行政院國家科學委員會專題研究計畫　成果報告

飛行式二次離子質譜儀(TOF-SIMS)在人體肝細胞中重金屬中毒的離子影像分析研究

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Imaging of single liver tumor cells intoxicated by heavy metals using ToF-SIMS

Abstract

Human liver tumor cells intoxicated with five different Cd, Cu, Cr, Hg, and Zn metals were analyzed using imaging time-of-flight secondary ion mass spectrometry (ToF-SIMS) to visualize their distributions in a single cell basis. A protocol was developed by combining rapid freezing, freeze-fracture, and imprinting for transferring the intoxicated cells to a silicon wafer. As shown in the ToF-SIMS images, the cellular morphology was preserved, which indicates that this protocol can be used to prepare a representative cell for ToF-SIMS imaging analysis. Among the five metal ions studies, our results indicate Cr and Cu ions preferentially diffuse into the cell after simulated intoxication.

Keywords: Liver cancer, Tumor cells, Heavy metals, ToF-SIMS, Imaging

Introduction

Living cells display rapid molecular responses when they are exposed to environmental and physiological stresses. Among these stress, the ubiquitous and persistent heavy metals are of particular interest due to their dual roles, both essential and lethal, in a biological systems, and pose a unique dilemma to cells. Heavy metals can function as essential co-factors (mostly as structural and catalytic components) for a wide range of the biochemical processes or even as signal transducers, and yet these same metals can be extremely toxic to cells at higher concentrations [1]. Some metals, such as arsenic and cadmium, currently display unknown cellular function but toxic effects. Other metals, such as copper, iron, and zinc are essential for normal cellular function, but become toxic under certain circumstances and/or above particular concentrations. [2,3]

To understand the cellular response to heavy metals, it is essential to identify and localize intracellular metal ions. Among the spectroscopic imaging techniques for biological samples, both dynamic [4] and static SIMS [5] imaging may become a useful tool for cancer research. Due to its central role in metabolism and its sensitivity to environmental pollutants, liver has always been the focus of toxicological study of heavy metals. Little is known about the effects of the heavy metals upon live tumor cells. The implication of heavy metals to the occurrence heptoma cells has inspired to conduct this study. To our best knowledge, this is the first such attempt using ToF-SIMS imaging analysis to locate he intoxicating metal ions in a single cell basis. Another aim of this research is to develop a protocol by combining rapid freezing, freeze-fracture, and imprinting technique modified from published methods [6,7] to prepare human liver tumor cells, i.e., HepG2 cell line, for ToF-SIMS imaging. Morphological and biological factors were both used to evaluate the feasibility of the protocol.

2. Experimental

2.1. Preparation of heavy metal ion solutions

Cd, Cu, Cr, Hg and Zn heavy metals were used as intoxicating agents to HepG2 cells. The respective intoxication solution was prepared by dissolving appropriate amount of $CdCl_2$, $CuSO_4$, K_2CrO_4, $Hg(NO_3)_2$, or $ZnSO_4$, respectively in de-ionized water to four different concentrations, i.e., 5, 10, 15 and 20 μM. Therefore, there were 20 different sets of experiments conducted in this study, with a 24-celle plate used in each set.

2.2. Preparation of HepG2 cells

HepG2 cells were placed on sterilized silicon wafer and cultured in Dulbecco's modified Eagle medium (DMEM; Trace MultiCel) containing 10% (v/v) fetal bovine serum (FBS; Trace MultiSer) or Hepatozyme- SFM (Gibco-BRL) supplemented with 4 m*M* L-glutamine (Gibco-BRL). The HepG2 cells were immersed in individual intoxication solution for 48 h at 37℃. To prevent the distortion of the cell morphology, published methods [6,7] were consulted and modified. A piece of silicon wafer was placed on top of the HepG2 cells destined to be fractured for making a 'sandwich' sample. The sandwiched samples were then subjected to rapid freezing and freeze-fracture under liquid nitrogen. A schematic plot of this sandwich process was illustrated in Fig.1. The fractured samples were freeze-dried overnight at room temperature prior to ToF-SIMS imaging.

Precautions are taken to preserve the cell in their native condition for ToF-SIMS imaging. First, the biological activity of HepG2 must be quenched rapidly by rapid freezing. Next, the HepG2 imprint must be introduced into the vacuum chamber of ToF-SIMS as soon as possible to minimize the exposure to ambient moisture.

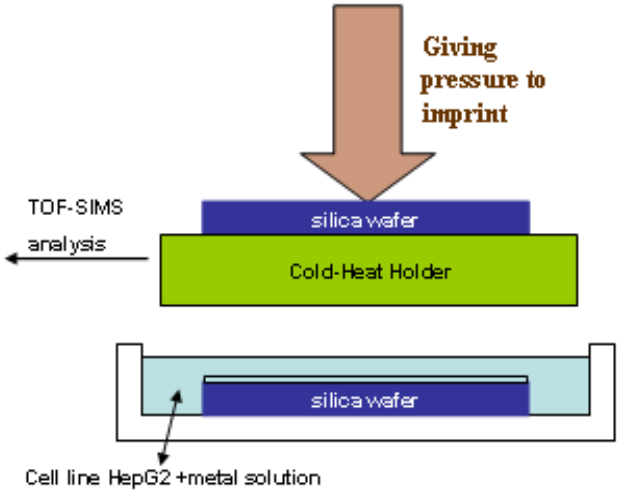

Fig. 1 Imprint HepG2 cells to a silicon wafer

2.3. ToF-SIMS analysis

The ToF-SIMS data were recorded on a ToF-SIMS IV instrument (ION-TOF GmbH, Germany). A focused primary ion beam of 25 keV Ga^+ ions was used at a typical current of 0.5-1 pA and with a 20-100 ns pulse width, oriented 45^o to the sample. The data were recorded by scanning the primary ion beam over an analysis area (50×50 μm^2 to100×100 μm^2) and stored in a raw data file for retrospective extraction of the selected ionic images and of spectra from the selected areas within the analysis area. Data of high-resolution mass spectra ($m/\triangle m$ = 2000-5000; lateral resolution, 3-4 μm) and high lateral resolution images (lateral resolution 0.5 μm, $m/\triangle m$ = 200-1000) were recorded separately to ensuring that the accumulated ion dose in the imprint was kept below the so-called static limit, 1×10^{13} ions/cm^2.

3. Results and discussion

Figure 2 shows two typical sets of ToF-SIMS images of the intoxicated HepG2 cells. The shape, size and surface densities of the circular structures in the TOF-SIMS images reveal that they originate from the HepG2 cells. Upon inspection of the K^+ and Na^+ ion images, we found the relatively low levels of intracellular Na^+ and relatively high levels of intracellular K^+, which indicates that the cells are healthy prior to sample pretreatment. Our result indicates the cells remain intact after the pretreatment process.

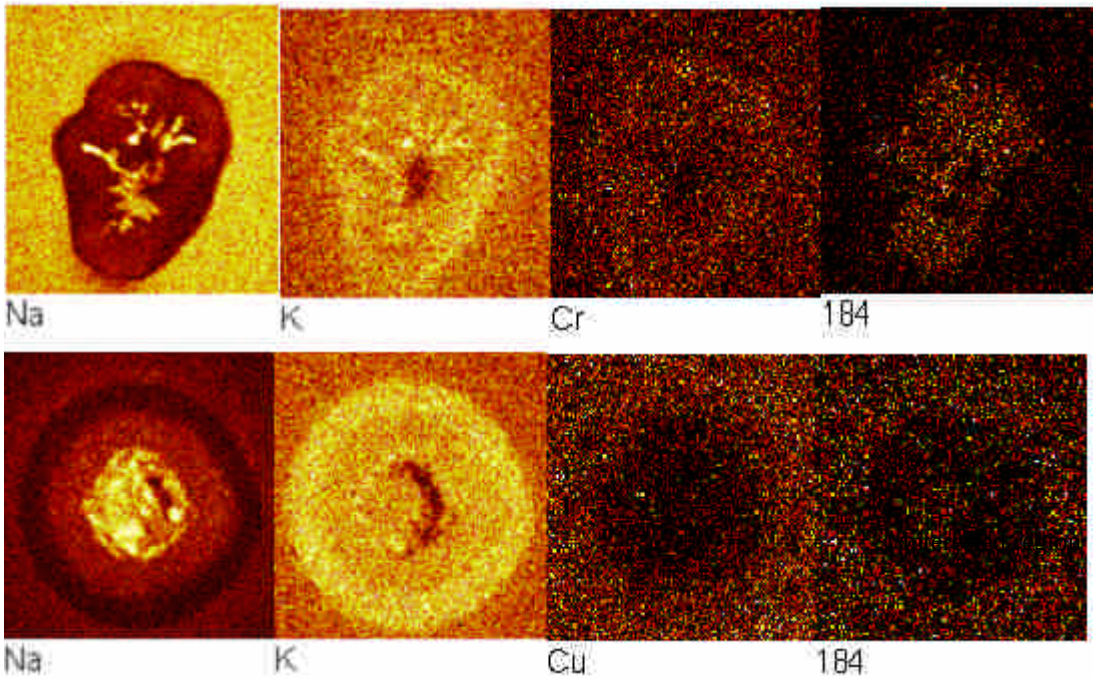

Fig. 2 Positive ion ToF-SIMS images of a single as-prepared HepG2 cell. Those images in the upper row are the images of a HepG2 cell immersed in K_2CrO_4 solution and the lower ones are in $CuSO_4$ solution, both with 15 μM concentration.

The peak at 184 u (Fig. 3a) is attributed to the PC ($C_5H_{15}NPO_4^+$) head-group [8] and is consistently present in every intoxicated cell, supporting the feasibility of the developed protocol. From ToF-SIMS images, a higher concentration of Cr ions (Fig. 3b) was found in the cell with simulated Cr intoxication, compared to the lower concentrations of Cr ions were found in the cells for the other simulated metal intoxications. The similar observation was found in the case of Cu ions (Fig. 3c). These indicate that Cr and Cu ions preferentially diffuse into the cell after the specific simulated intoxication. We were able to detect the Zn (Fig. 3d) ions inside the cells but the intensities of the signals were similar for all the simulated metal intoxications. We cannot exclude the observed signals of Zn ions as background signal. However, the signals of Cd (Fig. 3e) ions inside the cells were too low to detect and some alternative method should be adapted. As to Hg ions, HgH (Fig. 3f) ions were measured but the observations were unable to distinguish the signals from the background, similar to the case of Zn ions. In order to enhance the sensitivity of the detection, some alternative measurement for the particular metal ions should be adapted, such as the fragments associated with the metal ions. The identification of those peaks is also under investigation.

4. Conclusions

This study demonstrates that static SIMS imaging of elemental and molecular ions provides the foundation for a valuable insight into biochemical compositions of the intoxicated HepG2 cells. The proposed protocol of combining rapid freezing, freeze fracture, and imprint onto a Si wafer has been proved to be a valid preparation process for liver tumor cells before ToF-SIMS imaging. Future work on applying similar experimental conditions to normal liver cells is under investigated. The result would provide new insight about the different responses between the normal and tumor liver cells due to heavy metal intoxication.

5. Acknowledgements

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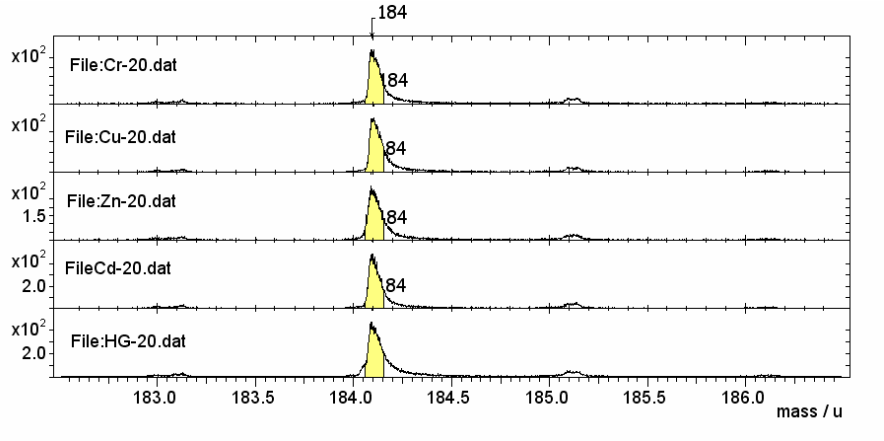
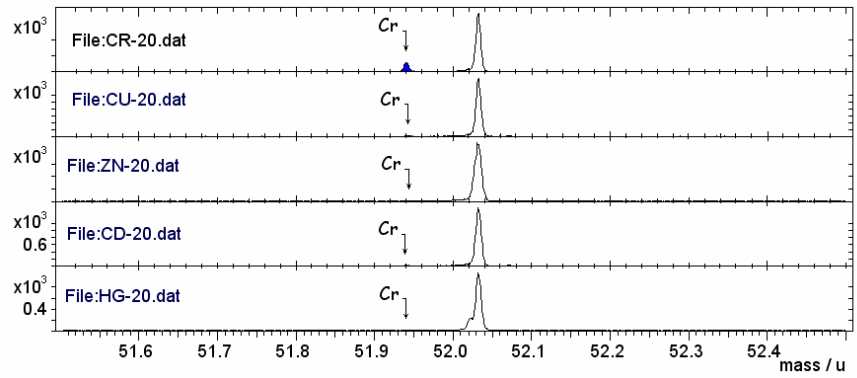
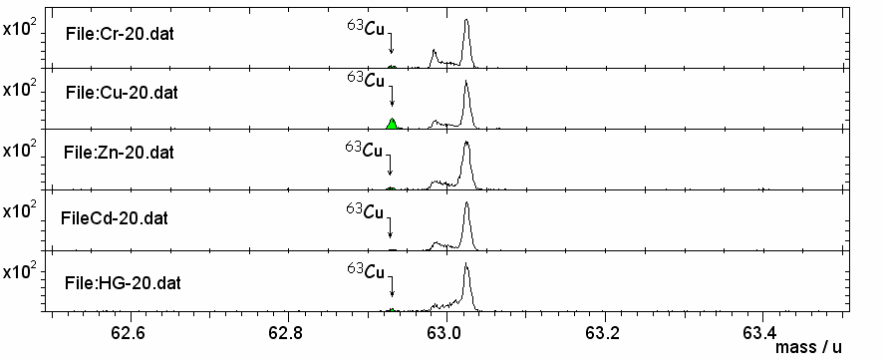
(a)



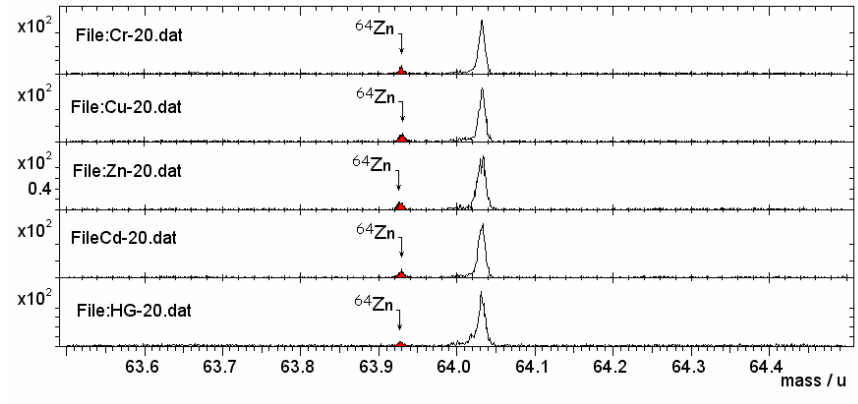
(b)



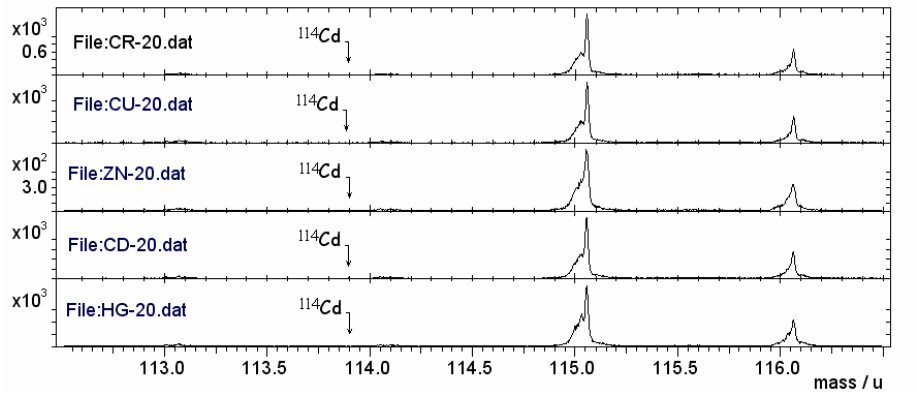
(c)



(d)



(e)



(f)

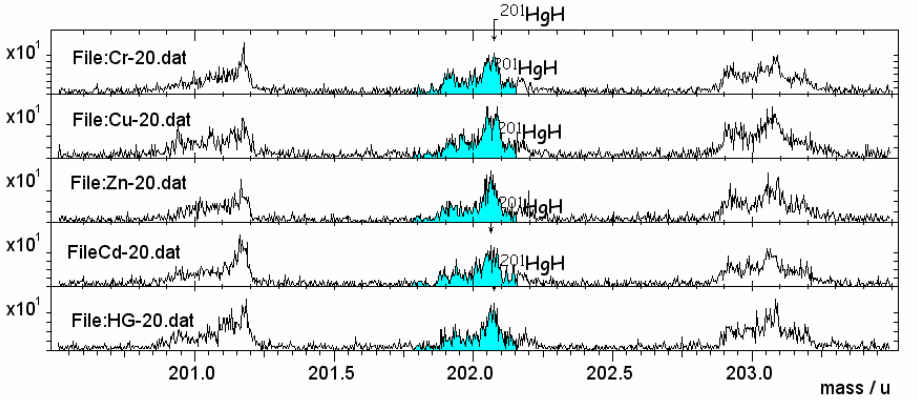


Figure 3 ToF-SIMS mass spectra of (a) PC head-group, (b) Cr, (c) Cu, (d) Zn, (e)Cd and (f) HgH ions.

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