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Comparison of Bi_1^+ , Bi_3^+ and C_{60}^+ primary ion sources for ToF-SIMS imaging of patterned protein samples

Manish Dubey^{1,2}, J. Brison^{1,2}, David W. Grainger^{4,5}, and David G. Castner^{1,2,3,*}

¹ National ESCA and Surface Analysis Center for Biomedical Problems, Box 351750, University of Washington, Seattle, WA 98195-1750 USA

² Department of Chemical Engineering, Box 351750, University of Washington, Seattle, WA 98195-1750 USA

³ Department of Bioengineering, Box 351750, University of Washington, Seattle, WA 98195-1750 USA

⁴ Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, Salt Lake City, UT 84112-5820 (USA)

⁵ Department of Bioengineering, University of Utah, Salt Lake City, UT 84112-5820 (USA)

Abstract

Imaging time-of-flight secondary ion mass spectrometry (ToF-SIMS) has been used to study protein bound to a photolithographically-patterned, commercial poly(ethylene glycol) (PEG)-based polymer film. The effect of different ion sources on the fragmentation pattern from this sample was analyzed with respect to the surface sensitivity of characteristic protein fragments and contrast in the ion images. The method demonstrates that, under similar fluence (below the static limit), Bi_3^+ provides better surface sensitivity for low mass fragments and the best image contrast as compared to Bi_1^+ and C_{60}^+ cluster sources. Principal component analysis (PCA) was utilized to process depth profiles for this sample and shows that a primary ion fluence of approximately 20×10^{12} ions/cm² is required to etch through the adsorbed protein layer.

Keywords

ToF-SIMS imaging; principal component analysis; image contrast; depth profile; surface analysis

Introduction

Recent advances in the field of ToF-SIMS, especially the introduction of cluster ion sources, have resulted in improved capabilities due to the enhanced secondary ion yields produced by primary cluster ions.[1,2] These advances have also resulted in an increased use of ToF-SIMS to study biological systems.[3] There has been much interest in this area as different ion sources produce differences in fragmentation patterns, spectral yields and efficiencies, as shown in studies that used multiple ion sources.[4] These advancements have also improved ToF-SIMS surface imaging capabilities, especially in biological applications.[5] ToF-SIMS imaging is especially attractive as a label-free technique, unlike fluorescence microscopy and immunochemistry methods where specific molecules must be tagged and targeted for analysis. We have recently reported the use of ToF-SIMS imaging to study high-fidelity

*Corresponding Author: castner@nb.engr.washington.edu.

surface patterns of functional groups on photo-lithographically-patterned, commercial polyethylene glycol (PEG)-based polymers.[6] We have also demonstrated that two different ligands co-patterned onto these non-fouling hydrogel surfaces can self-select their respective protein partners from a mixed solution. In this case, ToF-SIMS imaging was used to distinguish and map specific amino acids fragments originating from the two proteins.[7]

The aim of this work is to investigate the quality of ToF-SIMS images obtained with Bi_1^+ , Bi_3^+ and C_{60}^+ primary ion sources. A number of groups have studied and compared the spectral yields, damage cross sections, efficiencies etc. between various ion sources on test samples such as Irganox or self-assembled monolayers.[8–10] The current study is focused on examining ToF-SIMS imaging characteristics on a more complex surface: biological patterns on a polymeric substrate relevant to applications in biomedical diagnostics, microarray technologies, device miniaturization and drug discovery.[11] Differences in secondary ion yields and image contrast from Bi_1^+ , Bi_3^+ and C_{60}^+ primary ion sources will be discussed. Depth profiles on this substrate using the C_{60}^+ ion source were also collected and processed using PCA.

Experimental

Photolithographic Patterning of NHS Reactive Ester Polymer Coatings

The process used to fabricate amine-reactive N-hydroxysuccinimide (NHS) surface patterns on commercial polymer substrates by conventional photolithography has been detailed elsewhere and it has shown to produce alternating NHS- and methoxy (MeO)-terminated polymer coating patterns.[6,12] Amine-derivatized biotin (EZ-link, Cat. No. 21346, Pierce Biotechnology, Rockford, IL) was reacted with the NHS pattern and subsequently exposed to streptavidin (Prozyme No. SA-10, San Leandro, USA) to obtain patterned streptavidin samples that were then transferred to ultra-high vacuum for ToF-SIMS analysis.

ToF-SIMS Analysis of Patterned Surfaces

ToF-SIMS data were acquired on an ION-TOF 5–100 instrument (ION-TOF GmbH, Münster, Germany) equipped with a 25 kV LMIG source and a 10 kV electron impact source, both with an incident angle of 45 degrees to the surface. Ion images were acquired using Bi_1^+ , Bi_3^+ and C_{60}^+ primary ions in the high current bunched mode (i.e., high mass resolution mode). All data were collected by rastering the beams over a $500 \times 500 \mu\text{m}^2$ area on the sample surface while keeping the fluence below the SIMS static limit of 1×10^{12} ions/ cm^2 . All images contained 128×128 pixels. A low-energy electron beam was used for charge compensation on the polymer surface samples. The mass resolution ($m/\Delta m$) of positive secondary ion spectra was typically between 7000 and 8500 for the $m/z = 27$ peak for the Bi_3^+ and Bi_1^+ primary ions, whereas it was between 2500 and 4000 for the C_{60}^+ primary ions. Depth profiles were acquired in the dual-beam mode with Bi_3^+ for analysis and C_{60}^+ for etching. The analysis beam was rastered over a $500 \times 500 \mu\text{m}^2$ area that was centered inside the $1000 \times 1000 \mu\text{m}^2$ C_{60}^+ crater. Depth profiles were acquired in the non-interlaced mode (analysis and sputtering beams active in different ToF-cycles) by alternating imaging sequence (30 scans, 1.8×10^{10} Bi/ cm^2) and etching sequence (3.2 seconds, 2×10^{12} C_{60} / cm^2). For PCA, peaks were selected from m/z 1 to m/z 200; these peaks were mean-centered and normalized to the total ion intensity. PCA was then performed on this dataset as described previously using a series of scripts written by NESAC/BIO for MATLAB (MathWorks, Inc., Natick, MA). [13,14]

Results and Discussion

ToF-SIMS has been used to characterize proteins and peptides on various substrates by studying the ion fragments originating from constituent amino acids.[15,16] In the present

study, Bi_1^+ , Bi_3^+ and C_{60}^+ sources were used to collect secondary ion images from polymer surfaces patterned with proteins. Characteristic amino acid peaks originated only from the streptavidin regions of the sample, consistent with our previous studies.[6,7] This produced ToF-SIMS discrimination of the surface patterns. To compare the surface sensitivity of the three primary ion species, spectra were regenerated from $100\text{ }\mu\text{m} \times 100\text{ }\mu\text{m}$ areas within the streptavidin regions and PCA was utilized to highlight the differences among the spectra. PCA, a statistical tool used to analyze ToF-SIMS data from complex samples such as those in this study, identifies differences between spectra and which fragments, or fragment intensity differences, are responsible for those differences. The peak list used for analysis of the Bi_1^+ , Bi_3^+ and C_{60}^+ spectra included characteristic peaks from the streptavidin region (i.e., amino acids fragments) as well as the methoxy-capped region (i.e., PEG polymer fragments) as described previously.[7] Figure 1(a) shows the principal component 1 (PC1) vs. PC2 plot from nine streptavidin regions (three from each source). A clear separation is observed in PC1, due mainly to positive loadings of the polymer peaks. The amino acid fragments do not load either positively or negatively in PC1. PC2 (14% variance) shows an interesting separation wherein fragments corresponding to Bi_3^+ load negatively while fragments corresponding to C_{60}^+ and Bi_1^+ load positively. In this case the PC2 loadings plot shows most of the amino acid fragments load negatively, whereas fragment peaks characteristic of the underlying PEG polymer substrate load positively. Although the variance in PC2 is small (14%), it shows that the Bi_3^+ source produces fragment signals from the top-most protein layer more selectively than the Bi_1^+ and C_{60}^+ sources. This indicates that the energy, crater size, escape depth of intact molecules, and other parameters are optimum for Bi_3^+ source. PC2 does not provide sufficient separation for Bi_1^+ and C_{60}^+ spectra to compare their sensitivity for protein-specific fragments. This same observation is obtained by comparing the ratio of protein to polymer fragment intensities produced by the three ion sources (data not shown).

To quantify image contrast in the ToF-SIMS images, three ion fragment peaks were chosen from the PC analysis -- $\text{C}_2\text{H}_5\text{O}$ (characteristic of the underlying polymer), $\text{C}_4\text{H}_8\text{N}$ and $\text{C}_8\text{H}_{10}\text{NO}$ (both characteristic of streptavidin) -- to contrast the performance of the different ion sources. Ion images for these three fragments produced by the three ion sources used are shown in Figure 2a. The contrast for these images were calculated as described in our earlier publication.[7] Figure 2b shows the calculated contrast for each image in Figure 2a. These results clearly indicate that the Bi_3^+ source provides the best image contrast for these patterned samples. However, it should be noted that the images from two Bi sources have spatial resolutions (both ca. $4\text{ }\mu\text{m}$) that are significantly better than the images from the C_{60} source (ca. $30\text{ }\mu\text{m}$). The noticeably better spatial resolution of the Bi sources probably contribute to the improved contrast obtained with those sources. However, operating the C_{60} source in a mode that would provide a similar spatial resolution to the Bi sources, results in mass resolutions that are insufficient to separate the protein peaks from the polymer peaks. This would completely eliminate image contrast.

Because of its improved contrast and good surface sensitivity compared to Bi_1^+ , the Bi_3^+ beam source was used as the analysis beam for dual beam 3-D imaging of the patterned sample. C_{60}^+ was used as an etching beam for these experiments. For the surface under investigation, streptavidin sits on top of a polymeric substratum layer spin-coated onto a glass (silicate) substrate. Figure 3a shows the depth profile in the streptavidin region for characteristic fragments from the protein ($\text{C}_4\text{H}_8\text{N}$), polymer ($\text{C}_5\text{H}_4\text{O}_2$), and substrate (Si). It is clearly evident that initially the protein fragment (top-most layer) intensity decreases while the polymer fragment (intermediate layer) intensity increases with increasing C_{60}^+ fluence. Both fragment intensities decrease while the substrate fragment (Si) increases for C_{60}^+ fluences above $\sim 20 \times 10^{12}\text{ ions/cm}^2$.

PCA was used to estimate the fluence required to etch through the overlying protein layer. Figure 3b show the PC1 (98% variance) and PC2 (2% variance) scores for spectra generated after each C_{60}^+ etching cycle. The peak list for this analysis consisted of all fragments between m/z 1 and m/z 200. The sigmoidal-shaped PC1 curve (98% variance) indicates the presence of three components, as expected. For PC1, the substrate fragments along with the polymer fragments load positively, while some amino acid fragments load negatively. For PC2, the protein and substrate peaks load in the negative region while the polymer fragments load in the positive region. This results in the PC2 scores plot exhibiting a maximum in the polymer region. This plot indicates that a fluence of approximately 20×10^{12} ions/cm² and 90×10^{12} ions/cm² (indicated by the vertical lines in Figure 3b) are required to remove the protein and polymer layer, respectively.

Conclusions

ToF-SIMS imaging of biological systems is gaining increased attention with notable new progress. This study compares both the surface sensitivity and ion-generated image contrast for three different primary ion sources (Bi_1^+ , Bi_3^+ and C_{60}^+) for analysis of patterned biological samples. Bi_3^+ was the most surface-sensitive and also provided the best image contrast. Dual beam (Bi_3^+ analysis and C_{60}^+ etching) 3-D depth profiling was capable of distinguishing the layered nature of the patterned samples (protein/polymer/glass) and estimate the ion fluences required to etch through the overlying protein and polymer layers.

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Figure 1.

(a) PC1 vs. PC2 scores plot for ToF-SIMS data collected from three different ion sources, which clearly discriminates data from all ion sources. (b) The PC2 loadings plot showing the amino acids peaks load with the spectra from the Bi_3^+ source.

Figure 2.

- (a) Ion fragment images for fragments characteristic of methoxy ($\text{C}_2\text{H}_5\text{O}^+$) and streptavidin ($\text{C}_4\text{H}_8\text{N}^+$, $\text{C}_8\text{H}_{10}\text{NO}^+$) regions, respectively, as acquired using three different ion sources.
 (b) Image contrast calculated for each image in (a)

Figure 3.

(a) Surface depth profile from the streptavidin regions of the patterned sample for fragments characteristic of protein (C_4H_8N), polymer ($C_5H_4O_2$) and substrate (Si). (b) PC1 and PC2 scores plots for spectra from the streptavidin region of the patterned samples produced after each C_{60}^+ etching cycle.