Published in final edited form as:

Methods Mol Biol. 2010 ; 656: 113–130. doi:10.1007/978-1-60761-746-4_6.

Quantitative Imaging of Chemical Composition in Single Cells by Secondary Ion Mass Spectrometry: Cisplatin Affects Calcium Stores in Renal Epithelial Cells

Subhash Chandra

Cornell SIMS Laboratory, Department of Biomedical Engineering, Snee Hall, Cornell University, Ithaca, NY 14853, U.S.A. (sc40@cornell.edu)

Abstract

A detailed protocol for quantitative single cell mass spectrometry imaging (MSI) analysis is described in this chapter with examples of the treatment of cells with anticancer drug, cisplatin. Cisplatin, cis-diamminedichloridoplatinum (ii) (CDDP), is widely used for the treatment of many malignancies, including testicular, ovarian, bladder, cervical, head and neck, and small cell and non-small cell lung cancers. The possibility of renal injury by cisplatin treatment is a major dose-limiting factor in this cancer therapy. At present, the mechanisms of cisplatin-induced renal cytotoxicity are poorly understood. In this work, secondary ion mass spectrometry (SIMS) was used for investigating cisplatin-induced alterations in intracellular chemical composition in a well established model (LLC-PK₁ cell line) for studying renal injury. The cells were cryogenically prepared by the sandwich freeze-fracture method for subcellular imaging analysis of chemical composition (total concentrations of K⁺, Na⁺ and Ca²⁺) in individual cells. The single cell analysis of these diffusible ions necessitates the use of reliable cryogenic sample preparations for SIMS. The sandwich freeze-fracture method offers a simple approach for cryogenically preserving diffusible ions and molecules inside the cells for SIMS analysis.

A CAMECA IMS 3f SIMS ion microscope instrument capable of producing chemical images of single cells with 500 nm spatial resolution was used in the study. In cisplatin treated cells, SIMS imaging showed the presence of detectable amount of platinum at mass 195, as ¹⁹⁵Pt⁺ secondary ions in individual cells. SIMS observations also revealed that individual cells differed in their response to cisplatin. While the chemical composition of some cells was unaffected by cisplatin, others showed a reduction in cytoplasmic calcium stores that was not associated with changes in their intracellular K or Na concentrations. Another population of cells displayed an increased in cytoplasmic calcium concentration that was associated with higher levels of intracellular Na and a reduction in K concentration of the same cells. Since the loss of intracellular K and the gain of Na and Ca are typical symptoms of cell injury, it is plausible that the initial response of the cell to cisplatin treatment is the reduction in cytoplasmic calcium pool in stores. If, somehow, the calcium stores are compromised with cisplatin, then maintenance of free Ca²⁺ homeostasis would become uncontrollable in the cell. These observations open new avenues of research for understanding of the mode of action of cisplatin in cell injury. This study also demonstrates the need and vast potential of single cell imaging mass spectrometry techniques in cell biology and medicine.

Keywords

Cisplatin; Renal injury; Calcium stores; Freeze-fracture; Cellular potassium and sodium; SIMS; Imaging mass spectrometry; Cryogenic sample preparation for SIMS

1. Introduction

The chemotherapeutic cisplatin, *cis*-diamminedichloridoplatinum ii; (CDDP), is widely used for the treatment of many malignancies, including testicular, ovarian, bladder, cervical, head and neck, and small cell and non-small cell lung cancers (1). There is a large amount of literature on the mechanisms of cisplatin-induced cell death, but its mode of action still remains unclear. Cisplatin may initiate the programmed cell death (apoptosis) via its binding to DNA, which may activate p53-dependent apoptotic pathways. Cisplatin, however, can also kill cancer cells with mutated p53 (2-4). Cisplatin may kill cells via multiple modes like apoptosis, necrosis, and perturbation of calcium homeostasis (3-6). To understand the mechanisms of cisplatin resistance and toxicity is an area of active research in cancer therapy.

Due to the renal excretion of cisplatin, the kidney is exposed to a higher concentration of cisplatin than any other organ. Nephrotoxicity of cisplatin is one of the most important dose limiting factors in cancer therapy. A single therapeutic dose of cisplatin may cause kidney damage within 48 hours (7). Therefore, the chemotherapeutic dose for cancer therapy is limited by the risk of chronic or acute renal failure (8,9). Renal proximal tubular cells are the main target for cisplatin toxicity in the kidney, since cisplatin nephrotoxicity manifests primarily as proximal tubule dysfunction (8-10). Some of these dysfunctions may include the altered synthesis of nucleic acids and proteins, disorganization of intracellular cytoskeleton, and defective transport of organic and inorganic solutes (9). The *in vitro* models have been useful in studying the mode of action of cisplatin based on single cell observations with optical/laser confocal fluorescence microscopy (8,10,11-13). These studies have implicated cisplatin induced injury with DNA fragmentation and activation of apoptotic pathways, endoplasmic reticulum stress, mitochondrial dysfunction, and the production of reactive oxygen metabolites. The perturbation of intracellular calcium homeostasis has also been observed with cisplatin treatments (14). More work is needed for understanding cisplatin-induced alterations in intracellular chemical composition of physiologically relevant cations, such as K^+ , Na^+ , and Ca^{2+} , since they are intimately involved in the maintenance of membrane potential of the cell. This study shows that the SIMS based single cell imaging technique of ion microscopy can provide a valuable tool for this line of research in chemotherapy.

Imaging mass spectrometry techniques are becoming valuable tools in biology and medicine due to their capabilities of *in situ* measurements of elements, isotopes, and molecules in single cells and at subcellular scale resolution (15-20). As discussed in various chapters of this book, imaging mass spectrometry techniques differ widely in their capabilities of detecting elements (isotopes) or molecules and have wide range of applications in biological research. In magnetic sector dynamic SIMS instruments, the recent technological innovations allow isotopic imaging capabilities of negative secondary ions at 50nm spatial resolution with a liquid metal Cs^+ primary ion beam (18,21,22). However, in NanoSIMS the detection of positive secondary ions needed for the imaging of physiologically relevant cations (K^+ , Na^+ , etc.) has remained at submicron scale resolution with the O^- primary ion beam (22). A major challenge for imaging mass spectrometry originates from the sample preparation, which needs to immobilize "The analyte" faithfully in its native location to withstand the high vacuum conditions of SIMS instruments (15,17,22,23). This study focuses on the utility of a CAMECA IMS-3f SIMS instrument, which is capable of producing secondary ion images with a 500nm spatial

resolution in the ion microscope mode for studying changes in the chemical composition of renal epithelial cells in response to cisplatin.

2. Materials

2.1. Cell Culture, Growth medium, Cisplatin, Isopentane, Liquid Nitrogen, and compressed Dry Nitrogen

1. The pig kidney LLC-PK₁ cell line has been used as an effective model for testing renal injury upon exposure to cisplatin (12,13). This is a non-excitabile cell type with characteristics of mainly the proximal tubules (24). LLC-PK₁ cells (American Type Culture Collection (ATCC CRL1392, Manassas, VA, USA), were maintained in Medium 199 supplemented with 3% fetal bovine serum (ATCC, Manassas, VA, USA) in a humidified atmosphere of 95% air and 5% CO₂ at 37°C.
2. Cisplatin, cis-diamminedichloridoplatinum (ii) (Sigma, St. Louis, MO, USA).
3. Isopentane, 2-methylbutane (Sigma, St. Louis, MO, USA).
4. Liquid nitrogen
5. A tank of compressed dry nitrogen

2.2 Silicon substrate for cell growth and Latex beads as spacers

1. An electrically conducting cell growth substrate is required for imaging analysis with a CAMECA IMS-3f SIMS ion microscope because the sample is held at $\pm 4,500$ V in the high vacuum sample chamber of the instrument. Polished high purity N-type semiconductor grade silicon wafers were used for cell growth (Silicon Quest International, Santa Clara, CA, USA). For cell growth, the silicon wafers were cut into small pieces of random shapes of approximately 1 cm² surface area using a diamond-tipped scribe.
2. The latex beads serve as spacers in the sandwich freeze-fracture method of sample preparation (see later). Latex beads with approximate diameter of 11 μ m (Duke Scientific, Palo Alto, CA, USA) were used in the study.

2.3 Freeze-dryer, reflected light microscope, and air-tight sample chamber

1. A TIS-U-Dry freeze-drier (FTS Systems, Inc., Stone Ridge, NY, USA) is used for freeze-drying the cells.
2. An Olympus Microscope with reflected light Nomarski optics (Olympus America Inc., Center Valley, PA, USA) is used for photographing the freeze-dried cells on silicon substrates.
3. A silicon substrate containing the freeze-dried cells is transferred from a desiccator to a home made air-tight Teflon chamber to avoid any re-hydration during optical microscopy measurements. A photo of this simple chamber is shown in Fig. 1. The silicon chip containing the freeze-dried cells is placed in a rectangular flat indentation in the Teflon. A glass coverslip is placed over the indentation in Teflon and sealed with hot wax as shown in Fig. 1.

2.4 Scanning electron microscope (SEM)

For observing structural preservation of freeze-dried cells at greater details than provided by optical microscopy, a JOEL JSM 35 scanning electron microscope (JEOL Ltd., Tokyo, Japan) was used in this study. For improving the quality of SEM images, the freeze-dried cells were coated with a thin layer of Au/Pd in a sputter coater (Ted Pella, Inc., Redding, CA, USA).

2.5 Secondary ion mass spectrometry (SIMS) instrumentation

A CAMECA IMS-3f magnetic sector dynamic SIMS instrument (Cameca Inc., Paris, France), which is capable of producing isotopic (elemental) images with 500 nm spatial resolution in the ion microscope mode, was used in this study. The original 3f SIMS instrument has been upgraded and equipped with a primary ion beam mass filter, a 5f Hall Probe control chassis, and a Charles Evans & Associates model PC-1CS computer interface system for control of instrument operation. The instrument is integrated to a CCD camera capable of 14 bits per pixel image digitization (Photometrics, Tuscon, AZ, USA).

2.6 Computer image processing

Computer image processing was performed using DIP Station software (Haydon Image Processing Group, Boulder, CO, USA).

3. Methods

3.1 The Cell growth and Cisplatin treatment

1. The LLC-PK₁ cells were maintained in Medium 199 supplemented with 3% fetal bovine serum at 37 °C and 5% CO₂ atmosphere. The cells were grown on the polished surface of high-purity N-type semiconductor grade silicon pieces. The silicon substrate is not toxic to cells and has been used for SIMS studies of ion transport and localization of calcium stores and anticancer drugs at subcellular scale resolution (25-27). Silicon pieces were thoroughly washed in water and sterilized prior to cell seeding. The cells were seeded at a density of 2.5×10^5 cells per 50 mm plastic Petri dish (*see Note 1*). Each dish contained six to eight silicon substrate pieces. After the cells reached about 80% confluency on silicon substrates, approximately 25,000 non-reactive 11 µm latex beads were added to each Petri dish (*see Note 2*). The beads were allowed to settle for approximately 30 min. prior to the experiments. The addition of beads is a necessity because they act as spacers in the sandwich freeze-fracture method of sample preparation and protect the cells from squashing during cryogenic sampling (28). For exposure of LLC-PK₁ cells to the drug, 6 µM cisplatin was added to each Petri dish for 4 hr. The Petri dishes without the drug treatment contained control cells. The 4 hr. short exposure with clinically relevant low concentration of 6 µM cisplatin was chosen for investigating the early cytotoxic response of cells to cisplatin (9,14).

3.2. Cryogenic sample preparation

1. After respective treatments, the cells were cryogenically prepared with the sandwich freeze-fracture method (28). In brief, the method involved the following steps: (i) the silicon pieces containing the cells is removed from the nutrient medium; (ii) excessive nutrient medium is removed from the cells by touching the edges of the silicon piece with a filter paper (*see Note 3*); (iii) a new clean silicon piece is placed on top, sandwiching the cells (and beads) between the two silicon pieces, (iv) the sandwich is fast frozen in super-cooled isopentane (*see Note 4*); (v) the frozen sandwich is quickly transferred to liquid nitrogen (*see Note 5*), (vi) the fracture is exposed by prying apart the two halves of the silicon-cell and bead-silicon sandwich under liquid nitrogen with simple tools that can be immersed in liquid nitrogen (23,28). A photo of these simple tools is shown in Fig. 2.

2. For complementary replica or membrane fracture studies, the two silicon pieces from both sides of the sandwich can be analyzed in the frozen hydrated state with scanning electron microscopy techniques (29). The substrate side of the sandwich contains groups of cells fractured at the plasma membrane. The apical half-membrane and the overlaying growth medium were removed to the non-substrate side of the sandwich. The cells fractured at the dorsal surface (apical membrane), produced by this method are nearly the whole cells without

the EF-leaflet of the plasma membrane (29). These fractured cells are used for SIMS imaging studies of chemical composition after freeze-drying. The purity and simplicity of this method is recognized by the fact that cells remained unperturbed in their growth medium throughout the sampling procedure that takes about 15 seconds. This sandwich fracture method is equally feasible to cells grown on other planar surfaces such as germanium wafer pieces, glass, and plastic substrates.

3.3. Freeze-drying

1. The silicon substrates containing the fractured cells are transferred from liquid nitrogen and onto the pre-chilled sample stage of the Freeze-drier. For this step, a thin aluminum plate of about 3 square inches may serve as a base for transferring the silicon pieces first under liquid nitrogen and onto the aluminum plate. The aluminum plate is then transferred to the pre-chilled sample stage of the freeze-drier. Depending on the type of freeze-drier, the sample stage can be pre-chilled between the -65 to -90°C . In our laboratory a TIS-U-Dry freeze-drier is used for freeze-drying the cells. After the overnight freeze-drying, the temperature of the sample stage of the freeze-drier is gradually increased to 40°C to avoid any rehydration during venting of the freeze-drier. The freeze-drier is vented to dry nitrogen, and the aluminum plate containing the samples is quickly transferred to a desiccator. These samples can now be stored for a desired time for optical (and or SEM), and SIMS analysis.

3.4. Light microscopy measurements

A silicon substrate containing the freeze-dried cells is transferred from a desiccator to an air-tight Teflon chamber to avoid any re-hydration during optical microscopy measurements (Fig. 1). An Olympus Microscope with reflected light Nomarski optics was used for photographing the fractured freeze-dried cells. Figure 3 shows an example of how to find fractured cells on silicon substrates under reflected light Nomarski imaging. The low magnification image contains a fractured area on the silicon substrate, which is shown with a dotted line (Fig. 3a). This area appears clean since the fracture plane has passed through the cell surfaces and overlaying nutrient medium has been removed by this process. At least hundreds of cells are contained within this area. Such fractured areas scatter randomly on a silicon substrate and may occupy only a small fraction of the total surface area of the substrate (*see Note 6*). A higher magnification view of individual cells in fractured areas shows well preserved nuclei, nucleoli, and cytoplasmic structures (Fig. 3b).

3.5. Scanning electron microscopy (SEM) measurements

The SEM provides an invaluable technique for observing structural preservation of fractured cells at greater details and to correlate SEM observations of single cell analysis with SIMS imaging of chemical composition (30,31). An example of structural preservation of fractured cells is shown here. For improving the quality of SEM images, the cells were coated with a thin layer of Au/Pd in a sputter coater. Figure 4 shows SEM images of fractured cells. An arrow in the low magnification SEM image points to a spacer latex bead in the fractured area containing many cells (Fig. 4a). A higher magnification view of fractured cells, recorded after tilting the sample stage of the SEM by 40° , shows well preserved cytoplasmic structures of mitochondrial dimensions. A metaphase cell in the field of view, shows the presence of sister chromatids (arrow) in some chromosomes which are laying perpendicular to the incident beam (Fig. 4b). The tilting of the SEM sample stage can significantly improve the recognition of microfeatures in surface topography of fractured cells.

3.6. SIMS imaging of chemical composition in single cells

1. A CAMECA IMS-3f magnetic sector SIMS instrument, capable of producing isotopic (elemental) images with 500 nm spatial resolution in the ion microscope mode, was used in

this study. A 5.5 keV primary ion beam of O_2^+ (approximately 100 nA beam current with a spot size of 60 μm in diameter) was used in this study. The primary ion beam was raster scanned over a 250 μm^2 region. A 60 μm contrast aperture was used for imaging positive secondary ion images.

2. The cells were coated with a thin layer of Au/Pd to enhance their electrical conductivity for SIMS analysis. In the positive secondary ion detection mode, images of masses 12, 23, 39, 40, and 195 provided the subcellular distribution of $^{12}C^+$, $^{23}Na^+$, $^{39}K^+$, $^{40}Ca^+$, and $^{195}Pt^+$ secondary ions, respectively. A few minutes of pre-sputtering is required for secondary signal stabilization, fine image focusing, and the removal of Au/Pd coating from the cell surface prior to the recording of SIMS images on a CCD camera. These few minutes of pre-sputtering represent an initial step of the instrument tuning and signal stabilization under the bombardment of a reactive primary ion beam, like O_2^+ used here. In the CAMECA IMS-3f SIMS ion microscope instrument, SIMS images are recorded one image at a time with continuous sputtering of the cell surface in the Z-axis. In general, the sputtering times were 0.4 s for integration of $^{39}K^+$ and $^{23}Na^+$ images and 2 min for $^{12}C^+$, $^{40}Ca^+$, and $^{195}Pt^+$ images. It should be noted that topographic artifacts can be easily distinguished in the analysis of cultured cells, as they cause stretching and blurring of SIMS images.

3. An example of the SIMS imaging of chemical composition in control renal epithelial LLC-PK₁ cells is shown in Fig. 5. SIMS images represent spatially resolved distribution of the total concentration (both free and bound forms) of designated elements via the detection of their respective major isotopes. The level of brightness within a SIMS image is directly proportional to the local concentration of the analyte. The ^{39}K SIMS image reveals a nearly homogeneous distribution for intracellular potassium. The individual cells in the field of view are easily recognized as they are separated by dark lines representing inter-cellular spaces (Fig. 5a). These cells also reveal high K-low Na signals (Fig. 5b) with a K/Na ratio of approximately 11, which is indicative of the analysis of well preserved healthy cells. The ^{23}Na image appears dark since it was integrated for the same amount of time (0.4 s) as the ^{39}K image. This approach provides a direct visual comparison for evaluating the health status of the analyzed individual cells in the field of view. The ^{40}Ca image reveals lower concentration of total calcium in the nucleus as compared to the cytoplasm (Fig. 5c). The calcium storing organelle, the endoplasmic reticulum, is a major contributor to the cytoplasmic pool of calcium in SIMS images (31). A bright-intensity perinuclear region is also present in the cytoplasm of each cell in the ^{40}Ca image (Fig. 5c). This region has been identified as the Golgi apparatus in this cell line (32). A carbon image is also shown to illustrate the distribution of carbon in these cells (Fig. 5d). The ^{12}C image is recorded since spatially-resolved carbon intensities in relation to the signals of other analytes provide the foundation for quantitative imaging at subcellular scale resolution (see later). There was no detectable signal at mass 195, indicating that background for $^{195}Pt^+$ secondary ions was minimal in these control cells.

4. An example of the SIMS analysis of chemical composition in cisplatin treated LLC-PK₁ cells is shown in Fig. 6. Individual cells in SIMS images are identified by numbers for the ease of their matching between the ^{39}K , ^{23}Na , and ^{40}Ca images (Fig. 6a-c). Individual cells display great variations in their response to cisplatin treatment, as it is clearly visualized in ^{39}K , ^{23}Na , and ^{40}Ca SIMS images (Fig. 6a-c). For example, while the majority of cells have gained significant quantities of sodium in response to cisplatin, but some (e.g., cells 2 and 3) still maintain the high K-low Na signature. The distribution of calcium stores has been altered completely in these cells. For example, the higher concentrations of calcium in Golgi apparatus can no longer be visualized after cisplatin treatment. Also, in general, the cells with higher Na signals also show a higher level of accumulation in their cytoplasm. It is plausible that the cisplatin response is cell cycle dependent, and, therefore, such a large variation is observed in these asynchronously growing cells. A much better understanding of these cisplatin-induced

changes in chemical composition can be made by single cell analysis of each cell, quantitatively, by digital image processing (see below). It should also be noted that SIMS is capable of imaging Pt in cells. The presence of Pt was detected at mass 195, as $^{195}\text{Pt}^+$ secondary ions (Fig. 6d), even though SIMS' sensitivity for Pt detection is poor due to its high ionization potential.

3.7. Quantification of SIMS ion microscopy images

Quantification of SIMS ion microscopy images in fractured freeze-dried cells has been reviewed previously (15). In brief, the illumination of a dynamic SIMS ion microscopy image, I , is related to the concentration of the imaged element M by the equation (33):

$$I = t C_M S i_p a_0$$

where t is the practical ion yield of element M (the ratio of the number of M^+ ions collected to the number of M atoms removed from the sample), C_M is the atomic concentration of element M corrected for its isotopic abundance, S is the total sputtering yield (number of atoms of any kind removed from the sample per incoming primary ion), i_p is the primary beam current density, and a_0 is the analyzed area of the sample surface. While i_p and a_0 are controllable, t and S depend on many factors. The practical ion yield of an element is inversely proportional to the exponential of the element's first ionization potential and depends on the chemical state of the element, the chemical and physical properties of the sample matrix, instrumental transmission, and sampling conditions. Variations of t and S within the imaged field have been considered as matrix effects, along with mass interferences due to the matrix which can augment measured intensities. The basic requirements of any image quantification scheme for dynamic SIMS images are (i) the sample preparation method must preserve native elemental distributions of the sample, (ii) the evaluation of matrix effects must be made, (iii) the quantification standard should have the matching matrix composition as the sample, and (iv) calibration of the ion microscope's imaging system.

First, the reliability of sandwich freeze-fracture sample preparation method in preserving intracellular chemical composition has been confirmed in many studies (*e.g.*, see reviews in reference ¹⁵ and 23). Both structures and chemical composition are well preserved for subcellular scale SIMS studies in fractured freeze-dried cells. Second, the evaluation of matrix effects between the nucleus and the cytoplasm of freeze-fractured freeze-dried cells revealed no significant differences (34,35). This observation was not surprising since the major components of the cell matrix in mammalian cell cultures are C, H, N, and O, and their cellular distributions are largely homogeneous at the submicron scale (34). Third, the quantification standards were generated from cell culture samples themselves and the relative-sensitivity-factors (RSF) for desired analytes were determined with respect to the cell matrix $^{12}\text{C}^+$ carbon signals (36). In the last step of image quantification, the imaging system was calibrated for pixel-by-pixel image quantification by ratioing the analyte signals to the cell matrix $^{12}\text{C}^+$ signals in the same spatial registration (36). The absolute dry weight concentrations obtained by this method can be converted into wet weight millimolar concentrations by assuming 85% cell water content in mammalian cells (37). Subcellular quantification of SIMS ion images of ^{39}K , ^{23}Na , ^{40}Ca , and ^{10}B , from fractured freeze-dried cells has provided invaluable information in studies of organelle level calcium stores (27,31,32) and screening of boronated anticancer compounds in boron neutron capture therapy of cancer (20,26,38,39).

3.8. Digital image processing of SIMS images for quantitative imaging

SIMS isotope images were digitized directly from the microchannel plate/fluorescent screen assembly of the ion microscope with a slow scan CCD camera capable of 14 bits per pixel

image digitization. SIMS images of positive secondary ions of ^{12}C , ^{23}Na , ^{39}K , and ^{40}Ca were recorded from the same cell. SIMS image integration times varied according to their intensities. In general, the image integration times were 0.4 s for ^{39}K and ^{23}Na and 2 min. for ^{12}C and ^{40}Ca images. The variations in time of exposure for various SIMS isotope images were compensated for quantification in relation to the time of exposure of the ^{12}C image. Computer image processing was performed using DIP Station software. SIMS images were quantified using relative sensitivity factors to the cell matrix element $^{12}\text{C}^+$ signals in the same spatial registration (36). The registration of analyte signals to the $^{12}\text{C}^+$ signals in the same spatial location within a cell in SIMS images compensates for variations in microchannel plate response and primary ion beam heterogeneity. The concentrations of ^{39}K and ^{23}Na were calculated on the single cell basis since their distributions are nearly homogeneous. Total calcium was measured in the nucleus and the cytoplasm by selecting regions of interests (ROIs) in individual cells.

3.9. Cisplatin-induced alterations of K, Na, and Ca concentrations in single cells

The corresponding concentration of K, Na, and Ca in control and cisplatin-treated renal epithelial LLC-PK₁ cells shown in SIMS images in Fig. 5 and Fig. 6, respectively, are listed in Table 1. Physiologically relevant concentrations of K and Na were observed in control cells (Table 1). The cell cytoplasm contains nearly two fold higher concentration of calcium stores than nucleus in control cells (Table 1). The endoplasmic reticulum is mainly responsible for storing the majority of cytoplasmic calcium in this cell line (31). Cisplatin-treated cells display large variations in their chemical composition. For example, cells 2, 3, and 5 show intracellular concentrations of K and Na very comparable to the healthy control cells, but their cytoplasmic calcium stores have been reduced substantially (Table 1). Cells 1, 4, 6, 8, and 9 have gained toxic levels of intracellular Na (over 70 mM) and accumulated substantially higher Ca concentrations in both cytoplasm and nucleus (Table 1). Since these observations were reproducible (not shown), a few patterns of cisplatin induced cytotoxic response can be deduced from single cell observations. First, the cells with healthy K and Na levels but reduced cytoplasmic calcium stores (Cell 2, 3, and 5) probably reflect the early response to cisplatin as the reduction in ER calcium pool. This may be a defense mechanism of the cell against cisplatin, which causes a shift in fundamental calcium homeostasis. Second, a persistent perturbation of calcium homeostasis may raise intracellular ionized Ca^{2+} levels in the toxic range. At this stage, mitochondria will participate in Ca^{2+} buffering by unphysiological loading of calcium for protecting the cells from injury (or death). It is plausible that cells 1, 4, 6, 8, and 9 are at this stage of cisplatin-induced injury and demonstrate cytoplasmic loading of calcium in SIMS analysis. Further signs of toxicity are also evident in these cells by the gain of Na^+ and the loss of K^+ (Table 1). Such alterations in intracellular K^+ and Na^+ concentrations will result in destabilization of the membrane potential. SIMS observations shown here provide strong support to previous studies that have implicated ER stress, mitochondrial dysfunctions, and perturbation of calcium homeostasis in cisplatin-treated cells (12-14). This work also demonstrates that mass spectrometry based single cell imaging techniques can provide valuable tools in cell biology and medicine for characterization of chemotherapeutic agents.

4. Notes

1. In general, two Petri dishes per treatment are sufficient to provide enough number of silicon substrate pieces for cryogenic sampling and SIMS analysis.
2. After the beads are added, a gentle shaking of the Petri dish by rotating it in your hand is recommended for dispersing the beads evenly in the nutrient medium.
3. It is not recommended to dry the cells in this step. A thin layer of overlaying nutrient medium on top of the cells is a requirement for sandwich freeze-fracture.

4. Super-cooled isopentane can be made easily by taking about 50-80 ml of isopentane in a 200 ml beaker. The beaker containing isopentane is placed in a bigger container filled with liquid nitrogen to less than half the height of the isopentane beaker. This assembly is placed on a magnetic stir. The isopentane is stirred by putting a small magnet in the beaker. In approximately 60-90 seconds of stirring, such a cooling results in clouding of isopentane prior to its freezing. This cloudy isopentane is known as “super cooled isopentane”. Individual users may modify this procedure according to their needs. As a caution, it is recommended that this step be done after wearing safety goggles.

5. Frozen silicon sandwiches can be stored under liquid N₂ for desired periods of time.

6. It is also possible that any given silicon substrate may not contain any fractured areas. In general, six to eight substrate pieces per treatment would provide sufficient number of fractured cells for SIMS analysis. Also, the cells grown to higher confluency (over 70%) on silicon substrates improves the chance of fracturing them as a monolayer in large areas. Although we have characterized this fracture by matching compliments of the same individual cells on the substrate and the other side of the sandwich (29), it cannot be ruled out that some fractured areas contain cells where the fracture plane has pass through the cell surface without splitting the apical membrane.

Acknowledgments

This work was supported in part by Biological and Environmental Research Program (BER), U.S. Department of Energy, grant number DE-FG02-91ER 61138. Partial supports from a NIH grant R01CA129326 (National Cancer Institute), Cornell Core Facilities, and NYSTAR Program are also acknowledged. (The content discussed in this chapter is solely the responsibility of the author and does not necessarily represent the official views of the National Cancer Institute or National Institutes of Health). The NIH/NSF Development Resource for Biophysical Imaging Opto-electronics (DRBIO) is acknowledged in culturing the cells used for SIMS experiments in this study.

References

1. Rosenberg, B. Platinum complexes for the treatment of cancer: Why the search goes on, in *Cisplatin, Chemistry and Biochemistry of a leading Anticancer Drug*. Bernhard, Lippert, editor. Wiley-VCH; Basel, Switzerland: 1999. p. 3-27.
2. Zamble DB, Jacks T, Lippard SJ. p53-dependent and -independent response to cisplatin in mouse testicular teratocarcinoma cells. *Proc. Natl. Acad. Sci. USA* 1998;95:6163–6168. [PubMed: 9600935]
3. Pestell KE, Hobbs SM, Titley JC, Kelland LR, Walton MI. Effect of p53 status on sensitivity to platinum complexes in a human ovarian cancer cell line. *Mol. Pharm* 2000;57:503–511.
4. Gonzalez VM, Fuertes MA, Alonso C, Perez JM. Is cisplatin-induced cell death always produced by apoptosis? *Mol. Pharmacol* 2001;59:657–663. [PubMed: 11259608]
5. Lieberthal W, Triaca V, Levine J. Mechanisms of death induced by cisplatin in proximal tubular epithelial cells: apoptosis vs. necrosis. *Am. J. Physiol* 1996;270:F700–F708. [PubMed: 8967349]
6. Liang X, Shen D, Chen KG, Wincovitch SM, Garfield SH, Gottesman MM. Trafficking and localization of platinum complexes in cisplatin-resistant cell lines monitored by fluorescence-labeled platinum. *J. Cell Physiol* 2005;202:635–641. [PubMed: 15546142]
7. Osman AM, El-Sayed EM, El-Demerdash E, Al-Hyder A, El-Didi M, Attia AS, Hamada FMA. Prevention of cisplatin-induced nephrotoxicity by methimazole. *Pharmacol. Res* 2000;41:113–119. [PubMed: 10712836]
8. Nowak G. Protein kinase C- α and ERK1/2 mediate mitochondrial dysfunction, decrease in active Na⁺ transport, and cisplatin-induced apoptosis in renal cells. *J. Biol. Chem* 2002;277:43377–43388. [PubMed: 12218054]
9. Rabik CA, Dolan ME. Molecular mechanisms of resistance and toxicity associated with platinating agents. *Cancer Treat. Rev* 2007;33:9–23. [PubMed: 17084534]
10. Cumming BS, Schnellmann RG. Cisplatin-induced renal cell apoptosis: Caspase 3-dependent and -independent pathways. *JPET* 2002;302:8–17.

11. Horky M, Wurzer G, Kotala V, Anton M, Vojtesek B, Vacha J, Wesierska-Gadek J. Segregation of nucleolar components coincides with caspase-3 activation in cisplatin-treated HeLa cells. *J. Cell Sci* 2000;114:663–670. [PubMed: 11171371]
12. Park MS, Leon MD, Devarajan P. Cisplatin induces apoptosis in LLC-PK₁ cells via activation of mitochondrial pathways. *J. Am. Soc. Nephrol* 2002;13:858–865. [PubMed: 11912244]
13. Liu H, Beliga R. Endoplasmic reticulum stress-associated caspase 12 mediates cisplatin-induced LLC-PK₁ cell apoptosis. *J. Am. Soc. Nephrol* 2005;16:1985–1992. [PubMed: 15901768]
14. Spletstoeser F, Florea A-M, Busselberg D. IP₃ receptor antagonist, 2-APB, attenuates cisplatin induced Ca²⁺-influx in HeLa-S3 cells and prevents activation of calpain and induction of apoptosis. *Brit. J. Pharmacol* 2007;151:1176–1186. [PubMed: 17592515]
15. Chandra S, Smith DR, Morrison GH. Subcellular imaging by dynamic SIMS ion microscopy. *Anal. Chem* 2000;72:104A–114A. 2000.
16. Rubakhin SS, Jurchem JC, Monroe EB, Sweedler JV. Imaging mass spectrometry: fundamentals and applications to drug discovery. *Drug Discov. Today* 2005;10:823–837. [PubMed: 15970265]
17. Winograd N. The magic of cluster SIMS. *Anal. Chem* 2005;77:142A–149A.
18. Lechene C,F, Hillion F, McMahon G, Benson D, Kleinfeld AM, Kampf JP, Distel D, Lyuten Y, Bonventre J, Hentschel D, Park KM, Ito S, Schwartz M, Benichou G, Slodzian G. High resolution quantitative imaging of mammalian and bacterial cells using stable isotope mass spectrometry. *J. Biol* 2006;5:20–20. 30. [PubMed: 17010211]
19. Gazi E, Lockyer NP, Vickerman JC, Gardner P, Dwyer J, Hart CA, Brown MD, Clarke NW, Miyan J. Imaging ToF and synchrotron-based FT-IR microspectroscopic studies of prostate cancer cell lines. *Appl. Surf. Sci* 2004;231-232:452–456.
20. Chandra S, Tjarks W, Lorey DR II, Barth RF. Quantitative subcellular imaging of boron compounds in individual mitotic and interphase human glioblastoma cells with imaging secondary ion mass spectrometry (SIMS). *J. Microsc* 2008;229:92–103. [PubMed: 18173648]
21. Slodzian G, Daigne B, Girard F, Boust F, Hillion F. Scanning secondary ion mass spectrometry with parallel detection. *Biol. Cell* 1992;74:43–50. [PubMed: 1511246]
22. Boxer SJ, Kraft ML, Weber PK. Advances in imaging secondary ion mass spectrometry for biological samples. *Ann. Rev. Biophys* 2009;38:53–74. [PubMed: 19086820]
23. Chandra S. Challenges of biological sample preparation for SIMS imaging of elements and molecules at subcellular resolution. *Appl. Surf. Sci* 2008;255:1273–1284.
24. Hull RN, Cherry WR, Weaver GW. The origin and characteristics of a pig kidney strain, LLC-PK₁. *In Vitro* 1976;12:670–677. [PubMed: 828141]
25. Chandra S, Morrison GH. Imaging elemental distribution and ion transport in cultured cells with ion microscopy. *Science* 1985;228:1543–1544. [PubMed: 2990033]
26. Chandra S, Kabalka GW, Lorey DR II, Smith DR, Coderre JA. Imaging of fluorine and boron from fluorinated-boronophenylalanine in the same cell at organelle resolution by correlative SIMS ion microscopy and confocal laser scanning microscopy. *Clin. Cancer Res* 2002;8:2675–2683. [PubMed: 12171900]
27. Chandra S. Subcellular imaging of RNA distribution and DNA replication in single mammalian cells with SIMS: the localization of heat shock induced RNA in relation to the distribution of intranuclear bound calcium. *J. Microsc* 2008;232:27–35. [PubMed: 19017198]
28. Chandra S, Morrison GH, Wolcott CC. Imaging intracellular elemental distribution and ion fluxes in cultured cells with ion microscopy: A freeze-fracture methodology. *J. Microsc* 1986;144:15–37. [PubMed: 3795262]
29. Chandra S, Morrison GH. Evaluation of fracture planes and cell morphology in complementary fractures of cultured cells in the frozen-hydrated state by field-emission secondary electron microscopy: feasibility for ion localization and fluorescence imaging studies. *J. Microsc* 1997;186:232–245. [PubMed: 9226938]
30. Chandra S, Morrison GH. Sample preparation of animal tissues and cell cultures for secondary ion mass spectrometry (SIMS) microscopy. *Biol. Cell* 1992;74:31–42. [PubMed: 1511245]
31. Chandra S. Quantitative imaging of subcellular calcium stores in mammalian LLC-PK₁ epithelial cells undergoing mitosis by SIMS ion microscopy. *Eur. J. Cell Biol* 2005;84:783–797. [PubMed: 16218191]

32. Chandra S, Kable EPW, Morrison GH, Webb WW. Calcium sequestration in the Golgi apparatus of cultured mammalian cells revealed by laser scanning confocal microscopy and ion microscopy. *J. Cell Sci* 1991;100:747–752. [PubMed: 1814929]
33. Morrison GH, Slodzian G. Ion microscopy. *Anal. Chem* 1975;47:932A–943A.
34. Chandra S, Ausserer WA, Morrison GH. Evaluation of matrix effects in ion microscopic analysis of freeze-fractured, freeze-dried cultured cells. *J. Microsc* 1987;148:223–239. [PubMed: 3443979]
35. Ausserer WA, Chandra S, Morrison GH. Morphological and elemental integrity of freeze-fractured, freeze-dried cultured cells during ion microscopic sampling. *J. Microsc* 1988;154:39–57. [PubMed: 2746635]
36. Ausserer WA, Ling Y-C, Chandra S, Morrison GH. Quantitative imaging of boron, calcium, magnesium, potassium, and sodium distributions in cultured cells with ion microscopy. *Anal. Chem* 1989;61:2690–2695. [PubMed: 2619055]
37. Buchanan RA, Leapman RD, O'Connell MF, Reese TS, Andrews SB. Quantitative scanning transmission electron microscopy of ultrathin cryosections: subcellular organelles in rapidly frozen liver and cerebellar cortex. *J. Str. Biol* 1993;110:244–255.
38. Chandra S, Lorey DR II, Smith D. Quantitative subcellular dynamic SIMS imaging of boron-10 and boron-11 isotopes in the same cell delivered by two combined BNCT drugs: *In vitro* studies on human glioblastoma T98G cells. *Rad. Res* 2002;157:700–710.
39. Chandra S, Lorey DR II. SIMS ion microscopy imaging of boronophenylalanine (BPA) and $^{13}\text{C}^{15}\text{N}$ -labeled phenylalanine in human glioblastoma cells: Relevance of subcellular scale observations to BPA-mediated boron neutron capture therapy of cancer. *Int. J. Mass Spect* 2007;260:90–101.

Fig. 1.

Photograph of an air-tight sample chamber for making reflected light microscopy measurements on freeze-dried cells on a silicon substrate. A silicon chip containing the cells is isolated from the normal atmosphere by a glass coverslip which is glued to a Teflon support. Reproduced from reference ³⁰ (S. Chandra and G. H. Morrison. Sample preparation of animal tissues and cell cultures for secondary ion mass spectrometry (SIMS) microscopy. *Biol. Cell* 74, 31-42, 1992).

Fig. 2. Simple tools used for fracturing cell cultures grown on the silicon substrate under liquid nitrogen in sandwich freeze-fracture method. Reproduced from reference ²⁸ (S. Chandra, G. H. Morrison, and C. C. Wolcott. Imaging intracellular elemental distribution and ion fluxes in cultured cells with ion microscopy: A freeze-fracture methodology. *J. Microsc.* 144, 15-37, 1986).

Fig. 3.

Recognition of fractured renal epithelial LLC-PK₁ cells in freeze-dried samples with a reflected light microscope: Fractured cells can be recognized on silicon substrates from their clean appearance. (a) A low magnification reflected light Nomarski image illustrating a fractured area within the boundary highlighted by a dotted line, containing hundreds of fractured cells; (b) a high magnification reflected light microscopy image shows individual cells with discernible nucleus, nucleolus, and cytoplasmic structures.

Fig. 4. SEM images of fractured freeze-dried renal epithelial LLC-PK₁ cells (a, b). An arrow in image (a) points to a spacer bead in a fractured area. In image (b) sister chromatids can be recognized in some metaphase chromosomes of the dividing cell. The thread-like cytoplasmic structures plausibly represent mitochondria in cells.

Fig. 5. SIMS imaging of potassium, sodium, calcium, and carbon in fractured freeze-dried control renal epithelial LLC-PK₁ cells (panels a-d). Individual images of $^{39}\text{K}^+$ and $^{23}\text{Na}^+$ were integrated on the CCD camera for 0.4 s and $^{12}\text{C}^+$ and $^{40}\text{Ca}^+$ for 2 min.

Fig. 6. SIMS imaging of potassium, sodium, calcium, and platinum in fractured freeze-dried renal epithelial LLC-PK₁ cells treated with 6 μM cisplatin for 4 hr. (panels a-d). Individual images of ³⁹K⁺ and ²³Na⁺ were integrated on the CCD camera for 0.4 s and ⁴⁰Ca⁺ and ¹⁹⁵Pt⁺ for 2 min. Regions marked in the figure by arrows and numbers are individual cells measured and described in the text and in the Table 1.

Table 1

Total K, Na, and Ca concentrations calculated from SIMS ion microscopy images of single cells shown in control (Fig. 5) and cisplatin-treated (Fig. 6) renal epithelial LLC-PK₁ cells. Concentrations in control cells are listed as the mean $\pm$ S.D. for all cells shown in Fig. 5. For cisplatin-treated cells, concentrations are listed in individual cells identified by the cell numbers in Fig. 6. Total Ca concentrations reflect the level of calcium stores in designated subcellular compartments

Treatment	Total Ca (mM)		Cellular K (mM)	Cellular Na (mM)
	<u>Nucleus</u>	<u>Cytoplasm</u>		
Control cells	0.51 $\pm$ 0.07	1.03 $\pm$ 0.16	168 $\pm$ 13	15 $\pm$ 3
Cisplatin				
Cell 1	0.80	1.74	139	91
Cell 2	0.44	0.69	179	12
Cell 3	0.48	0.71	183	14
Cell 4	0.77	1.63	97	96
Cell 5	0.45	0.76	146	16
Cell 6	0.81	1.68	133	73
Cell 7	0.53	0.87	157	33
Cell 8	0.75	1.47	133	72
Cell 9	0.82	1.45	128	71