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MOLECULE SPECIFIC IMAGING OF HUMAN RED BLOOD CELLS USING TOF-SIMS

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1. Introduction

Time of flight secondary ion mass spectrometry (TOF-SIMS) has been employed to image membrane chemicals on freeze-fractured, frozen-hydrated human red blood cells. Red blood cells (erythrocytes), mainly due to their wide availability and uniform size, have been well characterized using a variety of techniques [1]. Static TOF-SIMS analysis of red blood cells demonstrates the feasibility of studying membrane-related events such as exocytosis (release of chemicals from a cell through vesicle fusion with the outer cell membrane) on cells of a small size (~8 μm). In addition, chemical imaging using mass spectrometers capable of molecular recognition provides a direct way of chemically probing the cell without the need for labeling with isotopes or other atoms, dopants, or dyes.

In order to fully exploit the power of static TOF-SIMS imaging, we have chosen to use freeze-fracturing and frozen-hydrated analysis for our studies. The rapid freezing of cells causes all cellular processes to be halted, creating a snapshot of the cell chemistry, so the chemical distribution of the living cell is preserved [2]. Conversely, natural cell death or slow freezing, is a dynamic process in which chemical reactions and relocalizations change the chemical picture of the cell [2]. Preparations such as freeze drying with chemical fixation, which can give excellent results for scanning electron microscopy experiments, are known to cause diffusion of chemicals within the cells [3]. A further advantage of studying frozen-hydrated samples is that volatile compounds, lost with other vacuum methods, are retained.

2. Experimental

Red blood cells (>48 days old) were obtained from the American Red Cross. The cells were rinsed in phosphate buffered saline (PBS) and centrifuged twice at 100 g for two minute cycles. At the end of each cycle, the yellowed buffer solution and the middle layer of liquid (light red) which contained the lysed cells were removed. The "washed" cells were then resuspended in PBS in a 1:2 ratio and mixed with 8 μm polystyrene beads that serve to aid in fracturing. The bead/cell suspension was then used for freezing.

Two types of sample holders were used for these experiments. The first consisted of two Si wafers between which the suspension was placed in a sandwich- type of formation. The second was a small metal disk (approximately 1 mm) which was dimpled in the center. The suspension was placed in the holder in droplet form. Samples were rapidly frozen in liquid propane condensed at liquid nitrogen temperature. They were then placed in liquid nitrogen for storage and were stored for up to six months.

The red blood cell samples were fractured in a home-built vacuum preparation chamber [4]. A cold knife held at approximately liquid nitrogen temperature was used for fracturing. The sandwich-type samples were fractured by prying the two Si wafers apart with the cold knife. Droplet-type samples were fractured by contacting the frozen droplet with the knife to initiate the fracture plane, and then moving the sample away from the knife to break off the top of the sample. The sample temperature was approximately -105°C at the time of fracturing.

Imaging was performed on a Kratos Prism mass spectrometer. The Ga^+ ion source was incident at approximately 45° to the sample surface. The 25 kV Ga^+ ions were pulsed to a time of 16-30 ns. The reflectron-based flight tube had a path length of 4.5 m. Charge compensation was achieved with a 30 eV electron flood gun pulse for 50 μs fired 50 μs after the primary ion pulse. The primary ion spot size was 200 nm and the ion current was 500 pA. The sample was held at liquid nitrogen temperature during analysis.

3. Results and Discussion

It is imperative to carefully choose the fracturing conditions that produce artifact-free surfaces to accomplish imaging of frozen-hydrated cells with static SIMS analysis. The fracturing conditions were specifically chosen to occur immediately after sublimation begins, so sublimation begins to dominate over condensation as the sample warms in vacuum. If the temperature of the sample is too cold (< -110°C), and condensation is the main process, then a freshly fractured surface is immediately covered with many layers of water from the residual water vapor in the vacuum chamber. The water can cover all species of interest related to the cellular matter and is identified by a spectrum dominated by water-based clusters. A sample fractured at an ideal temperature (approximately -105°C) still exhibits evidence of water in its spectrum, but the water peaks no longer dominate. Instead, a variety of other species related to the cells, including many low-mass hydrocarbon peaks and several high mass peaks of interest, can be seen. Water was still observed in the spectrum due to the natural water content of cells and the surrounding media. In contrast, a sample that is fractured at a temperature that is too warm (approximately -95°C) contains no observable water related peaks, and even more importantly, images from these samples display little or no chemical heterogeneity. The reason for the chemical homogeneity is that the nonvolatile components of the media and cells do not sublime in vacuum. Therefore they are left behind and can form a layer of salts and other debris thick enough to cover cell components. In fact,

fracturing at a temperature that is too warm essentially freeze dries the sample surface.

When a fracture plane passes in close proximity to a cell, it can be exposed at the sample surface in different ways. The first way is that the cell can merely be uncovered. It is similar to "peeling" off the media layer on top of the cell. This fracture is an important type of fracture in membrane-based studies because it exposes the outside membrane of the cell. The polar headgroups exposed at the surface are thought to impart function to a membrane, and this type of fracture brings them directly to the surface for analysis. A second type of exposure occurs when the cell is sliced through the middle. In this case, the cytoplasm and organelles of the cell are exposed.

Characteristic images of cells fractured through the middle are displayed in figure 1. Figure 1a is a total ion image of an area which is representative of the area depicted in the SIMS images (figure 1b-e). Note the cross-section of the biconcave-disk cell shape of the cell in the upper left of the image a. Figure 1b shows the water chemical map. The water is homogenous across the surface of the image in contrast to C, CH, CH_2, CH_3 (C_1 Hydrocarbons) and sodium (both not shown) and C_5H_9 (an intense ion, 1c), potassium (1d) and C_2 hydrocarbons (1e). Since there is very little membrane exposed to the ion beam, the spectrum from this area shows no evidence of membrane molecules. A similar fracture, on cells that contain organelles would undoubtedly expose the organelles in some instances for ion beam analysis.

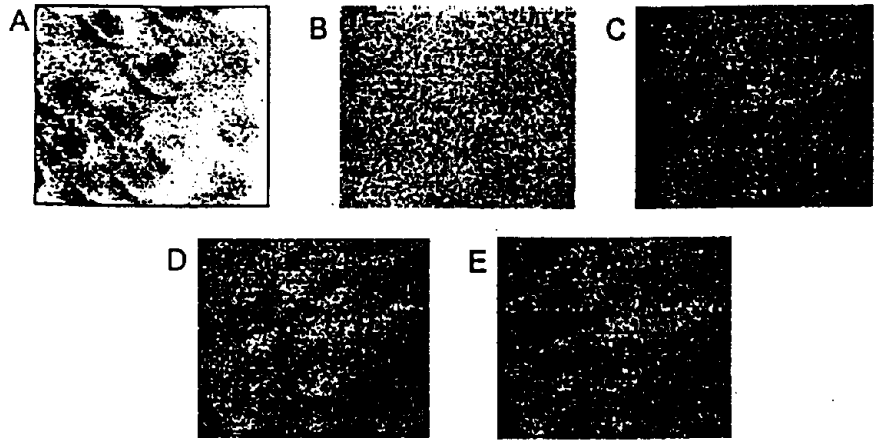

Figure 1. Freeze-fractured frozen-hydrated red blood cells fractured across the middle. A. Total ion image of representative area, (B-E of second area) B. Water, C. C_5H_9. D. Potassium, E. C_2 Hydrocarbons Field of view is 50 μm. (+SIMS mode)

Chemical morphology in figure 1 is proven because water (1b) is homogeneous compared to C_5H_9, C_2 hydrocarbons and potassium (figure 1c-e). If the chemical localization in figure 1c-e were due to surface morphology (bumps on the surface enhancing the SIMS yield), the water image would show increased intensity in the same areas.

Characteristic images of cells fractured in the "peeling" scenario can be seen in figure 2. This type of fracture is more common than the fractures through the middle of cells. Erythrocytes appear round with a dimple in the middle as seen in the total ion image in 2a. The Na signal is depicted in figure 2b and shows several holes. The high Na signal around the "peeled" cells is most likely from the Na-based PBS buffer solution. The "holes" in the Na images or areas of low intensity are in direct contrast to the phosphocholine headgroup intensity (figure 2c), much like a photographic negative. The phosphocholine headgroup originates from sphingomyelin, phosphatidylcholine or other choline-containing phospholipids, all of which have been documented as significant components of the protoplasmic membrane leaflet of the human erythrocyte [5].

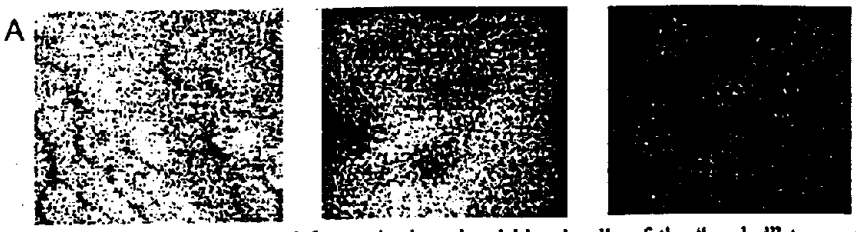

Figure 2. Freeze-fractured frozen-hydrated red blood cells of the "peeled" type of fracture. A. Total ion image Field of View is 50 μm., (B-E of second area) B. Sodium. C. Phosphocholine headgroup (m/z 184) Field of View is 66 μm. (+SIMS mode)

4. Conclusions

Membrane-based imaging offers chemical information from unmodified cells which has typically been very difficult to obtain directly. Distribution of membrane compounds, both homogeneous and heterogeneous, could indicate if a cell is undergoing exocytosis or is under membrane rearrangement. Furthermore, chemical imaging of internal organelles and vesicles could indicate their origin or function within the cell. Future research will involve the study of excoytosis model cell lines of neurons. Membrane-based imaging studies could lead to a greater understanding of chemical communication between cells.

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