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Static Time-of-flight Secondary Ion Mass Spectrometry Imaging of Freeze-fractured, Frozen-hydrated Biological Membranes

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The study of cell membrane lipid and steroid composition and distribution is important for the understanding of membrane dynamics and function. Here we present efforts to chemically image phospholipid distributions on a submicron scale on freeze-fractured and frozen-hydrated liposomes and red blood cells using time-of-flight secondary ion mass spectrometry. Sample preparation by freeze fracturing of membranes is described. Fragments representative of phospholipid headgroups are found to be localized on both liposomes and red blood cells. In addition, the cholesterol molecular ion [M+H] is localized on liposome surfaces. © **1998 John Wiley & Sons, Ltd.**

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The advent of imaging secondary ion mass spectrometry (SIMS) has opened new opportunities for the chemical mapping of biological cells. Presently these experiments are quite popular using dynamic SIMS, where the probe ion beam possesses a high enough flux ($>1 \times 10^{13}$ primary ions/cm^2) such that the sample surface is eroded in time.[1–3] These studies have successfully probed the near-surface spatial distribution of atomic species and small molecular fragments, although beam damage obliterates most of the molecular information.

In recent years, it has become clear that with time-of-flight detection, sufficient sensitivity is available to acquire spatially resolved images in the static SIMS mode, where the primary ion flux is less than 10^{13} ions/cm^2.[4–7] In this mode, a plethora of molecular information is available in the mass spectra, adding a new dimension to the challenge of characterizing biomaterials. To acquire meaningful molecule specific images, a new range of sample preparation issues present themselves.

Most importantly, samples must be characterized in the high vacuum environment of the mass spectrometer. To overcome this obstacle, scanning electron microscopy (SEM) and dynamic SIMS experiments commonly employ freeze drying. This strategy may be adequate to study unvarying conditions in the cell. Our experiments have suggested, however, that to maintain intrinsic chemical localization of small molecules and to study changing cell processes, fast freezing followed by freeze fracture of frozen-hydrated samples is required.

Freeze fracturing is particularly challenging for static SIMS due to the inherent sensitivity of the signal to surface composition. Water condensation onto samples held at cryogenic temperatures in vacuum can easily mask the presence of target chemicals. Some of these issues have been recently addressed through imaging of single paramecia exposed to cocaine.[6] In these studies, it was shown that the cellular membrane with associated cocaine molecules could be observed if the fracture temperature of the frozen sample was adjusted to be high enough to prevent water condensation, but low enough to inhibit freeze etching of the freshly exposed surface, similar to techniques used in the cryo-SEM community to prepare some replicas.[8]

In this paper, we further extend these procedures to characterize frozen-hydrated, freeze-fractured biological cells using imaging TOF-SIMS by examining two membrane systems;[4,5] liposomes, where the membrane composition can be controlled,[9] and human red blood cells, where the membrane chemistry is well characterized.[10] Liposomes are typically spheres of one or more layers of lipid bilayers. They can be synthesized without proteins and cholesterol, although both may be included. The interior of the liposome contains an aqueous solution. Since the membrane composition and the content of the inner aqueous core are controllable, liposomes are much simpler than whole cells that contain organelles, chemical gradients, proteins and complex chemistry. Red blood cells are well suited to this study since they are also well defined systems that do not contain internalized membranes, are of a uniform size (about 8 μm) and are easy to obtain.

Our results show that both synthetic and naturally occurring cellular membranes may be freeze-fractured to expose their surfaces such that they are directly amenable to molecule-specific imaging TOF-SIMS experiments. Liposomes constructed from phosphatidylcholine dipalmitoyl and phosphatidyl-*n*-monomethylethanolamine dipalmitoyl and cholesterol are presented to illustrate this localization. Moreover, we show images acquired from freeze-fractured red blood cells which yield chemical maps of the choline derived fragment at *m/z* 86.

EXPERIMENTAL

Mass spectrometric imaging is performed on a Kratos (Manchester, UK) PRISM time-of-flight secondary ion mass spectrometer. Ion formation is achieved with a pulsed

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25 kV Ga^+ primary ion beam incident at approximately 45° to the surface normal. The Ga^+ ion beam spot size used in these experiments is 200 nm and the current was 50 pA. The resulting secondary ions are mass analyzed in a reflectron-based time-of-flight analyzer with a path length of 4.5 m. The typical mass resolution of the analyzer is 4000 with 50% transmission efficiency. The primary ion beam is subsequently rastered over the surface. Imaging is accomplished by selecting masses of interest and recording their intensities with respect to the position of the primary ion beam. A pulse of 30 eV electrons fired for 50 μs after each Ga^+ pulse provides charge compensation. The target stage is cooled to approximately liquid nitrogen temperature.

Samples are prepared by plunge freezing in rapidly stirred liquid propane. (Extreme caution must be used when handling liquid propane. An explosion-proof hood should be used and care should be taken to avoid frostbite.) The sample is a sandwich of two Si pieces with the liposome or cell suspension placed between them. The upper piece of Si is a thin strip, and the lower piece is a square. The sandwich is grasped between metal forceps with insulated handles and manually plunge frozen in liquid propane. The samples are then stored in liquid nitrogen.

Liposomes are prepared by dissolving lipids and other components, if desired, in an organic solvent and evaporating the solution to dryness, under vacuum, on the sides of a round-bottomed flask. Water is then added to the flask and swirled for several minutes. This preparation creates multilamellar liposomes with varying dimensions of up to 100 μm in diameter.[9] Single component liposomes containing one phospholipid and multicomponent liposomes containing one or more phospholipids and/or cholesterol may be synthesized in this fashion.

Red blood cells are obtained from the American Red Cross and are more than 48 days old. The cells are suspended in phosphate buffered saline (PBS) and centrifuged twice at 100*g* for two minute cycles. The yellowed supernatant, which contains the buffer and damaged cells, is removed after each centrifugation. The 'cleaned' cells are then resuspended in PBS buffer in a 1:2 ratio with 8 μm polystyrene beads (Duke Scientific, Palo Alto, CA, USA). The beads serve to act as spacers so the cells are not crushed between the Si wafers and help to initiate cleavage planes during fracturing, since they are approximately the same size as the red blood cells.[11]

RESULTS AND DISCUSSION

Several steps must be taken to achieve the goal of molecular imaging of membranes in their native state. First, cell processes must be rapidly and simultaneously stopped by fast freezing. Next, the frozen sample must be introduced into the vacuum chamber and exposed to the ion beam with as little contamination from water as possible due to the surface sensitivity of static SIMS. The sample preparation steps are therefore crucial to obtaining images representative of membrane surfaces.

To preserve the native state of a cell, it is imperative to freeze the cell quickly ($>10^4$ °C/s) to stop cell processes as fast as possible.[12] Manual plunge freezing in liquid propane is a very rapid method of freezing (1×10^4 °C/s).[13] In contrast, chemical fixation is a slow process, occurring on a time scale of seconds to minutes, and therefore should only be used to study static conditions in a cell.[14] Rapid freezing is desired since the faster a sample is frozen, the closer the ice will be to an amorphous state.[12] Crystals growing in the ice due to slow freezing may result in the reorganization of salts at the edge of the crystals, and as they grow in size, crystals may damage the physical structure of the cell.[12]

Fracturing of the frozen sample in vacuum is necessary to produce an artifact-free surface. Freeze fracturing is a process whereby a cleavage plane is initiated in a frozen sample, and the sample is split along that plane.[8] Cutting or microtoming using a cold knife is not an acceptable method for exposing surfaces for static SIMS because the knife may drag across the surface and smear, or otherwise displace, chemical species. In addition, the knife may cause local heating due to friction as it moves over high spots on the surface as it cuts.[15] Fracturing at the proper temperature and pressure is essential to successfully prepare a frozen-hydrated sample for static SIMS analysis. The conditions must be as close as possible to those of a 'no-etch' situation where there is no net water build up or removal after fracturing.

If a frozen-hydrated sample is fractured at a temperature that is too warm, the water at the surface of the sample will rapidly sublime. Even though the extent to which the surface is dried is unknown, only the top few molecular layers are of significance to the static SIMS probe and hence, the sample will appear to be completely dried. A dried surface may indicate that the cells have been damaged due to the rapid water loss. Fracturing at conditions that are too cold results in the freshly fractured surface being covered with residual water from the vacuum chamber as water condenses on the cold surface. If the temperature of fracturing is at a point where the forces of sublimation and condensation are at equilibrium, the fractured sample will be exposed to vacuum with minimal contamination from the chamber (a 'no-etch' situation).

Although freeze drying would be a simpler preparation for cells in vacuum because they could be analyzed at room temperature, freeze drying of a surface can cause redistribution of the molecules present in the cells or liposomes as water is removed.[16] The nonvolatile components of the media and cells remain behind when the water sublimes, coating cells with a homogenous layer of salts and other debris. The 'water vapor wind phenomenon' also implies that molecules can move across a surface in the 'wind currents' of the subliming water as the sample is dried in vacuum.[15] In addition, freeze drying is inherently a state that is not native to the cell or liposome because both naturally contain water.

Previous studies in our laboratories of paramecia[6] utilized a brass sample holder with a central raised portion (Balzers, Hudson, NH, USA). In the center of the raised portion, there was a dimple (approximately 1 mm in diameter and 0.7 mm deep) that held the droplet of cell suspension. Fracturing was accomplished by bringing a cold knife up to the sample to initiate the cleavage plane, and then moving the sample away from the blade to remove the upper, fractured piece of frozen cell suspension. Using this procedure, atomic ions (Na^+, K^+, Ca^+) and small hydrocarbon species were observed.[6] Paramecia were then doped with dimethyl sulfoxide (DMSO) and cocaine hydrochloride to develop molecular imaging techniques. In these cases, DMSO and cocaine molecular ions were imaged in the paramecia.[6]

Here we employ a sandwich-type holder which has been shown to produce satisfactory results for liposome suspensions.[5] These devices consist of a 5 mm by 5 mm Si wafer of which about 18 mm^2 is covered by fractured liposome

25 kV Ga^+ primary ion beam incident at approximately 45° to the surface normal. The Ga^+ ion beam spot size used in these experiments is 200 nm and the current was 50 pA. The resulting secondary ions are mass analyzed in a reflectron-based time-of-flight analyzer with a path length of 4.5 m. The typical mass resolution of the analyzer is 4000 with 50% transmission efficiency. The primary ion beam is subsequently rastered over the surface. Imaging is accomplished by selecting masses of interest and recording their intensities with respect to the position of the primary ion beam. A pulse of 30 eV electrons fired for 50 μs after each Ga^+ pulse provides charge compensation. The target stage is cooled to approximately liquid nitrogen temperature.

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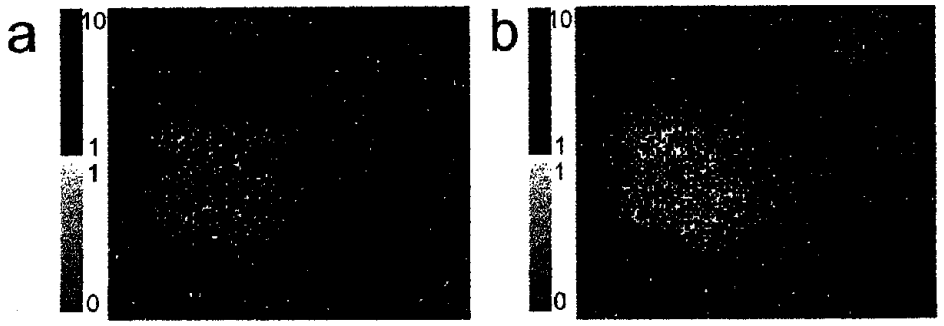

Plate 1. Freeze-fractured, frozen-hydrated phosphatidylcholine dipalmitoyl liposome. Field of view is 143μm by 117μm. (a) Water in blue (*m/z* 18, 19), phosphocholine fragment in yellow (*m/z* 166) (b) Water in blue (*m/z* 18, 19), phosphocholine headgroup in yellow (*m/z* 184) Note that the intensity of water is decreased over the liposome where the intensities of *m/z* 166 and 184 are greatest. +SIMS mode.

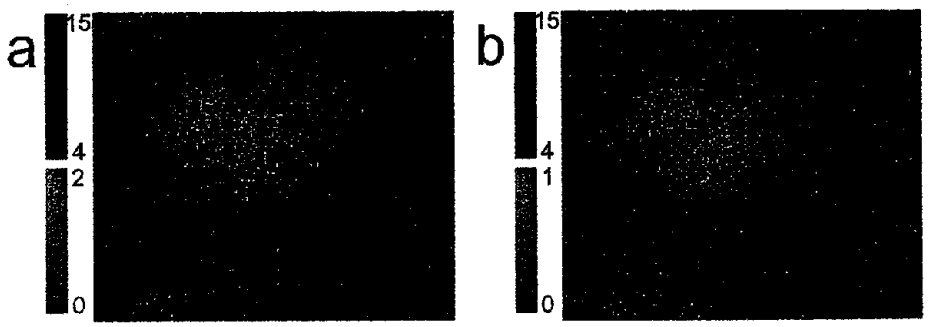

Plate 2. Freeze-fractured, frozen-hydrated phosphatidyl-*n*-monomethyl ethanolamine dipalmitoyl/cholesterol liposome. Field of view is 66μm by 54μm. (a) Water in blue (*m/z* 18, 19), phospho-*n*-monomethyl ethanolamine headgroup in green (*m/z* 156) (b) Water (*m/z* 18, 19) in blue, cholesterol molecular ion in green (*m/z* 386), +SIMS mode.

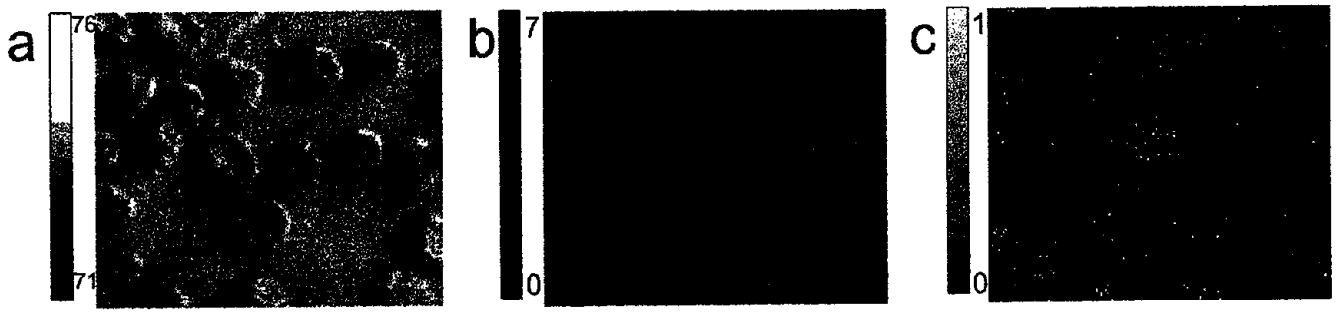

Plate 3. (a) Total ion image of freeze-fractured, freeze-dried red blood cells. Field of view is 50μm by 42μm. (b) and (c). Freeze-fractured, frozen-hydrated red blood cells. (b) Water in blue (*m/z* 18, 19) (c) Choline fragment in yellow (*m/z* 86) ($(CH_3)_3NCH_2CH$) Field of view 100μm by 84μm. +SIMS mode.

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