Evaluation of internal distribution and extracellular action of the cell via TOF-SIMS

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Abstract

Cisplatin is one of the most popular and traditional platinum-based anti-cancer drugs. Additionally, it is known for its effect on different types of cancers. To clarify the reaction mechanism of anti-cancer drugs in a cell, visualization of drugs in a single cell is required. In this study, we investigated a secondary ion species obtained from cisplatin, which was bounded to the nucleus in a cell and its intensity. PtCl$_2^-$ was mainly detected via SIMS during the analysis of pure cisplatin reagent. In contrast, a high-intensity signal for PtCN$^-$ was detected from the cultured cells that administered cisplatin. However, the signal was not detected from the cisplatin in the reagent state. Chlorine in the cisplatin structure is replaced with water when it is combined with the cell nucleus. Therefore, PtCN$^-$ was mainly detected from intracellular because the structure was changed by cisplatin binding to the nucleus and it exhibits anti-cancer activity. The results showed that the cisplatin selectively combined with the nucleus. Through TOF-SIMS, we achieved a visual distribution of cisplatin intracellular nucleus.

Keyword TOF-SIMS, Cisplatin, Anti-cancer drugs, Mechanism of action
Introduction

Recently, research on the diagnosis and treatment of the living body has been conducted in the medical and pharmaceutical department. Additionally, there is increased need for visualization of the distribution of drugs. Moreover, there is significant interest to study cancer diagnosis. Because it is difficult to determine a proper of anti-cancer drugs that are more harmful compared to other drugs, it is necessary to determine an appropriate dose of anti-cancer drugs. Various factors in terms of tumor type, size, nature, and growth rate need to be considered. To determine the correct doses in pharmacological tests, appropriate dose and condition were studied. However, it is difficult to evaluate the appropriate dose and condition at the single-cell level. It is possible to confirm the safety and effect of the drug in the preclinical stage if the clinical dose is tested via bio-imaging. In another case, molecular imaging is necessary for the elucidation of drug delivery systems (DDS) and non-target side effects. Matrix assisted laser desorption/ionization (MALDI) and fluorescence microscopy are popular methods for direct imaging of biological samples. MALDI is an analysis technique with soft ionization of biological molecules such as protein and polysaccharide via laser irradiation. However, because the resolution is limited by diffraction limit of the laser probe, the spatial resolution of MALDI is not sufficient for the single-cell analysis. Fluorescence microscopy has a high enough spatial resolution for single-cell analysis. However, because of the overlapping wavelengths of fluorescence labeling reagents and damage to the sample by UV photon irradiation and the fluorescence reagent, multi-component imaging is difficult. Secondary ion mass spectrometry (SIMS) is one of the analytical techniques for visualization of multi-component analysis with a single-cell scale. Notably, some important elements such as Ca, K, P, and N, which trigger cell activation, were visualized on a single-cell scale by high spatial resolution dynamic-SIMS, such as nano-SIMS (Ametek Co. Ltd.). In contrary, TOF-SIMS using a pulsed primary ion beam is widely used for the molecular imaging technique. Moreover, TOF-SIMS has a high spatial resolution that enables individual imaging of single cells. Component
analysis can be expected in the single-cell level. Additionally, an imaging method of a biological sample with low damage and high sensitivity by gas cluster ion beam (GCIB) has been studied.\textsuperscript{8} Because of their soft sputtering nature that results in a high useful yield for high mass region, Ar cluster and C\textsubscript{60} (fullerene) are frequently used as primary ion beams for biological sample imaging.\textsuperscript{9,10} GCIB does not have high lateral resolution because the primary ion beam is extracted from a large ion source. A TOF-SIMS apparatus developed by Sakamoto used a focused ion beam (FIB) as the primary ion beam and had the best spatial resolution of 40 nm.\textsuperscript{11} In addition, it is capable of imaging intracellular for \textit{in situ} cross-sectioning using the same FIB. Therefore, it is possible to perform single-cell level imaging and visualization of drug intracellular by FIB-TOF-SIMS of high lateral resolution analysis.

Cisplatin (Pt(NH\textsubscript{3})\textsubscript{2}Cl\textsubscript{2}) is the anti-cancer drug used in this study. Cisplatin is one of the most popular and traditional platinum-based anti-cancer drugs. Additionally, it is known for its effect on different types of cancers. Cisplatin is a platinum chloride complex in the solid state. The mechanism of action of cisplatin is thought that it binds intracellular DNA and inhibits replication. The cross-linking reaction is considered important for antitumor activity.\textsuperscript{12} Chlorine in the cisplatin molecule is replaced with water molecules when cisplatin passes into the cell. Therefore, cisplatin is a structure in which chlorine is replaced with water when it is combined with the cell nucleus.\textsuperscript{13,14} In this study, cisplatin administered for cultured lung cancer cells was analyzed via the high spatial resolution TOF-SIMS for the visualization of the mechanism with a single-cell scale.

\textbf{Experimental}

\textit{Sample}

To evaluate secondary ion species obtained from pure cisplatin molecules, which were accurately introduced into the cancer cell as an anti-cancer drug, the mass spectra of cisplatin in a cancer cell were compared with those of a pure reagent. The cisplatin reagent powder on an indium
plate was prepared as a reagent sample. Human lung cells (line H526) were cultured in a CO₂ incubator at 37 °C and 100% humidity in 5% CO₂ atmosphere. The cells were transferred into a 24-well plate before administration of the anti-cancer drug. Cisplatin was diluted with DMSO solvent at a concentration of 1 mM to 100 μM, and then it was administered to the cells. Thereafter, the cells were incubated in a CO₂ incubator for 24 h. The supernatant was absorbed and casted on a Si wafer (6×6 mm², Nilaco Corporation) after incubation. After floating cells in the medium were deposited on the Si wafer, most of the medium was removed using a cotton swab, and then the cells on the Si wafer were dried in the atmosphere at room temperature. To remove the medium solution from the cells, the cells were washed by casting water onto the Si wafer. Cells were dried in vacuum (1.0×10⁻⁵ Pa).

**TOF-SIMS apparatus and analysis**

Dried cells were analyzed using the homemade TOF-SIMS. Our TOF-SIMS utilizes an FIB of 30 keV Ga⁺ as a primary ion beam for a high spatial resolution of approximately 40 nm. To avoid surface contamination from residual gas in the chamber, the DC mode and pulsed mode (200 ns pulse width, 10 kHz repetition rate) of the FIB irradiates the cells, respectively. Secondary ions are collected in the TOF-MS via a delayed-extraction method that maintains both high spatial resolution and high mass resolution. The mass imaging was performed with an image resolution of 256 × 256 pixels in each field-of-view. The primary ion dose was above 10¹³ ions/cm² in all images Fand fragments.

**Results and discussion**

Cisplatin is known to form cross-linked structure with the cell DNA. For the visualization of cisplatin distribution within a single-cell scale, cross-sectioning and/or surface etching of the sample by FIB was necessary. In this study, the inside of a single cell was imaged using surface
etching process for spreading in two-dimensional direction. Secondary ions obtained from the cisplatin and can be detected even when the ion beam is irradiated beyond the static limit (10\(^{12}\) ions/cm\(^2\)) in TOF-SIMS should be investigated. Therefore, the mass imaging of the reagent was analyzed using an ion dose beyond the static limit. Figures 1 and 2 show the positive and negative secondary cisplatin reagent ion images on the indium plate, respectively. The main secondary ion species that were detectable at high intensity for high spatial resolution mass imaging are shown in these images. Figure 3 shows a secondary ion mass spectrum in the negative ion mode extracted from the negative secondary ion image. There are five stable isotopes of platinum, and the four major abundant isotopes can be observed easily via mass spectrometry. The natural isotopic abundances of the four major isotopes of \(^{194}\)Pt, \(^{195}\)Pt, \(^{196}\)Pt, and \(^{198}\)Pt are approximately 32.97\%, 33.83\%, 25.24\%, and 7.16\%, respectively. The other element of cisplatin that has multiple major isotopes in natural abundance is chlorine. Therefore, the secondary ion derived from cisplatin can be identified from the isotope pattern in the mass spectrum by considering the isotope ratios of platinum and chlorine. The mass spectrum in figure 3 shows that the secondary ions are obtained from cisplatin and the isotope ratio of molecular ions corresponds to the natural abundance of platinum and chlorine. It was evident that PtCl\(_2^+\) was mainly detected in the cisplatin reagent. Secondary ions obtained from cisplatin were detected in the positive ion mode. However, the main signals were detected in the negative ion mode. Figure 4 shows secondary ion images of cultured cancer cells (H526) with 100 μM cisplatin. Figure 5 shows its secondary ions mass spectrum. Additionally, Figure 4 shows intracellular images after pre-sputtering with the FIB. Generally, the nucleus in the cancer cells is enlarged and it occupies a large part of the cells. Since the surface was sputtered enough to remove the cytoplasm, the bright region in figure 4(a) shows the nuclei. Additionally, cisplatin ion fragments were detected to be present in the negative ions. Secondary ion imaging showed that PtCN\(^-\) was mainly detected from the intracellular region. In addition, PO\(_2^\)\(^-\) was detected from same position as PtCN\(^-\). PO\(_2^\)\(^-\) is one of the fragment ions from phosphoric acids in the cellular nucleus. Therefore, the distribution of PO\(_2^\)\(^-\) shows a nuclear position.
detected as a fragment ion during the analysis of cisplatin in the reagent state. However, PtCN⁻ was detected by changing the cell structure. Figure 4 illustrates that PtCN⁻ is cisplatin component detected in the nucleus and at a similar position where PO₄⁻ is detected. During the mechanism of action of cisplatin, chlorine is replaced with water molecules when cisplatin passes in the cell and binds intracellular DNA, and inhibits replication. DNA in the nucleus found at the base. Since the structure was changed by cisplatin bound to DNA and it exhibited anti-cancer activity, PtCN⁻ was mainly detected in the intracellular. Figure 3 shows that the amount of cisplatin ions detected from the cell was significantly higher than that in Figure 5. This is because the residual components of anti-cancer drugs after washing the cell surface were detected. The mass spectra of Figure 3 include cisplatin, which was diluted with the DMSO solvent to 100 μM. Additionally, PtNH₃Cl⁻ was detected from intracellular. PtNH₃Cl was detected from cisplatin in the reagent state. Therefore, this result shows the detection of components from the cell surface by diluting and cisplatin, which does not bind intracellular DNA. In this study, the anti-cancer drug was administered at 100 μM. IC50 of H526 is around 10 μM¹⁵, where IC50 is half maximal inhibitory concentration. This indicates the concentration that results in a 50% survival rate when a drug is administered to cells. Therefore, it is concluded that PtNH₃Cl⁻ was detected for cisplatin not bound to the nucleus via cisplatin overdose.

Conclusions

The results show us the mechanism of action of cisplatin. Additionally, cisplatin in the cell was detected different structure compare to reagent and selectively combine with nucleus. Typically, bulk analysis method has been used for detecting intracellular anti-cancer drugs. However, it is impossible to show the position of the drug in the cell via this method. In this experiment, an anti-cancer drug was detected from a particular organelle in the cell, and the fragment ion of the anti-
cancer drug showed binding to the organelle. We confirmed the consistency of the mechanism of action from the visualization of the cisplatin intracellular nucleus distribution via TOF-SIMS.

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Figure captions

Fig. 1  Secondary positive ions images of cisplatin reagent. (a) All positively charged ions, (b) Pt\(^+\), \(m/z=194-196\), (c) PtO\(^+\), \(m/z= 210-212\) (d) PtCl\(_2^+\), \(m/z= 264-266\).

Fig. 2  Secondary negative ions images of cisplatin reagent. (a) All negatively charged ions, (b) Cl\(_2^-\), \(m/z=70\), (c) Pt\(^-\), \(m/z= 194-196\), (d) PtO\(^-\), \(m/z= 210-212\), (e) PtCl\(^-\), \(m/z=229-231\), (f) PtClNH\(_3^-\), \(m/z=246-248\), (g) PtCl\(_2^-\), \(m/z=264-266\).

Fig. 3  Secondary negative ion mass spectrum of the cisplatin reagent extracted from mass imaging in Fig. 2.

Fig. 4  Secondary negatively ions images of intracellular of culture cell treated cisplatin after pre-sputtering (area of 90×90 \(\mu\text{m}^2\)). (a) Total negative ions image, (b) PO\(_2^-\), \(m/z=63\), (c) Pt\(^-\), \(m/z= 194-196\), (d) PtNH\(_3\)Cl\(^-\), \(m/z= 246-248\), (e) PtCN\(^-\), \(m/z=220-222\).

Fig. 5  Secondary negative ions mass spectrum of intracellular of culture cell treated cisplatin after pre-sputtering (area of 90×90 \(\mu\text{m}^2\)).
Fig. 4

Fig. 5
Graphical Index

(a) [Image]
(b) [Image]
(c) [Image]
(d) [Image]
(e) [Image]