensity of the Ca(II) spectral lines. To evaluate the influence of the desolvation system, we recorded the emission intensities of the Ca(I), Ca(II), and H-β spectral lines with and without inclusion of the desolvation system. The emission intensity of the H-β spectral line reduces and the magnitude of the Ca(II)/Ca(I) emission intensity ratio increases four-fold with inclusion of the desolvation system. Finally, the elements Ca, Mg, and Fe present in a single cell of *Pseudococcomyxa simplex* are simultaneously determined by coupling the M-DIS equipped with the desolvation system to ICP-AES.

Keywords ICP-AES, desolvation system, single-cell analysis, micro-droplet injection system

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Introduction

The ability to analyze trace elements present in a single cell allows for the elucidation of metabolic machinery and pathogenic mechanisms of various diseases. The term metallomics was coined by Haraguchi[1] to encompass the study of trace inorganic elements in living tissues. In addition to the observation of biological activities in a single cell in response to changes in its environment, the quantitative and qualitative analysis of elements in a single cell also finds application in drug discovery; such analysis can help to evaluate if therapeutic agents are effective in specific cells. Amongst the several elements contained in a single cell, trace levels of elements (ppb) have a significant influence on biological activities.

Microanalytical techniques based on electron microbeams have been developed to analyze the elemental distribution in single cells.[2-3] Using a recently developed technique based on scanning X-ray fluorescence microscopy (SXFM), Shimura *et al.*[4] determined the intracellular elements after treating the cell with a platinum (Pt)-based anticancer agent, *cis*-diamminedichloro-platinum(II) (CDDP). Analysis of the images acquired using SXFM (element array) revealed that the average Pt content in CDDP-resistant cells is 2.6-times less than that in the sensitive cells, while the zinc content is inversely correlated with the intracellular Pt content.[4] However, the limited analytical sensitivity (only several fg of a metal present in a single cell can be measured by SXFM), the requirement of time-absorbing and complicated sample preparation processes, the expensive nature, and the limited scope of elements measured by this technique make it less appealing.[5]

Widely used methods for the simultaneous determination of elements in biological samples are inductively coupled plasma–atomic emission spectrometry (ICP-AES) and ICP–mass spectrometry (ICP-MS).[23,24] A typical ICP-MS can measure sub-ppt levels of metals; however, it requires large quantities of the samples solution (≥1 mL). Various sample introduction systems have been developed to facilitate measurements of metals in smaller amounts of sample solutions; for example, a direct-injection high-efficiency nebulizer (DIHEN) introduces the sample solution directly into ICP, precluding the use of a spray chamber.[6,7] To determine the trace-element composition of single yeast cells, Groombridge *et al.*[8] have used a time-resolved ICP-MS, which is equipped with a high efficiency cell introduction system (HECIS) comprising the high performance concentric nebulizer (HPCN) and a low-volume (15 mL) on-axis spray chamber utilizing a sheath gas flow. Hence, several elements (Mg, P, Ca, Mn, Fe, Cu, and Zn) present in the cell suspension are measured by the quadorpole mass analyzer ICP-MS at its lowest integration time (10 ms). Furthermore, the analysis of elements present in a single cell by

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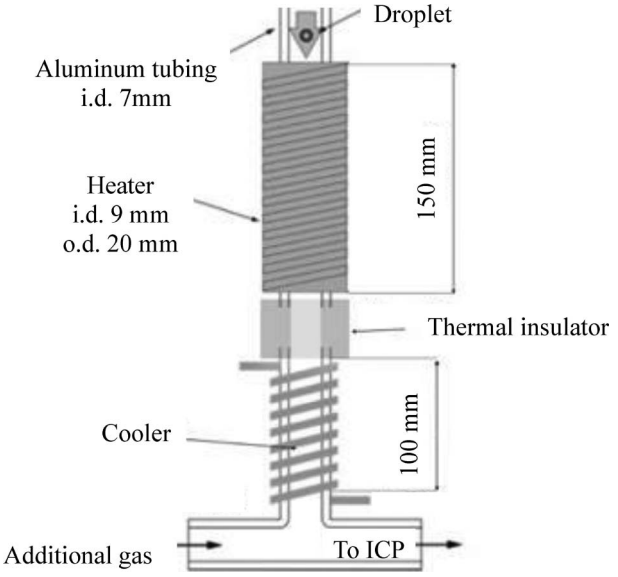

Fig. 1 Schematic representation of the desolvation system for use with M-DIS.

time-of-flight ICP-MS (ICP-TOFMS) have resulted in the simultaneous acquisition of the spectra of Mg, P, Ca, Mn, Fe, Cu, and Zn, with a time resolution of 1 ms.[8] However, the large volumes of samples introduced in the ICP by these sample systems lead to an impairment in the analytical sensitivity due to a reduction in the temperature of ICP.[9–13] Furthermore, traditional sample introduction systems are not suitable for transporting particular cells to the plasma, since the solution is nebulized in the sample-introduction system.

Our laboratory has developed a micro-droplet injection system (M-DIS) for highly sensitive analysis of elements in a very small amount of the sample. M-DIS transfers the sample as micro-droplets (diameter, 30 – 70 μm). In this system, the sample solution is not nebulized, but is introduced at the center of the ICP as a single droplet (approximately 100 pL).[14-16] Spatial and temporal control over the delivery of the sample is expected to yield high sensitivity. Furthermore, M-DIS allows for the inclusion of cells in the droplet, therefore facilitating the elemental analysis of a particular cell. By coupling the M-DIS sample-introduction system to a sector field ICP-MS instrument, we have previously investigated the analytical figures of merit with respect to single-cell analysis.[14] While a 100% transport efficiency of droplets is retained, the diameter of the droplet can be reduced to 23 μm when the M-DIS is operating in a triple-pulse mode. Absolute limits-of-detection (LODs) at the ag (10^{-18} g) levels are realized for 1000 single-droplet injections into ICP.[14] At a concentration of 100 ppb (0.1 mg/L), the absolute amount of element contained in a single cell of 5 μm diameter is equal to 6.5×10^{-18} g (6.5 ag). Highly sensitive analytical techniques with LODs at the ag level are required to detect trace elements in a single cell.

By downsizing of the droplets by gas heating, it is possible to reduce the vaporization load to the plasma. However, water vapor is introduced into the plasma, so the atomization and ionization load still exist. In this study, a desolvation system for M-DIS was developed. It traps the water vapor by placing a cooling part to the introduction route downstream of the heating. The desolvation system for M-DIS was tested by ICP-AES, and single cell AES was carried out using unicellular algae *Pseudococcomyxa simplex*.

Table 1 Average values of the absolute amount and concentration of elements in a single cell

Element	Absolute amount/fg	Concentration, ppm
Fe	80	300
Mg	57	210
Ca	11	41
Mn	2.9	11
Zn	0.63	2.4
Mo	0.32	1.2

Experimental

Ultrapure water used in the experiments was purified by a Milli-Q water purification system (resistivity, 18 MΩ cm; Direct-Q UV 3, Millipore, Bedford, MA).

Desolvation system for M-DIS

Figure 1 displays a schematic of the desolvation system developed for the M-DIS. Water in the droplet was evaporated by heating (200°C). The solvent, *i.e.*, water vapor, was removed, and only the dried sample was introduced into the ICP by additional gas flow.

Sample preparation

In this study, a standard solution of calcium (Ca, 1001 mg/L in 0.1 mol/L; Kanto Chemical Co., Ltd., Tokyo, Japan) was used. Ca was chosen as the analyte, since it is readily ionized and the wavelengths of the atomic and ionic spectral lines are close to each other. This standard solution was diluted to 100 mg/L with an appropriate volume of ultrapure water prepared by the Milli-Q system (Direct-Q UV 3, Millipore, Bedford, MA).

Unicellular alga *Pseudococcomyxa simplex*, an aerial alga found on terrestrial trees and rocks, was used as a sample for single-cell analysis. The diameter of the *P. simplex* cell varies between 3 and 8 μm, which is approximately the same as that of a human cell. Therefore, a cell can be included in a droplet with a diameter of 70 μm. Plant cells contain various essential elements. Average values of the concentrations of the different metals present in a single algal cell were measured. A unicellular alga sample (9.44×10^6 cell/mL) was decomposed in a mixed-acid solution, and quantitative analysis of the elements was performed by ICP-AES (ICPS-8100, Shimadzu Corp., Kyoto, Japan). As is evident from the values shown in Table 1, the amounts of iron, magnesium, calcium, manganese, zinc, and molybdenum were at the fg (10^{-15} g) level in a single cell. The cell volume was estimated based on a diameter of 5 μm. The diameter was obtained from the average value of the cell diameters in an optical microscope observation.

Unicellular algae were cultivated in suspended cell cultures. The cell samples were washed in ultrapure water (3 times) by re-suspension and centrifugation at 4000 rpm for 3 min to remove the broth. This broth can interfere with the ICP-AES determination of the elements. These washings were performed just before the analysis so as to minimize any possible alterations in the cell samples. The final solution used for analysis was the standard solution of yttrium (Y, 1001 mg/L in 0.1 mol/L; Kanto Chemical Co., Ltd., Tokyo, Japan), which is as an internal standard; it was diluted to 10 mg/L using ultrapure water. Since large cells and impure substances can impair efficient functioning of the M-DIS during droplet generation, the samples were

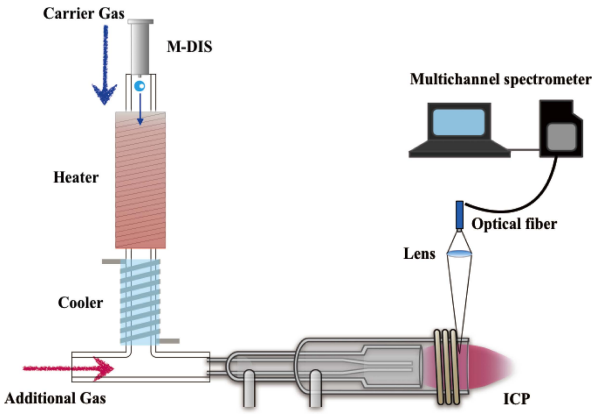

Fig. 2 Schematic representation of the droplet-injection ICP-AES set-up.

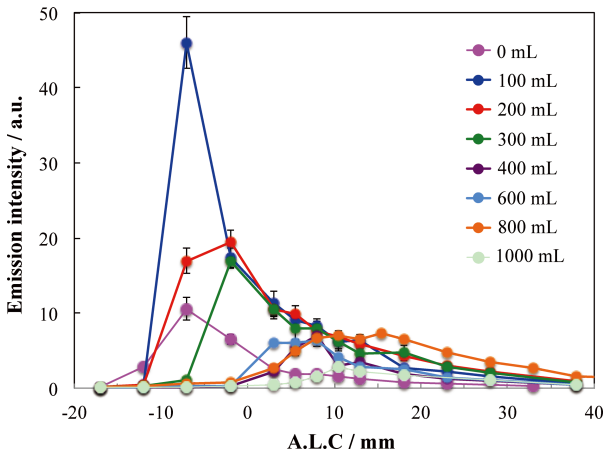

Fig. 3 Relationship between the ion emission intensity of Ca(II) spectral lines recorded at different heights above the load coil (ALC) and the flow rate of additional gas.

filtered through a Millipore filter (8 µM). The concentration of cells in the suspension was diluted such that 1 cell was present in approximately 40 droplets; such high dilution precludes the agglomeration of cells in a single droplet. The number of cells in the droplets was counted using a hemocytometer (OneCell Counter, BMS Inc., Tokyo, Japan).

ICP-AES

A desolvation system developed for the M-DIS was coupled to the ICP-AES, as shown in Fig. 2. ICP was generated using a Matching Box (Matching Box, Plasma Concept Tokyo Inc., Tokyo, Japan).[17–19] The RF generator power supply was maintained at 1200 W, the plasma gas-flow rate was 15 L/min, and the auxiliary gas-flow rate was 1 L/min. A multichannel spectrometer (HR 4000, Ocean Optics Inc., FL) was used for recording the emission spectra. The integration time of the spectrometer was set at 100 ms, and five replicates of each measurement were recorded. Emission from the plasma was focused as a 2:1 image in front of the optical fiber at a radial observation; a 50-mm-diameter plane-convex quartz lens with a focal length of 101.07 mm was used.[20–22] To investigate the spatial emission distribution of the introduced droplets in ICP, the measurement position was scanned by 5 mm increments from the ICP torch in the radial direction. Droplets (30 µm) of the standard Ca solution (100 mg/L) were injected continuously at 100 Hz. The cell sample applied to M-DIS for the analysis of a single cell was transported to the ICP-AES for the analysis.

Results and Discussion

Evaluation of the desolvation system for M-DIS

In a previous study, simultaneous multi-element analysis was performed by coupling M-DIS with ICP-TOFMS (GBC Optimass 9500). The absolute LOD using that system was at the pg (10^{-12} g) level, a sensitivity that is insufficient to detect trace elements in a single cell. Since the volume of the droplet generated in M-DIS is 1000-times greater than that of the mist generated in the traditional nebulizer, we presumed that the sample in the droplet is not ionized efficiently. Therefore, our efforts toward modulating sensitivity involve changing the volume of the droplet, resulting in the development of the desolvation system depicted in Fig. 1. Solvent of the droplet was evaporated by heating, and then the solvent was removed by cooling and the load of water vapor was removed using the desolvation system. It is conceivable that the solvent load of the droplet for ICP is reduced and sample is efficiently ionized.

The optimal flow rate of the additional gas in the desolvation system for M-DIS, *i.e.*, the gas required to transport the samples to ICP (see Figs. 1 and 2), and the position of the measurement above-the-load-coil (ALC) in the ICP-AES are varied. The flow rate of the additional gas is changed from 0 to 1000 mL/min, and intensity data is collected at different positions (–17.5 to 37.5 mm) ALC (Fig. 3). When the flow rate of the additional gas is increased to 100 mL/min, the signal intensity of the data recorded increases as the measurement height increases from –17.5 to –7.5 mm ALC. As the position of the measurement increases beyond –7.5 mm ALC, the signal intensity gradually decreases. At gas flow rates higher than 100 mL/min, a similar trend is observed; however, the signal intensity at the corresponding positions of measurements are lower than that observed when the flow rate is 100 mL/min. The decrease in the signal intensity at higher flow rates of the additional gas can be attributed to the short transit time of the sample in the ICP, which leads to inefficient ionization of the sample. The signal intensity is assumed to be impaired due to a precarious condition at the ICP. The maximum intensity of the Ca(II) spectral line is observed when the data is collected at –7.5 mm ALC and the flow rate of additional gas is 100 mL/min. In all subsequent experiments, the flow rate of the additional gas is maintained at 100 mL/min.

Evaluation of analytical sensitivity with desolvation

To evaluate the effect of desolvation on the ionization process, the intensities of the Ca(I), Ca(II), and H-β spectral lines were measured using only a heating system and a heating and cooling system. When compared to the signal intensity of the Ca(II) spectral lines observed in the absence of using a heating and cooling system, the observed signal intensity of the same is 10-fold higher when the heating and cooling system is used before the sample is introduced to the ICP (Fig. 4). Furthermore, as is evident from Fig. 4, when the heating and cooling system is implemented, the difference between the background spectrum and the droplet spectrum is minimized.

The intensity data is collected for each analyte at discreet intervals ALC in the analytical zone using only heating system and heating and cooling system in the injection unit (Fig. 5). As evident from Fig. 5, in the absence of desolvation, the highest

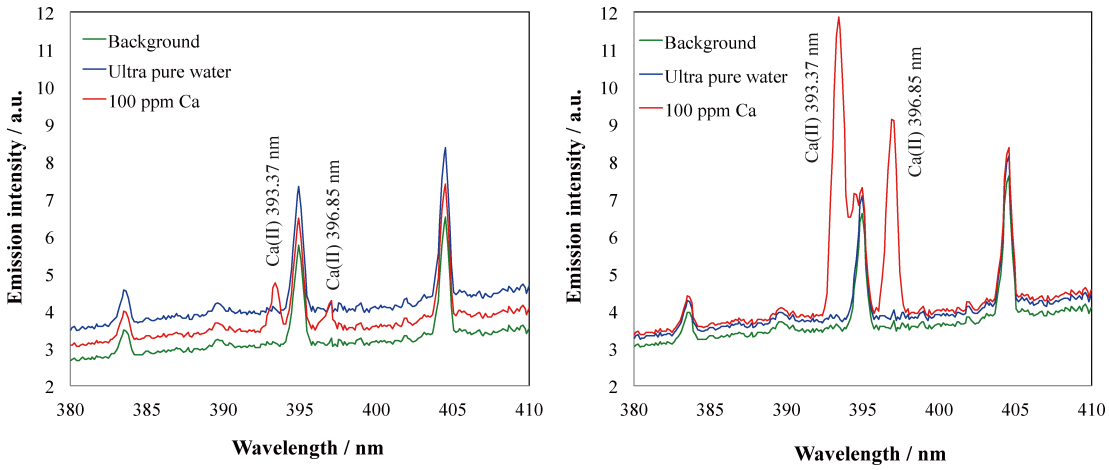

Fig. 4 Signal intensity of Ca(II) spectral lines recorded using only a heating system (left) and a heating-and-cooling system (right).

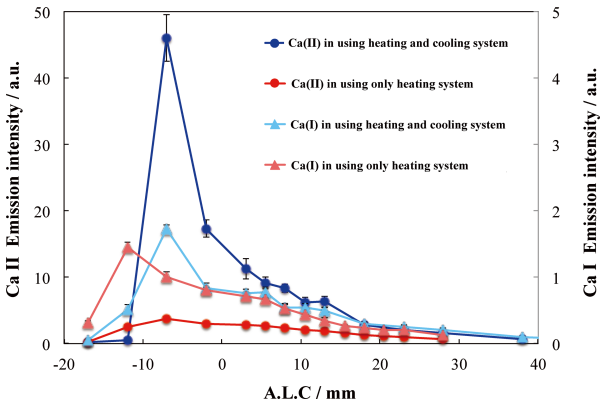

Fig. 5 Relationship between the intensities of Ca(II) (left axis) and Ca(I) (right axis) spectral lines recorded at different heights ALC using only a heating system and a heating-and-cooling system.

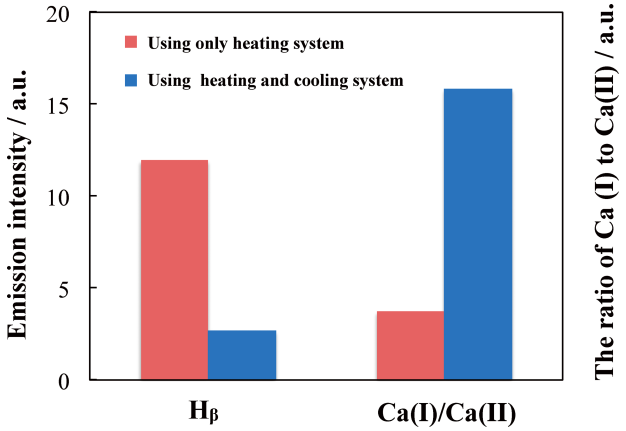

Fig. 6 H_β emission intensity and the ratio of Ca(II)/Ca(I) emission intensities using only a heating system and a heating and cooling system in the injection unit.

signal intensity of the Ca(II) spectral line is acquired at a distance that is 5 mm downstream to that observed for Ca(I). However, with the implementation of the desolvation system, the emission spectra of both Ca(II) and Ca(I) can be efficiently acquired at the same position ALC. Figure 6 shows the influence of the desolvation system on the intensity of the Ca(I), Ca(II), and H-β spectral lines. The intensity of the H-β spectral line is shown to decrease with the implementation of the desolvation system, while the Ca(II)/Ca(I) ratio of the emitted signal intensities is shown to increase four-fold with the introduction of desolvation in the injection system. It is assumed here that with implementation of the desolvation system, the solvent load of the droplet for ICP is reduced, and the sample is efficiently ionized.

Single cell elemental analysis in unicellular alga

The unicellular algae *Pseudococcomyxa simplex*, the elemental composition of which has been shown in Table 1, is used to validate the developed desolvation system. Unicellular algae contained in a 70 µm droplet are introduced to ICP by M-DIS. Elemental analysis of the single cell is performed by ICP-AES.

A multichannel spectrometer (HR 4000, Ocean Optics Inc., FL), with an integration time of 3.8 ms, is used for recording the emission spectra. As depicted in Fig. 7, the emission spectra of Ca, Mg, and Fe present in a single cell are detected simultaneously. This result suggests that multi-elements present at fg levels in a single cell can be effectively determined using the described system.

Conclusions

In this paper, we developed an M-DIS and a desolvation system for M-DIS. M-DIS can be used to introduce the sample as a droplet into the ICP. With inclusion of the desolvation system, the solvent load in the droplet can be decreased, since water vapor is readily removed. This system is implemented with the ICP-AES.

First, the optimal conditions for the flow-rate of additional gas and the measurement point ALC are determined to be 100 mL/min and –7.5 mm ALC. Next we evaluate any variations in the analytical sensitivity with inclusion of the desolvation system. When compared to the intensity recorded in the absence of the desolvation system, the signal intensity of the Ca(II) spectral lines is 10-fold higher when recorded after implementation of the desolvation system. Meanwhile, the emission intensity of

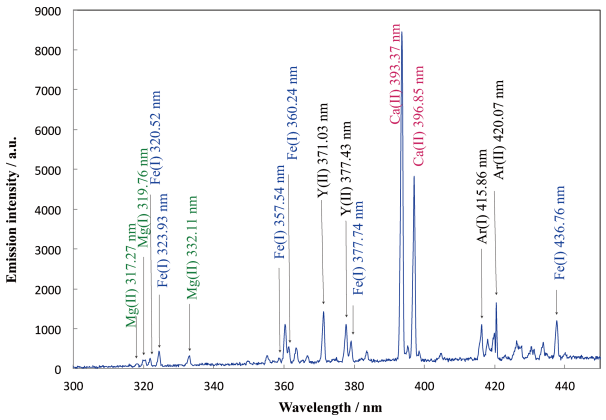

Fig. 7 Emission spectrum recorded from a single cell of *Pseudococcomyxa simplex* using the developed desolvation system coupled to the ICP-AES.

H_β reduces with the inclusion of the desolvation system. Taken together, these results indicate that the inclusion of the desolvation system effectively improves the analytical sensitivity toward the detection of metals. To validate the method, the developed method is used to determine the composition of a unicellular alga of known compositions. Multiple elements present in the cell can be simultaneously determined with coupling of the desolvation system to ICP-AES. This method successfully records the emission spectra of Ca, Mg, and F contained in a single cell; however, the emission spectra corresponding to the metals Mn, Zn, and Mo are not detected.

Studies to couple M-DIS and a desolvation system to ICP-MS or ICP-TOFMS for the analysis of trace elements are being pursued further in our laboratories.

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