行政院國家科學委員會專題研究計畫 成果報告

利用分子轉印技術在人體腎臟細胞中重金屬中毒的飛行式二次離子質譜儀(TOF-SIMS)離子影像分析研究
研究成果報告(精簡版)

計 畫 類 別 ：個別型
計 畫 編 號 ：NSC 94-2113-M-040-002-
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計 畫 主 持 人：麥富德

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處 理 方 式 ：本計畫可公開查詢

中 華 民 國 96 年 01 月 27 日

附件一

行政院國家科學委員會補助專題研究計畫 □V成果報告 □期中進度報告

（計畫名稱）

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計畫編號：NSC94－2113－M －040 －002－
執行期間：2005 年 08 月 01 日至 2006 年 10 月 31 日

計畫主持人：麥富德
共同主持人：
計畫參與人員： 陳柏榮、陳俊豪、江妮臻、楊筱蕙

成果報告類型(依經費核定清單規定繳交)：V精簡報告　□完整報告
本成果報告包括以下應繳交之附件：
□赴國外出差或研習心得報告一份
□赴大陸地區出差或研習心得報告一份
□出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

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執行單位：中山醫學大學應用化學系

中　華　民　國　96 年　01　月　27　日

Imaging of Human Liver and Kidney Cell Intoxicated by Heavy Metal Through Time-of-flight Secondary Ion Mass Spectrometry (ToF-SIMS)

Abstract Human liver cell lines HepG2 and kidney cell lines HEK-293 were intoxicated by five different heavy metals. Both of HepG2 and HEK-293 were analyzed by spatially resolved time-of-flight secondary ion mass spectrometry (ToF-SIMS) to directly visualize the distribution of metal ions on a single cell basis. In order to enhance the lateral resolution, a protocol was developed by combining the freeze-fracture sample pretreatment with molecular imprinting techniques to transfer the intoxicated cells to a silicon wafer. HepG2 and HEK-293 were immersed in solutions containing six different heavy metal ions of various concentrations and time durations to simulate the intoxication process. Reliable imaging data library associated with the metal intoxication of HepG2 and HEK-293 were created as a reference for future studies. We have quantified the metal concentrations as well as depth profile of the HepG2 and HEK-293 on a single cell basis. This study can pave the way to study the time-evolution damage to the HepG2 and HEK-293 by heavy metals.

Keywords： HepG2、 HEK-293 、Heavy metals 、ToF-SIMS、 freeze-fracture 、molecular imprinting imaging.

Introduction

Living cells display rapid molecular responses when they are exposed to the environmental and physiological stresses. Among these stresses, the ubiquitous and persistent heavy metals are of particular interest due to their dual roles, both essential and lethal, in a biological system, 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 the same metals can be extremely toxic to cells at high concentrations [1]. Some metals, such as arsenic and cadmium, currently display not only some unknown cellular functions but also toxic effects. Other metals, such as copper, iron and zinc, are essential for normal cellular functions, but become toxic under certain circumstances and/or above particular concentrations [2, 3].

To understand the cellular response toward 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 sensitivity to environmental pollutants, both liver and kidney have always been the focus for the toxicological studies of heavy metals. Little is known about the effects of the heavy metals upon live tumor cells. We use human kidney cells intoxicated by heavy metals, and compare with the pridian study of human liver cells. Another aim of this research is to develop a protocol by combining rapid freezing, freeze-fracture and imprinting technique modified from the published methods [6,7] to prepare human liver and kidney tumor cells, i.e., HepG2 cell line and HEK-293 cell line, for ToF-SIMS imaging. Morphological and biological factors were both used to evaluate the feasibility of the protocol.

Experimental

A、Preparation of heavy metal ion solutions

Cd, Cu, Cr, Hg, Pb and Zn heavy metals were used as intoxicating agents to HepG2 and HEK-293 cells. The respective intoxication solution was prepared by dissolving the appropriate amount of $CdCl_2$, $CuSO_4$, K_2CrO_4, $Hg(NO_3)_2$ $ZnSO_4$ or $Pb(NO_3)_2$, respectively, in de-ionized water with four different concentrations for HepG2, i.e., 5, 10, 15 and 20 μM, and four different concentrations for HEK-293, i.e., 10, 20, 50 and 100 μM, Therefore, there were 40 different sets of experiments conducted in this study, with a 24-cell plate used in each set.

B、Preparation of HepG2 cells and HEK-293 cells

Both HepG2 and HEK-293 cells were placed on a 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) supple-mented with 4 mM L-glutamine (Gibco-BRL). And the HEK-293 cells were placed on a sterilized silicon wafer and cultured in minimum essential medium (MEM) containing 10% (v/v) horse serum with 4 mM L-glutamine (Gibco-BRL). The HepG2 cells and HEK-293 cells were then immersed in the individual intoxication solution for 48 h at 37°C. To prevent the distortion of the cell morphology, published methods [6,7] were consulted and modified. A piece of the silicon wafer was placed on top of the HepG2 cells and HEK-293 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 is illustrated in Fig. 1. The fractured samples were freeze-dried overnight at room temperature prior to ToF-SIMS imaging.

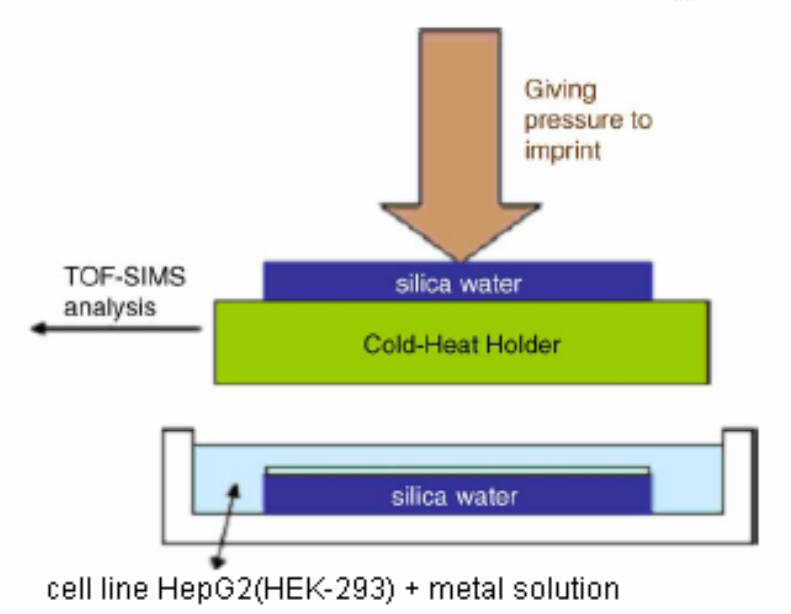

Fig. 1 Imprint HepG2 and HEK-293 cells to a silicon wafer.

Precautions are taken to preserve the cell in their native condition for ToF-SIMS imaging. First, the biological activity of HepG2 and HEK-293 must be quenched rapidly by rapid freezing. Next, the HepG2 and HEK-293 cells imprint must be introduced into the vacuum chamber of ToF-SIMS as soon as possible to minimize the exposure to ambient moisture.

C、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 45º to the sample. The data were recorded by scanning the primary ion beam over an analysis area (50μm × 50μm to 100μm × 100μm) and stored in a raw data file for the 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/Δm = 2000-5000; lateral resolution, 3-4 mm) and high lateral resolution images (lateral resolution 0.5 mm, m/Δm = 200-1000) were recorded separately to ensure that the accumulated ion dose in the imprint was kept below the so-called static limit, 1×10^{13} ions/cm^2.

Results and discussion

Fig. 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 the 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 results indicate the cells

remain intact after the pretreatment process. Fig. 3 shows two of ToF-SIMS images of the intoxicated HEK-293 cells, we found the levels of intracellular Na^+ is much higher than K^+, which indicate that the HEK-293 cells died before ToF-SIMS analysis.

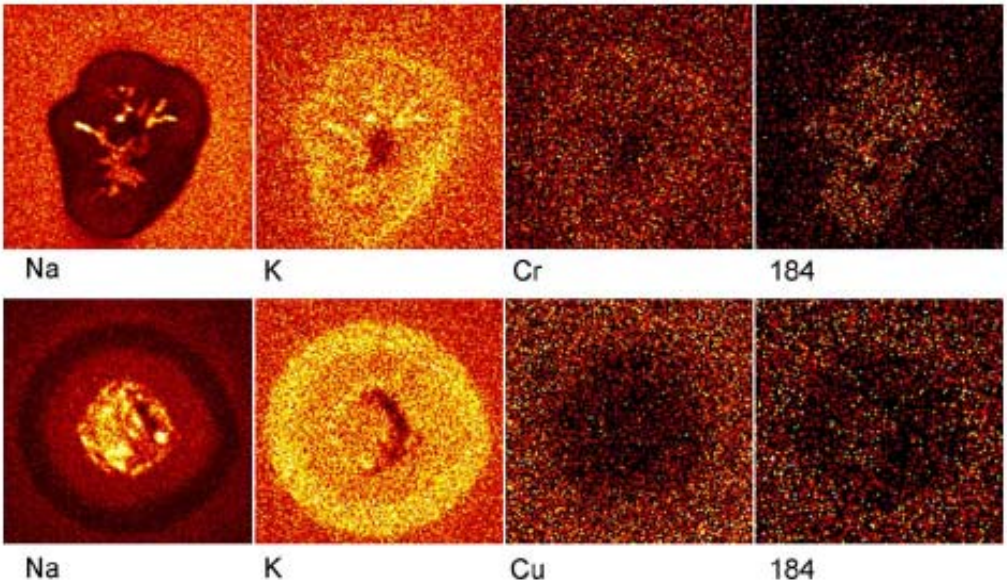

Fig. 2 Positive ion ToF-SIMS images of a single as-prepared HepG2 cell. Those images in the upper row are the images of a HepG2 cell immersed in K_2CrO_4 solution and the lower ones are in $CuSO_4$ solution, both with 15 μM concentration.

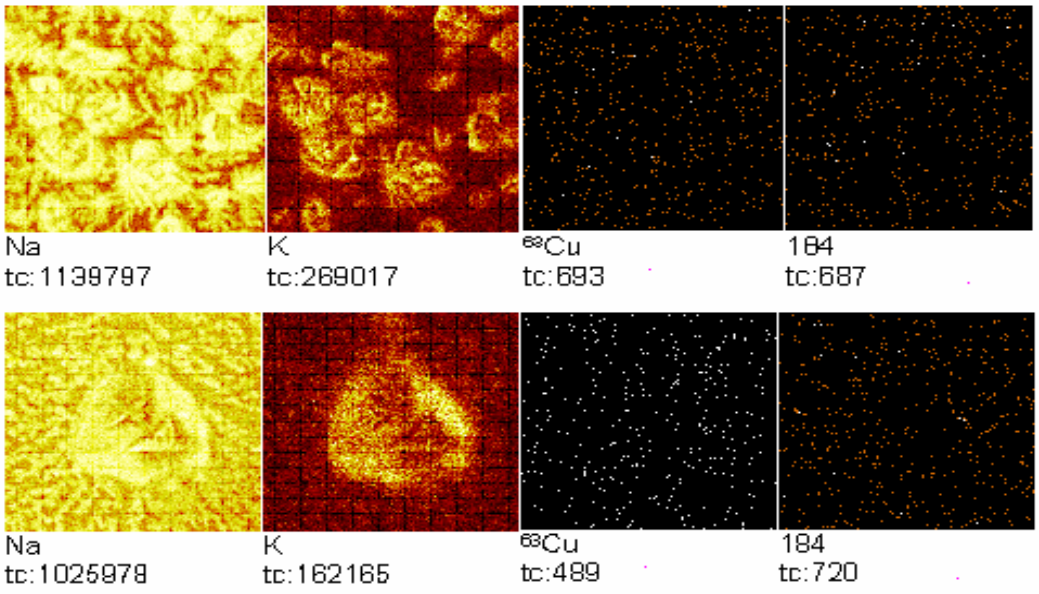

Fig. 3 Positive ion ToF-SIMS images of as-prepared HEK-293 cells. Those images in the upper row are the images of HEK-293 cells immersed in K_2CrO_4 solution with 20 μM concentration. and the lower ones are in $CuSO_4$ solution with 50 μM concentration.

The peak at 184 mm (Fig. 4a) 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. 4b) 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. 4c). These indicate that Cr and Cu ions can preferentially diffuse into the cell after the specific simulated intoxication. We were able to detect the Zn (Fig. 4d) 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. 4e) ions inside the cells were too low to be detected and some alternative method should be adapted. As to Hg ions, HgH (Fig. 4f) 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 measurements for the particular metal ions should be adapted, such as the fragments associated with those metal ions. The identification of those peaks is also under investigation. In Fig. 5, We cannot exclude the observed signals of Pb, Cd and Hg ions as background signal and the signals of Cr and Cu ions inside the cells were too low to be detected.

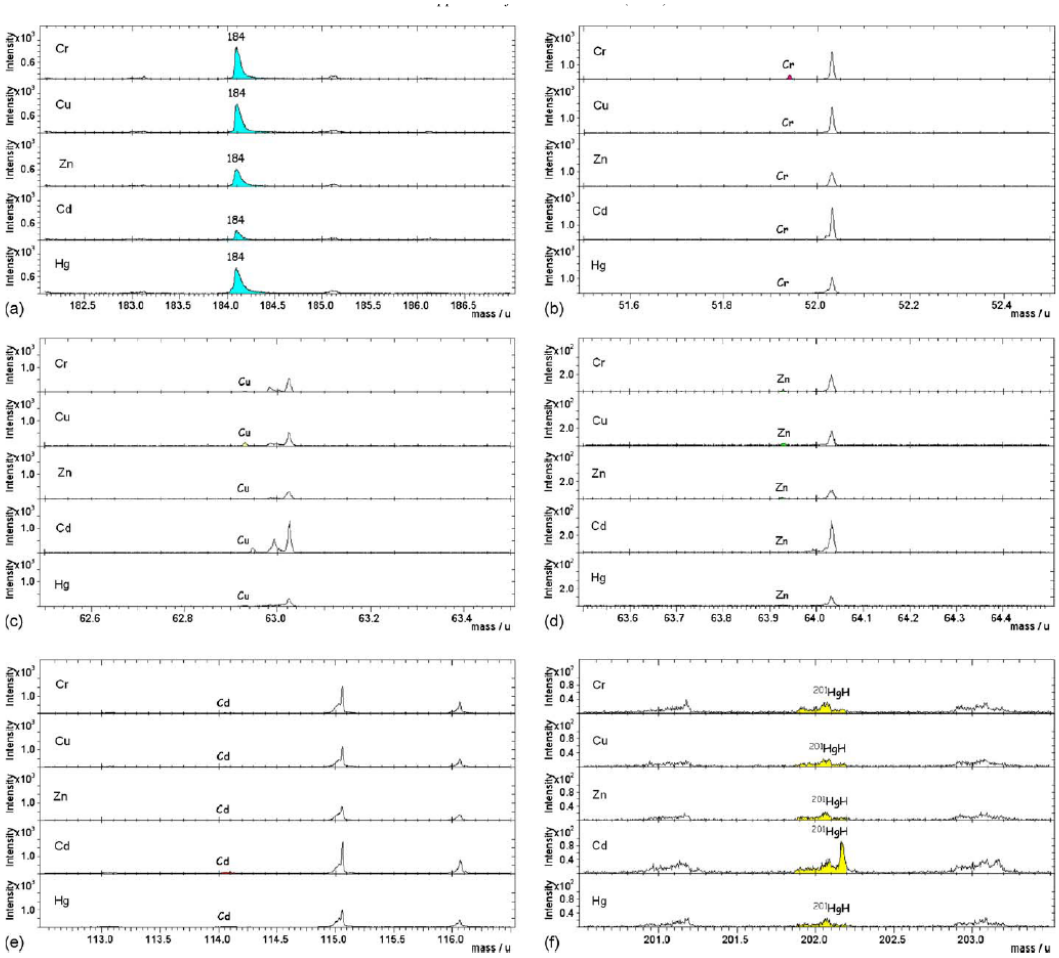

Fig. 4. ToF-SIMS mass spectra of (a) PC head-group, (b) Cr, (c) Cu, (d)

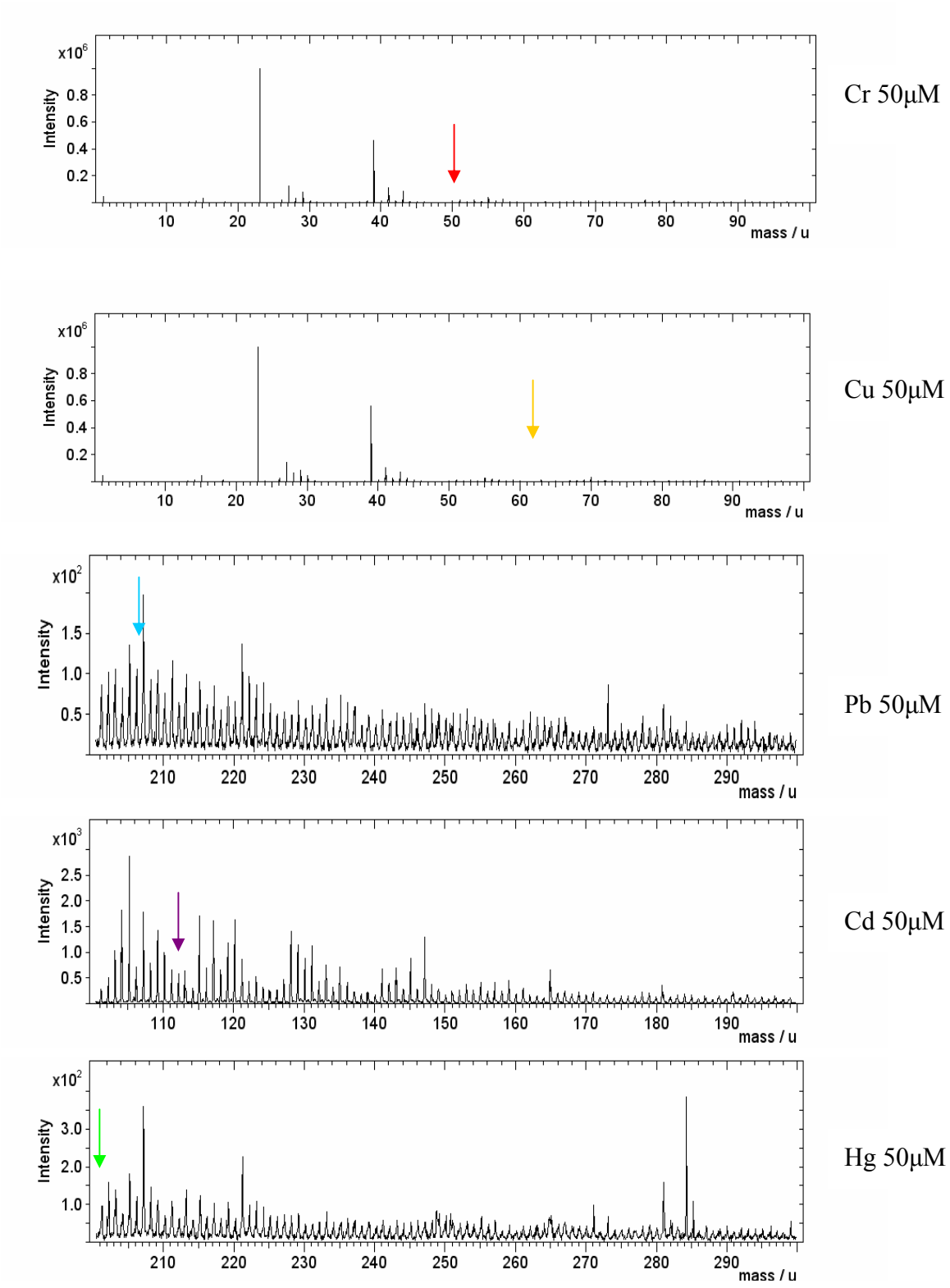

Fig. 5 ToF-SIMS mass spectra of Cr, Cu, Pb, Cd and Hg ions.

Conclusions

This study demonstrates that the static SIMS imaging of elemental and molecular ions can provides sound foundation for a valuable insight into the biochemical compositions of the intoxicated HepG2 cells. But the results of HEK-293 cells are not so good. We suppose that the kidney prefers to excrete rather than to absorb heavy metals.

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 the liver tumor cells before ToF-SIMS imaging. Future work on applying similar experimental conditions to normal liver cells is now under investigated. The result would provide a new insight about the different responses between the normal and tumor liver cells due to heavy metal intoxication.

References

1. S.J. Lippard (1993) Science 261: 699–700.
2. J. Koropatnick (1995) M.E.I. Leibbrandt, Effects of metals on gene expression, in:Toxicology of Metals, CRC Press, Springer, Berlin.
3. Agency for Toxic Substances and Disease Registry, Public health statement,(1989).
4. S. Chandra (2003) Appl. Surf. Sci. 203: 679.
5. N.P. Lockyer, J.C. Vickerman (2004) Appl. Surf. Sci. 231: 377.
6. S. Chandra, G.H. Morrison (1992) Biol. Cell 74:31.
7. T.P. Roddy, D.M.S.G. Ostrowski, N. Winograd, D.M. Cannon Jr., A.G.Ewing (2002) Anal. Chem. 74: 4020.
8. P. Sjovall, J. Lausmaa, H. Nygren, L. Carlsson, P. Malmberg (2003) Anal. Chem.75: 3429.