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MOLECULAR IMAGING OF FROZEN-HYDRATED MODEL MEMBRANE SYSTEMS

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1. Introduction

Biological membranes are integral components needed to sustain biological function. These membranes provide the necessary boundaries for individual cells and allow cellular components to interact with their environment. The chemical composition, as well as the spatial heterogeneity of membrane species, such as phospholipids, is of interest in the study of cellular function. Biochemical theories have proposed that the composition and the changes associated with the chemical species of the membrane impart function to cells [1]. One example of this is exocytosis, where vesicles (membrane bound packets of neurochemicals) inside a neuron fuse with the cellular membrane thereby releasing the vesicle contents into the extracellular space, allowing for neuronal communication to take place. Various areas of membrane study have extensively used model membrane systems, such as multilamellar and unilamellar liposomes, and have led to such innovations in fields such as drug delivery [2].

In this paper, static time-of-flight secondary ion mass spectrometry (TOF-SIMS) molecular imaging on liposome systems with cellular dimensions has been accomplished, showing the feasibility of investigating cell membranes. By using rapid freezing and freeze-fracturing techniques [3], snapshots of membrane chemical composition and location (before and after membrane events) can be studied. Frozen-hydrated liposomes have been used as a proof of concept model for single cell membranes where it is essential to preserve the native state of the membrane without introducing sample preparation artifacts. Sample preparation artifacts such as reduction of size or the movement of components of single cells due to dehydration, have been shown [4,5]. Molecular ions of membrane species and fragments unique to these various phospholipids (i.e. polar headgroups and non-polar fatty acids) and cholesterol have been identified allowing the molecular imaging of different frozen-hydrated liposome systems.

2. Experimental

Analysis was performed on a Kratos Prism TOF-SIMS spectrometer equipped with a gallium liquid metal ion gun with a pulsed 25 kV, 500 pA beam focused to a 200 nm spot. A liquid nitrogen (LN_2) cooled stage was used for analysis. The reflectron based time of flight path length was 4.5 m. Charge compensation was accomplished by an electron flood gun that pulsed a beam of

30 eV electrons for 50 μs after each ion pulse. Images were produced by individually recording numerous mass spectra across a rastered area of interest.

Liposomes were made by a rotary evaporation process [2]. Briefly, membrane components were dissolved in organic solvent, then rotary evaporated to dryness such that there was a thin coating on the flask. The membrane chemicals were then resuspended with doubly distilled water. Different phospholipids and cholesterol were used for liposomes of mixed composition. Liposome formation was verified by optical microscopy. A small drop of liposome suspension was placed on a 5 x 5 mm silicon wafer. A smaller piece of silicon was placed across the diagonal, on top of the liposome suspension. This sandwich was then immersed into liquid propane for several seconds and then stored in LN_2. The complete cold chain freeze-fracture method has been described in detail elsewhere [6].

3. Results and Discussion

Static TOF-SIMS analysis of standard phospholipid samples show characteristic ion fragments, such as phosphorylated headgroups and various tailgroups (i.e. monoglyceride and diglyceride ions) that have been reported previously [7,8]. Earlier studies from this laboratory reported on TOF-SIMS molecular mapping of frozen hydrated single paramecium cells [6]. These studies showed molecular mapping capabilities for a lipophillic dopant such as dimethyl sulfoxide (DMSO). However, no native membrane chemicals were identified, only small mass hydrocarbons.

Here, liposomes are used as a model membrane system to further study the sample preparation requirements since liposome composition is easily varied with a variety of components. The first liposomes analyzed are basic one-component samples consisting of phosphatidylcholine dipalmitoyl (DPPC). The results show the capability of molecular imaging for various fragment ions from model membranes with dimensions similar to single cells (10μm - 100μm).

Sample temperature during fracturing needs to be optimized in order to preserve the hydrated sample state and to minimize interference from residual vacuum water composition. The critical factor is the temperature at which molecules, especially water, begin to rapidly sublime in vacuum. Sublimation can be monitored via the pressure. The optimum temperature is around -105°C. If sample temperature is too cold during fracturing, water immediately condenses back down on the freshly exposed surface, interfering with SIMS analysis. When the sample temperature is too warm during fracturing, most, if not all, of the water is sublimed away. In the case of liposomes, water sublimation, and thus dehydration, damages the liposomes such that the phospholipid chemical images are homogeneous.

The second system of liposomes studied are two component systems that incorporated cholesterol because of the abundance in most cellular membranes. DPPC or phosphatidyl-N-mono-methylethanolamine dipalmitoyl (DPPNME) is mixed in a 1:1 molar ratio with cholesterol before dissolving in the above liposome preparation method. Cholesterol molecular ions as well as $[M\text{-}OH]^+$ are mapped along with the phospholipid molecular and fragment ions. These studies show that two different molecules within a membrane can be spatially mapped. Chemical

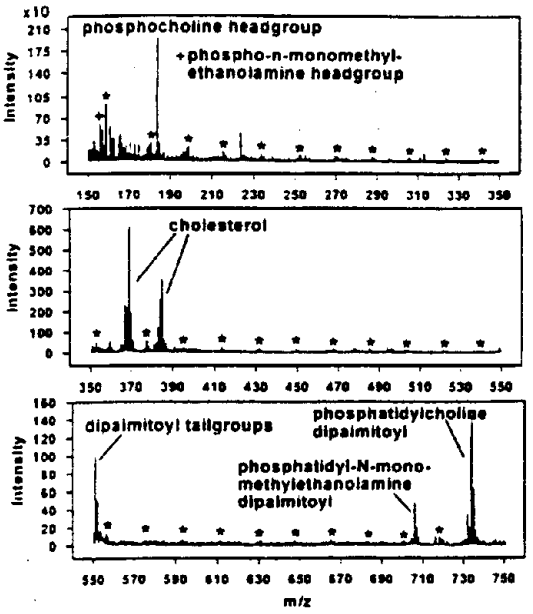

Figure 1. Positive mass spectrum of freeze-fractured frozen-hydrated DPPC/cholesterol liposomes mixed with DPPNME/cholesterol liposomes. Water clusters ($H_3O(H_2O)_n^+$) are indicated with *.

morphology, versus surface morphology, is confirmed by a homogeneous distribution of the small mass hydrocarbons (C^+, CH^+, CH_2^+, CH_3^+), and either a homogeneous image of water or a hole in the water corresponding to the signal from the liposome.

To further test TOF-SIMS ability to spatially analyze membrane components, two different liposomes systems (DPPC/cholesterol and DPPNME/cholesterol) are made separately and mixed before freezing. Figure 1 shows a representative spectrum. Headgroups, tailgroups, and molecular ions from both phospholipids, as well as cholesterol and its fragment are detected. Water is indicated by the clustering pattern appearing within the spectrum ($H_3O(H_2O)_n^+$).

Liposome images vary in molecular composition. Some liposomes show a single system consisting of cholesterol and one phospholipid, but not both. This would indicate mixing does not result in any exchange of material for those particular liposomes. However, several liposomes show a homogeneous distribution for all three components (DPPC/DPPNME/cholesterol) in the system perhaps from the collision induced redistribution of the molecules. A third case is also found and is shown in Figure 2. There is a clear demonstration of chemical spatial heterogeneity between the two phospholipid headgroups. This is possibly the result of a collision-induced fusion of two liposomes caught in the act of exchange. The decreased signal on the left side of the total ion and cholesterol images shows evidence of topography effects expected when two different sized liposomes are next to one another. However, the DPPNME headgroup image shows clear evidence for the capability of TOF-SIMS to image a heterogeneous distribution of membrane components in a frozen-hydrated system.

4. Conclusions

Fast-frozen model membrane systems, such as liposomes, have been used to demonstrate the feasibility of investigating frozen-hydrated membranes for both

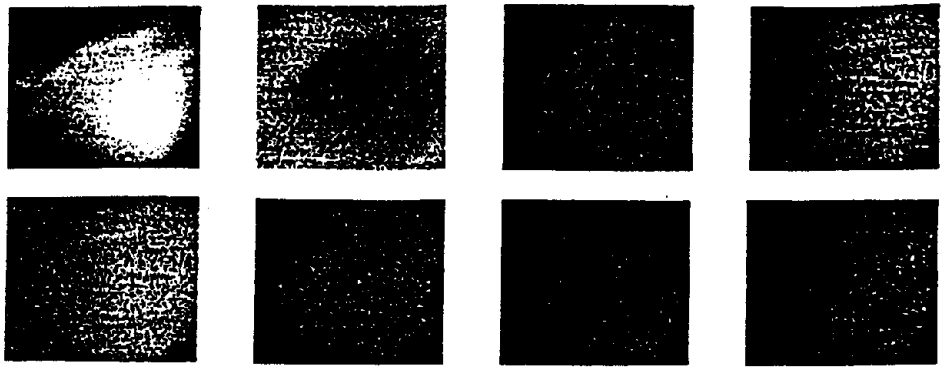

Figure 2. TOF-SIMS images of freeze-fractured frozen-hydrated DPPC/cholesterol liposomes mixed with DPPNME/cholesterol liposomes. Images (100μm x 100μm) are from left to right: (top): total ion (1-1220): water (18-19): DPPNME headgroup (156): DPPC headgroup (184): (bottom): cholesterol (382-387): dipalmitoyl tailgroup (550-553): DPPNME molecular ion (702-708): DPPC molecular ion (730-736). Nominal mass to charge ranges are indicated in parenthesis.

the molecular composition as well as the chemical spatial heterogeneity. Ongoing studies include variation of liposome composition and time dependent mixing studies to further investigate and enhance TOF-SIMS ability to spatially resolve membrane heterogeneity. Future studies will include the analysis of cell lines that are model systems for exocytosis events from neurons.

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