LIPID-SPECIFIC IMAGING OF MEMBRANE DYNAMICS USING MASS SPECTROMETRY

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1. Introduction

Biological membranes are essential in defining cellular boundaries and sustaining cellular functions [1]. However, a lack of total understanding concerning the vast complexity of membranes still remains, especially at the molecular level. The fluid mosaic model of membranes has been extended to recognize the importance of chemical domains in influencing membrane shape and function [2]. Many cellular functions, such as fusion (i.e. exocytosis) and fission (i.e. cell division and endocytosis), rely on membrane dynamics and are likely to be dependent upon formation of membrane domains. To allow fusion of two cellular membranes, a transient local alteration from the bilayer structure is required. In model systems, the hexagonal (H II) cylinder structure, observable under certain conditions, has been implicated as an intermediate model for localized membrane deformations that result in contact points between two interacting bilayers [3]. Factors that induce H II lipid polymorphism, such as calcium and cholesterol, are also important in dynamic membrane events. Chemical specific domains to create the proper environment for the inducement of the H II intermediate structures have also been proposed. However, it is still unclear whether chemical heterogeneity associated with the two merging or diverging membranes drives these fundamental membrane processes.

Molecule-specific imaging mass spectrometry is uniquely suited for spatially resolving the distribution of native molecular surface species at the micrometer scale. Time-of-flight secondary ion mass spectrometry (TOF-SIMS) molecular imaging has been carried out in our laboratories on model membrane systems [4-6]. Preliminary work suggests that it is feasible to characterize domains of specific lipid molecules [5], to assess their molecular orientation [6] and to monitor dynamic behavior, all at the micrometer spatial dimension [5]. By using rapid freezing and freeze-fracture techniques [7,8], snapshots in time of the spatially resolved membrane chemical compositions during dynamic membrane events have been taken at various time intervals after contact. Frozen-hydrated systems have been studied to preserve the native state of the cell without introducing severe sample preparation artifacts. We have captured each stage of the fusion event between two liposomes and have shown that membrane structure during fusion ranges from specific domains that then migrate across possible contact points to produce a homogeneous, fluid-mosaic membrane. Reported here, H II cylinder aggregates, in the presence of salt, show the inherent SIMS competition in the sputtering and ionization processes. Initial trials have proven the possibility of culturing model neuronal cells (rat pheochromocytoma (PC12)) on coated SIMS targets while still maintaining ability for induced cellular function. Further validation of sample preparation
improvements and optimization of analysis conditions described here hold the promise to define the cellular membranes at the molecular level.

2. Experimental

All analyses were performed on a Kratos Prism TOF-SIMS spectrometer equipped with a gallium liquid metal ion gun (LMIG - FEI Co.) with a pulsed 25 kV, 500 pA beam focused to a 45° incident, 200 nm spot. A liquid nitrogen (LN₂) cooled stage was biased at ±2.5 kV, along with an extraction lens biased at 4.7 kV. The horizontal TOF reflectron path length was 4.5 m with a microchannel plate detector. Charge compensation involved an electron flood gun pulse of 30 eV electrons for 50 µs after each LMIG pulse. The LMIG was rastered over the surface while collecting the corresponding TOF spectra for each point to generate a pixel image of selected ions. Scanning ion micrographs (SIMs) were taken after TOF-SIMS analysis with a channeltron detecting both electrons and ions.

Multilamellar, micrometer-sized liposomes and HII cylinders were formed in aqueous solutions by a rotary-evaporation technique [1] and characterized by optical microscopy. A small drop (3-10 µL) of suspension was placed on a 5 x 5 mm silicon wafer with a smaller shard of Si placed across the diagonal to form the sample sandwich. This was then immersed into liquid propane for several seconds and then stored in LN₂. Time-resolved analysis was accomplished by fast freezing in liquid propane at given time intervals after an event.

The complete cold-chain freeze-fracture method used in previous studies has been described elsewhere [4] with a newly constructed design presented here (Fig. 1). A two-stage fast-entry port (A) was evacuated for sample entry in approximately 1 min. The specimen was transferred onto a pre-cooled (B, 100 K) horizontal transfer arm (C) and then to another transfer arm (D) for positioning the specimen on a freeze-fracture stage (E). Once the sample was securely clamped (F) the sample temperature was precisely controlled (0.1 K) with LN₂ flow and cartridge heaters. An X, Y, Z manipulated LN₂ cooled knife (G) fractured the specimen. After fracturing, the sample was transported into the TOF-SIMS chamber via the horizontal transfer arm (C) for imaging. Pressures were typically kept in the 10⁻¹⁰ torr range by utilizing a turbo pump (J) and a LN₂ cooled cryoshroud (H) that encompasses the entire inner surface of the chamber. An IR lamp (I) was available for rapid baking after each completed analysis.

PC12 cells were successfully cultured on Si chips that were thinly coated with type I mouse collagen (Sigma). Differentiation was induced in a few days by adding 100 ng / mL nerve growth factor (NGF, Sigma) to the media when the cells were initially plated. Cells were then stained for two hours with DiI (Molecular Probes) for fluorescence imaging verification.

Figure 1. Schematic of freeze-fracture chamber specifically designed for molecular-imaging experiments. (A) fast-entry port, (B) cold-clamp for (C) horizontal transfer rod, (D) vertical transfer rod, (E) fracturing stage, (F) stage clamp feedthrough, (G) cryo-knife, (H) cryoshroud (inside vacuum chamber), (I) infrared lamp, and (J) turbo pump.
3. Results and discussion

Direct molecule-specific TOF-SIMS imaging of cellular membranes requires sample preparation protocols based on proven cryo-microscopy methods, but optimized for measurements of the top molecular layer. Imaging has been achieved by probing the inner portion of a sample, frozen as a sandwich that has been fractured in vacuo [5]. Fracturing criteria for preserving chemical distributions have been shown to be much more stringent than for morphological cryo-electron microscopy studies. The act of fracturing is suggested to be intrinsically violent, thus creating high, localized partial pressures of water at the fracture surface. The goal of providing a clean, undisturbed fracture surface in vacuo involves rapid equilibration of the surface condensation and sublimation fluxes just after fracturing. To this end, a newly constructed freeze-fracture unit is presently being characterized. A two-stage fast-entry system allows rapid sample introduction into the vacuum to reduce ambient water condensation and sample warming (further minimized along the cold-chain pathway to the fracturing stage). Fracturing temperatures are now more precisely controlled, for better reproducibility. Through the use of an internal cryo-shroud and an internal infrared light source, lower pressures can be maintained. The ability to maintain lower pressures is desired because of decreased residual water, a greater force to rapidly move water away from a freshly exposed surface, a reduction in the contamination of the analysis chamber, and the possibility for a higher throughput of fractured samples.

Molecule-specific TOF-SIMS imaging has directly measured the chemical and spatial distribution of lipid molecules on the surface of single multi-lamellar, micrometer-size liposomes. These studies have suggested that the lipid structure of model membranes during fusion proceeds through a discrete domain stage, where diffusion appears limited through a contact point, to eventually end in a completely homogeneous fused liposome. These segregated areas appear to be similar to what is expected for membrane domains in cellular systems. These studies recently have been extended to include various lipid structures along with high-salt aqueous environments. As discussed earlier, the HII cylinder phase is an interesting model for investigating membrane fusion with the physical structure consisting of minute aqueous-filled hexagonal cylinders embedded in a hydrocarbon environment, thus imparting poor hydration and favored aggregation. Figure 2 shows TOF-SIMS images (A-D) of frozen-hydrated aggregated phosphatidylethanolamine dipalmitoyl (DPPE) HII cylinders in a high NaCl environment. The total ion image (A) suggests relative ionization enhancement at the edges of the structure. Furthermore, the Na+ image (B) is the dominant

![Figure 2](image-url)
signal, not only around the edges as expected with cation stabilized structures, but over the entire analysis area. The water image (C) verifies the hydrated nature of the sample surface around the relatively frost-free lipid structure, indicative of a desirable fracture. However, the C$_3$ hydrocarbon image (D) illustrates the inability, thus far, of detecting lipid species within a high-salt environment. Comparison of SIMs E and F exemplifies beam-induced damage (less than 1 min. DC beam exposure) that is seemingly more prevalent in aqueous salt environments. Although these systems have extreme amounts of associated cations like Na$^+$ with high sputter and ionization relative to lipids, these investigations show the possible analysis interferences that need to be minimized through proper sample preparation and experimental design for successful lipid-specific imaging of membrane composition.

4. Summary and future directions

Development and optimization of freeze-fracture methodology specific to static TOF-SIMS molecular imaging of native chemical species of cellular membranes is discussed. It is apparent from the data that salt interferences could present difficulties if not accounted for. As noted above, PC12 cells represent a target model for these investigations. The promise of near-future studies on PC12 cells is shown in Figure 3, by not only the attachment to a substrate, but of the ability to influence membrane function during culturing such as differentiation. The ability to image the heterogeneous molecular spatial distribution of membranes captured during dynamic events represents a unique approach in our development of membrane bioanalytical chemistry.

Figure 3. Fluorescence image of a differentiated, as noted by axonal-like growths, PC12 cell on collagen-coated Si wafer. NGF treatment for 3 days. Average PC12 diameter is ~ 15 µm.

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5. References