SINGLE-CELL STUDIES OF MEMBRANE LIPIDS USING SECONDARY ION MASS SPECTROMETRY

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by
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Investigation of the spatial distribution of biological molecules in cell membranes can lead to an improved understanding of the role of lipids and other membrane species in biological function and disease. Time-of-flight secondary ion mass spectrometry (ToF-SIMS) is capable of imaging biological molecules across single cells and its potential for examining the functional segregation of lipids in cell membranes has been demonstrated. This thesis reviews SIMS as it pertains to biological samples and addresses relevant progress in SIMS including fundamental ionization studies of numerous lipids, instrumental and experimental developments, and novel applications of SIMS imaging to single-cell analyses. The objective of this thesis is to provide the foundation for future studies to understand the structural and biophysical aspects of single exocytosis events.

Specifically, in chapter 1 the biophysical properties of lipids and the analytical techniques commonly used to probe the role of lipids in biological function are discussed. In this chapter, ToF-SIMS theory and instrumentation are also reviewed and examples of biological applications with SIMS are presented from the literature.

In the second chapter, the ionization mechanism of phosphatidylcholine (PC) and the influence of water in SIMS experiments are examined. A frozen water matrix, as that found in freeze-fractured frozen-hydrated cellular samples, is shown to enhance the ionization of PC with ToF-SIMS. Isotopic profiles of the PC fragment ion, phosphocholine, from deuterated forms of PC are examined under various sample preparation conditions to show that ionization occurs through protonation from the matrix
and is enhanced by the water present in freeze-fractured samples. Freeze-fracture techniques have been used to expose lipid in frozen aqueous suspensions for static ToF-SIMS analysis. Because of the importance of surface water during SIMS analyses, sources of gas phase water resulting from freeze-fracture have been examined. Under proper fracturing conditions water vapor, resulting from water in the sample and water that condenses onto the sample surface, is released into the vacuum but does not condense back onto the surface. The combination of the demonstrated enhancement of PC signal from water with freeze fracture preparation techniques provides a potential advantage for the study of biological samples in a frozen-hydrated state.

In addition to PC, the cell membrane is composed of a complex assembly of many types of lipids. To enable SIMS analyses of such intricate samples, chapter 3 presents a series of fundamental ionization studies for numerous lipids. Standard SIMS spectra are analyzed for phosphatidylethanolamine, phosphatidylglycerol, phosphatidylserine, phosphatidylinositol, cholesterol, and sulfatide. Importantly, mass spectrometry of each of the lipids results in signature peaks that allow them to be identified. These signature peaks are also shown to be useful for imaging experiments and have been utilized to simultaneously image lipids on a micrometer-scale in picoliter vials. Because the low secondary ion signal achieved for lipids from an atomic primary ion source makes cell-imaging experiments challenging, improving signal with cluster primary ion sources is of interest. The secondary ion yield for seven lipids using atomic (Ga⁺ or In⁺) ion sources and a buckminsterfullerene (C₆₀⁺) primary ion source have been compared. A 40- to 1000-fold improvement in signal is obtained with C₆₀⁺ relative to the other two ion
sources, indicating great promise for future cellular imaging applications using the $C_{60}^+$ probe.

One particularly prevalent class of lipid is phosphatidylethanolamine (PE) because the shape of this lipid is believed to favor membrane fusion. Data in chapter 4 shows that PE is difficult to detect in a mixture containing PC and possible causes for the signal suppression have been tested. In this chapter, it is shown that, like PC, ionization of PE in SIMS occurs largely from extra-molecular proton sources. Mass spectra of DPPE and DPPE methylated derivatives reveal that the magnitude of signal suppression is directly related to the number of hydrogen atoms available for hydrogen bond donation and inversely related to the degree of steric hindrance on the phosphate headgroup. These results suggest that the relative basicities of lipids impact their final ion abundances, possibly by an equilibrium of preformed ions on the surface prior to primary ion impact. The data also suggest that hydrophobicity may affect the final ion distributions of PE and PC. However, the observed effect may only reflect the better shielding capabilities of longer tailgroups in a disordered lipid film and should not occur in experiments with ordered bilayers. Finally, solutions for improving the detection of PE are explored.

Along with these fundamental ionization studies, this thesis presents two SIMS imaging applications that showcase either experimental developments or the role of lipids in biological function. Chapter 5 introduces the combination of *in-situ* two color fluorescence and SIMS. *In-situ* two-color fluorescence imaging has been used to extend the types of biological applications possible with SIMS to include the analysis of fluorescently labeled, morphologically similar cells, originating from separate
populations. As an example, SIMS imaging and two color fluorescence microscopy have been used to compare macrophage cells treated to contain elevated levels of cholesterol compared to control cells. SIMS images of the fluorescently classified cells reveal that the two populations of cells have distinct outer leaflet membrane compositions. Relative quantification of treated versus control cells has established that the cholesterol-treated macrophage membranes contain an average of twice the cholesterol level when compared to the control macrophages. Expanding upon the *in-situ* fluorescence capability of SIMS with two-color fluorescence is an important advance because it will enable simultaneous molecule-specific imaging of cells from separate populations. By directly comparing the chemical effects of different drug treatments and incubation conditions, single-cell comparative studies with SIMS and two-color fluorescence can provide valuable information about normal biological functions, causative agents of diseases and drug resistance, and possible therapies for diseases.

Another single-cell SIMS study is detailed in chapter 6, which aims to understand biological fusion on a molecular-level. SIMS images of mating *Tetrahymena thermophila* have been used to support a role for lipids in membrane fusion during sexual reproduction. The images demonstrate that the low-curvature lipid, PC, is diminished in the membrane regions between fusing *Tetrahymena*, where a multitude of highly curved fusion pores exist. Additionally, mass spectra and principal component analysis indicate that the fusion region contains elevated amounts of 2-aminoethylphosphonolipid (2-AEP), a high curvature lipid. This evidence suggests that biological fusion involves and might in fact be driven by a heterogeneous redistribution of lipids at the fusion site.
The progress that is reported in this thesis sets the groundwork for many future endeavors using SIMS imaging and the seventh chapter will describe preliminary results for several forthcoming projects. SIMS images of protozoa taken with In$^+$ and C$_{60}^+$ primary ion sources have been compared to highlight the improved SIMS imaging capabilities of cluster projectiles for single-cell analysis. Furthermore, the possibility of generating 3-D molecule-specific images of a single cell with C$_{60}^+$ is supported by demonstration of molecular depth profiling through a layered system of water, lipid, and sugar. In addition to continued development of the instrumental aspects of biological SIMS, chapter 7 introduces new applications and novel cell systems for SIMS experiments. Two ideas for future investigations are discussed which use *Tetrahymena* to help understand the signaling mechanism that triggers lipid rearrangement during mating and to examine lipid distribution during exocytosis. Beige mouse mast cells are another type of cell poised for future SIMS experiments and a preliminary SIMS image shows promise for using these cells to explore the spatiality of lipids and biogenic amines during exocytosis. Finally, standard spectra of many biogenic amines and a chemotherapy drug are discussed with reference to broadening SIMS imaging applications to include investigations involving other biological molecules and drug agents.
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PREFACE

The work presented in this thesis is largely the product of collaborative work to which numerous colleagues have contributed. Portions of this thesis have been adapted from publications in journals and textbooks.

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Chapter 1
Investigations of Lipid Involvement in Biological Events Using Mass Spectrometric Imaging

Introduction

This thesis will discuss recent progress in the interdisciplinary areas of bioanalytical chemistry and surface science. Specifically, the seven chapters will focus on examining the role of cell membrane components in biological events using time of flight secondary ion mass spectrometry (ToF-SIMS). This chapter will provide detailed background to aid the reader in understanding the relevant biology and the analytical methods used to investigate cell membranes. The second half of this chapter addresses ToF-SIMS theory and provides generalized information about ToF-SIMS instrumentation, as well as a comprehensive discussion of the Kratos (Manchester, U.K.) Prism ToF-SIMS spectrometer used in most of the investigations in subsequent chapters. Hopefully, the specific information about the Kratos instrument will serve as a valuable resource to future researchers using the instrument. Finally, the end of this chapter reviews significant application-based contributions and instrumental advances in this area of research.
The Biological Role and Chemical Composition of Cell Membranes

Cellular membranes perform a vast array of dynamic biological functions and therefore are not simply stagnant barriers that enclose organelles and separate the intercellular and extracellular space. Specifically, membranes play an active role in signal transduction, membrane trafficking, and cell-cell communication. Regulated exocytosis is one example of cell-cell communication. It involves membrane bound organelles, called vesicles, fusing with the cell membrane and partially or completely releasing their chemical contents to the extracellular space, where an adjacent cell may receive the chemical signal (Figure 1-1). Various membrane components, such as membrane lipids, protein receptors, and cytoskeletal elements, collaborate to allow this process to occur. Neurotransmission is an especially important type of regulated exocytosis because abnormalities in neurotransmission have been observed in many neurological dysfunctions and disease, such as depression, Alzheimer’s Disease, and Parkinson’s Disease. Better understanding the cellular membrane and the multifaceted membrane interactions involved in exocytosis will pave the way to neurological disease treatment and prevention.
The chemistry of the cellular membrane is very complex and includes a medley of lipids, proteins, and carbohydrates. The phospholipid molecules found in the cell membrane contain hydrophilic and hydrophobic moieties and are arranged into a bilayer structure (Figure 1-2). In the lipid bilayer, phospholipids are stacked into two layers in a thermodynamically favorable manner: the hydrophilic phosphate headgroups interact with the hydrophilic extracellular and intercellular material and the hydrophobic fatty acid tailgroups interact with each other between the adjacent layers (Figure 1-3). The remaining cellular membrane components (proteins, carbohydrates, glycolipids, and sterols) are intercalated throughout this bilayer structure.
The fusion of lipid bilayers occurs in many biological events including exocytosis, viral infection, and sexual reproduction. Biological membrane fusion is believed to involve lipids and several classes of proteins including soluble N-
ethylmaleimide sensitive factor attachment protein receptors (SNAREs), Rab proteins, and Sec1/Munc-18 related proteins (SM proteins). These proteins may either direct the necessary bilayer structural rearrangement or may simply dictate the fusion site and provide a thermodynamic driving force for fusion to occur. Regardless of the precise function of these proteins in membrane fusion, their involvement in the process has been well documented and studied.

Based on research with liposomes, it is likely that lipids are also key players in biological fusion. Liposomes consist of a lipid bilayer that encases an aqueous solution, and therefore liposomes serve as simple model systems for cells. Liposomes, void of any proteins, have been observed to fuse under favorable conditions, thus lipids may control the fusion process to some degree. The exact structural rearrangement of lipids during membrane fusion is a subject of intense debate, however there are commonalities between the various hypotheses. Most theoretical models suggest that the two adjacent bilayers come into close proximity (<2-3 nm) and are destabilized to form various intermediate structures in which the proximal lipid monolayers merge. The most widely accepted intermediate structure, in terms of experimental and theoretical work, is “the stalk” (Figure 1-5). Finally, the distal monolayers also merge to create a channel, called a fusion pore, which joins the aqueous volumes that were initially separated by the membranes. In some cases, the fusion pore fully expands for complete fusion of the two bilayers and in other cases it transiently fuses and then reseals.
Lipid Polymorphism and Biological Fusion

During biological fusion, the lipid bilayer undergoes considerable structural rearrangement, adopting nonbilayer shapes, such as the stalk intermediate, of very high...
radius of curvature. The formation of contoured nonbilayer shapes likely requires the involvement of specialized lipids. Phospholipids can be divided into six main categories: phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, phosphatidylinositol, and phosphatidic acid (Figure 1-6). The respective category a phospholipid belongs to is determined by the alcohol attached to the hydrophilic phosphate headgroup. The phosphate moiety is attached to a hydrophobic tailgroup consisting of a glycerol backbone and one (lysophospholipid) or two fatty acid side chains. The acyl chains found in cell membranes are quite varied in carbon chain length and degree of saturation, although every membrane type seems to maintain a fairly consistent fatty acid composition among a given phospholipid class. The fact that a cell synthesizes such a complex assortment of lipids suggests that membrane lipid composition is important to biological function.
Figure 1-6: Structures for different categories of phospholipids. A. Phosphatidylcholine
B. Phosphatidylethanolamine C. Phosphatidylserine D. Phosphatidylglycerol E. Phosphatidylinositol F. Phosphatidic acid. $R_1$ and $R_2$ are hydrocarbon chains
Interestingly, in an aqueous environment phospholipids can self-assemble into different structures, which is a characteristic known as lipid polymorphism (Figure 1-7). The three most common lipid polymorphisms are the bilayer structure already described, the hexagonal II (H_{II}) structure, and the micellar structure. In the H_{II} structure, lipids are arranged into cylindrical tubes with the acyl chains facing the exterior of the tube. These tubes are hexagonally packed on top of each other such that the hydrophobic moieties interact. Micellar structures, another lipid polymorphism, form when lipids aggregate into spheres with the hydrophilic headgroup facing outwards. Upon hydration, the different lipid classes favor different lipid polymorphisms. For example, isolated phosphatidylcholine preferentially forms a bilayer structure whereas phosphatidylethanolamine adopts the H_{II} structure and lysophospholipids aggregate into micelles. The propensity of the lipid classes to self-assemble into different structures is significant because this ability may reflect how lipid composition can induce the nonbilayer structural changes that occur during membrane fusion.
One simple explanation for the different structures adopted by hydrated, isolated lipids is based on the shape of the lipid molecules (Figure 1-7). This hypothesis maintains that the relative cross-sectional areas of the hydrophilic and the hydrophobic regions of the molecule give each class of phospholipids a different dynamic shape. The
cross sectional area is determined not only by the physical size of the region of the molecule, but also by the degree of hydration, charge, hydrogen-bonding, and counterion effects. The different lipid shapes cause lipids to aggregate into different structures in order to best minimize the exposure of the hydrophobic regions of the molecule to the aqueous environment. Thus, lipid polymorphism is thermodynamically driven.

Lipid shapes can be generalized into three main categories: cylindrical, cone-shaped, and inverted cone-shaped (Figure 1-7). If the overall area of the lipid headgroup is comparative in size to the tailgroup, the lipid is referred to as cylindrical in shape. Phosphatidylcholine is an example of a cylindrical lipid, and therefore cylindrical lipids tend to self-assemble into a bilayer structure. A lipid is cone-shaped when the headgroup section is smaller than the tailgroup section; conversely a lipid is inverted-cone shaped when the opposite is true. Phosphatidylethanolamine is typically a cone-shaped lipid, and thus aggregates into \( H_{II} \) structures upon hydration. Lysophospholipids belong to the inverted-cone shaped class and these lipids adopt micelle structures. The cellular membrane contains a complex composition of lipids of different shapes which may allow variations in biological membrane curvature. It follows that cone-shaped lipids, such as phosphatidylethanolamine, are adept at forming highly contoured structures, like \( H_{II} \), and may therefore play a role in forming membrane fusion intermediate structures. In particular, membrane regions enriched in cone-shaped lipids may be preferential sites for biological fusion because the formation of contoured membrane, such as the stalk, structures would be energetically feasible (Figure 1-5).
**Functionality of Lipid Domains**

The original cell membrane model, the fluid mosaic model, stated that lipids freely transverse laterally and vertically through the membrane. Shortly thereafter, the model was modified to address observations of long-term order in the cell membrane and further experimentation has repeatedly shown that the cell membrane contains regions of restricted lipid mobility. These regions, called lipid rafts or domains, have a well-defined lipid composition and typically are concentrated in certain lipids. For instance, lipid rafts, which are more precisely defined as membrane entities that are insoluble in cold Triton X-100 detergent, are composed of glycosphingolipids, glycoprophosphoinositol (GPI)-anchored proteins, other constitutive proteins, cholesterol, and some phospholipids. The prevailing viewpoint is that these lipid rafts have biological functionality with respect to membrane trafficking and signaling. Lipid rafts are very small (<50 nm) but may cluster when the cell receives an activation signal. If two lipid rafts contain complementary signaling proteins, the coalescence of the smaller lipid rafts may allow and mediate the interaction of these proteins. Lipid rafts might therefore amplify protein signaling by restricting the proteins to the reaction site; lipid rafts might similarly prevent protein interactions by excluding molecules from the signaling site.

The role of lipid rafts in protein signaling also implicates them in the pathogenesis of a variety of diseases. For example, elevated levels of cholesterol, which results in a higher affinity of proteins for lipid rafts, have been observed in Alzheimer’s disease. Alzheimer’s disease is a neurological disorder trademarked by accumulations of amyloid-
β-peptide (Aβ)-containing plaques in the brain. Aβ is a protein fragment that is formed from the cleavage of the amyloid precursor protein (APP) by the enzymes β-secretase and γ-secretase. In healthy individuals, Aβ is then further cleaved by α-secretase into a nonamyloidogenic form. Ehehalt and Simons suggest that APP is present in both the fluid membrane regions and lipid raft regions and that APP favors the lipid rafts at elevated cholesterol levels. They propose that α-secretase cleavage occurs outside of lipid rafts and β-secretase cleavage occurs within the rafts. Therefore, elevated cholesterol would restrict a greater amount of APP to the β-secretase cleavage site and increase the production of Aβ, whilst preventing further cleavage by α-secretase.

Similarly, lipid rafts are suspected in many other diseases including Parkinson disease, HIV-1, and Epstein-Barr virus.

Along with signal transduction and disease pathogeny, lipid rafts may have a role in membrane fusion events, like exocytosis, by controlling protein interactions and by directing appropriately shaped lipids to the fusion sites. An additional or alternative argument is that domains with a lipid composition different from that of lipid rafts drive membrane fusion. These fusogenic membrane regions would be concentrated in cone-shaped lipids to enable the progression of the membrane through contoured intermediate structures. Because of the relationship of lipid domains with biological processes and disease, it is of interest to learn more about them.
Current Detection Methods for Lipid Domains

Studying lipid domains has been in vogue for several decades, but our understanding of the size, stability, and functionality of these domains is still in its infancy. Although there is a lot to be learned about the characteristics of lipid domains, scientists have already gained valuable information about them using a myriad of analytical techniques. These analytical techniques include a variety of optical methods, such as fluorescence recovery after photobleaching (FRAP), two-photon fluorescence microscopy, near field scanning optical microscopy (NSOM), and single particle trapping (SPT). Additionally, techniques, such as atomic force microscopy (AFM) and scanning electron microscopy (SEM), which probe sample topography, are common methods for examining lipid domains.

Optical Methods

FRAP is an optical method often used to identify lipid domains in single cells. As the name implies, FRAP involves photobleaching a section of a fluorescently labeled cell membrane and monitoring the recovery of fluorescence signal. Fluorescence recovery indicates lateral movement of nearby, labeled lipids into the photobleached region. If lipid domains did not exist, the fluorescence recovery would be 100%; however researchers have observed only partial fluorescence recovery for biological membranes. For example, FRAP measurements of fluorescently labeled human skin fibroblasts found that only 25% of the labeled lipids were mobile in a bleaching radius greater than the size of the domains. A smaller bleaching radius was not used because it might probe only a
single domain or fluid region, within which lipids diffuse freely. The results indicated that the fibroblast cell membranes contain protein-rich regions, which correlated with limited lipid diffusion between the boundary of the protein-rich domain and the surrounding fluid region.\textsuperscript{23}

Two-photon fluorescence microscopy is another popular optical tool for imaging lipid domains and investigating their properties. One such study with liposomes reported fluorescence images of phase specific dyes segregated between two domains of unique lipid composition.\textsuperscript{24} Importantly, the data in this experiment showed a clear correlation between membrane curvature and dye segregation, and thus domain composition. In a separate study, Weiss and coworkers utilized 2-photon 3D wide-field fluorescence microscopy and phase-specific fluorophores to image lipid segregation in liposomes of various binary compositions.\textsuperscript{25} Whereas this experiment demonstrated the utility of fluorescence microscopy for imaging domains, it also alluded to fundamental difficulties with the use of this technique. First, fluorescence microscopy is an indirect method of chemical imaging based on the assumption that the fluorophores partition selectively with different lipids. Additionally, phase specific fluorescent dyes are not available for all lipid compositions. Weiss and colleagues were unable to fluorescently label membrane regions composed of dipalmitoylphosphatidyglycerol (DPPG) and therefore were forced to deduce that non-fluorescent membrane regions contained DPPG. Despite these disadvantages, two-photon fluorescence can provide valuable information about lipid domains and is not limited to studies with model systems. For instance, this technique has been employed to image the formation of large lipid domains (up to 2 $\mu$m) in cholesterol depleted Chinese hamster ovary (CHO) cells.\textsuperscript{26} Unfortunately, conventional
microscopy techniques have a limited resolution (< 250 nm) based on the wavelength of light and therefore are ill-suited for imaging the nanometer-sized lipid rafts.

There are higher resolution optical techniques that are amenable to lipid raft studies in single cells. NSOM can image features smaller than the diffraction limit for traditional optics and thus is a particularly attractive analytical method for examining lipid rafts. Using NSOM and spatial autocorrelation analysis, lipid domains that are tens to hundreds of nanometers in size have been resolved and measured in human fibroblast cells in which endogenous transmembrane proteins, HLA class-I (HLA-I) molecules, have been labeled with a fluorescent antibody. Additionally, this study estimated that each of the domains contain between 25 and 125 HLA-I molecules, based on fluorescence intensity, quantum yield of the fluorophore, and the ratio of fluorophore to antibody.

SPT is another optical method that has been applied to lipid domain studies. Fluorescent beads or highly scattering gold beads are conjugated to membrane constituents and the movement of the beads is optically monitored. One such experiment combined SPT and epifluorescence to study the motion of labeled phosphatidylethanolamine through a Langmuir-Blodgett film. The random walk of the beads was clearly influenced at distinct boundary regions in the film indicating limited lateral mobility of phosphatidylethanolamine and suggesting the presence of domains.

The commonality between all of these optical methods is that they incorporate fluorophores in lipid bilayer. As previously stated, fluorophore-dependent methodologies suffer from indirect chemical localization and from the limited variety of fluorophores available. Also, it has been shown that fluorescent tags can alter the lipid packing and
phase of the membrane. Despite these disadvantages, the optical methods can indirectly monitor lipid diffusion through domains in real time and indirectly detect the chemical constituents of lipid domains. To obtain an accurate and more complete picture of lipid domains, these optical methods can be coupled with other complementary techniques.

**Topographical Methods**

Analytical techniques that investigate surface topography and morphology represent a second category of methods commonly used to investigate lipid domains. One such analytical technique, electron microscopy following freeze-etching, directs a focused beam of electrons onto a sample. The interactions of the impinging electrons and the sample cause electron scattering, which can be detected. Sample topographic and compositional heterogeneity cause differences in electron scattering which result in amplitude contrast in the images. In lipid domain experiments with electron microscopy, macromolecular markers are employed to label target lipids or proteins. These labels influence sample thickness and mass, and therefore create image contrast at their binding site. For example, Rock and coworkers imaged lipid domains in freeze-etched liposomes with this methodology. The liposomes contained a specific target lipid, Forssman glycolipid, and the binding specificity of Fab fragments of anti-Forssman allowed visualization of the location of the lipid by electron microscopy. Lipid domains of Forssman glycolipid were evident because of the clustered distribution of anti-Forssman in the electron micrographs.
A second example of a topographical technique is AFM which operates by scanning a probe across a sample surface and measuring either the repulsive or attractive forces between the probe and the sample. The measurements are then translated into topographical images with as good as 0.1 nm resolution. AFM has been frequently used to measure the size and shape of lipid domains. For instance, a 1.4 nm height difference was measured between the domain and fluid regions of ternary lipid monolayers. The domains were irregularly shaped with a median lateral size of 37 nm, which was dependent on cholesterol concentration. AFM has also been coupled with an optical method, total internal reflectance fluorescence microscopy, to deduce the chemical composition of domains in artificial lipid bilayers. In this study, the bright regions in the fluorescence images correlated with the shorter regions measured by AFM imaging. Because the fluorescently labeled lipid was known to partition into domains of less ordered lipids, it followed that the shorter regions contained domains of disordered lipids.

Topographical detection methods provide valuable information about the lateral size, shape, and height of lipid domains. However, these techniques lack chemical specificity and at best, chemical composition can be indirectly deduced by correlating the topographical data with optical images. Further pursuit of the properties and functionalities of lipid domains will require innovative technology and, importantly, a means to directly probe the spatial chemistry of lipid bilayers.
Secondary Ion Mass Spectrometry

Time of flight secondary ion mass spectrometry (ToF-SIMS) can directly image the chemical distribution across a surface and therefore is an attractive alternative to the traditional techniques used for studying lipid domains. SIMS has a proven history in biological applications as it has been used to image dopants in cells and lipids in biological tissue and single cells.\textsuperscript{36-39} Therefore, it is prudent to further develop this methodology to tackle complex biological questions, such as the functional role of lipid domains in cell membranes.

SIMS mass analyzes ionic species which are emitted (or sputtered) from a sample following the impact of an incident, or primary, projectile. More specifically, SIMS analysis uses a pulsed beam of primary projectiles which is focused to a small diameter (down to 50 nm) onto a sample (Figure 1-8). Depending on the desired application, a variety of primary projectiles (Cs\textsuperscript{+}, Ar\textsuperscript{+}, Ga\textsuperscript{+}, In\textsuperscript{+}, SF\textsubscript{5}\textsuperscript{+}, Au\textsubscript{n}\textsuperscript{+}, C\textsubscript{60}\textsuperscript{+}) may be suitable. The impinging primary particles transfer energy and momentum to the sample and these energetic interactions cause sample electrons, neutral species, and ions (or secondary species) to be ejected from the sample (Figure 1-9). The secondary ions are then electrostatically collected and directed into a mass analyzer.
Figure 1-8: Time of flight secondary ion mass spectrometry (ToF-SIMS) instrumental setup.
Theory behind SIMS

The crux of SIMS lies in two physical phenomena: the sputtering and the ionization of secondary particles. Neither of these phenomena is completely understood and considerable experimental and theoretical studies are ongoing in order to address the ambiguities. A clearer comprehension of the SIMS process may allow further development of the technique.

Sputtering

The original model for sputtering is Sigmund’s collision cascade theory, which uses classical mechanics to explain the interactions of primary, secondary, and recoiled particles. This theory states that the primary projectile induces cascades of binary collisions, which cause a large amount of energy and momentum transfer within the
The collisions may be between the primary projectile and target particles or between recoiled target particles and other target particles. Ultimately, the collision cascades may result in energy and momentum transfer to a particle at the sample surface. The particle will be ejected if the energy acquired by the collision exceeds the binding interactions at the surface and if the momentum vector is directed away from the surface.

Sigmund’s “hard sphere” collision cascade theory failed to sufficiently explain sputtering, particularly under low primary ion doses and for sputtering of multi-elemental organic molecules with numerous bonds. This theory oversimplified the sputtering process by not accounting for electronic interactions between the incident and target particles. Therefore, molecular dynamic (MD) simulations have been invaluable in accurately depicting the sputtering process under complex conditions. This theoretical tool analyzes the impact of a primary projectile on a crystal composed of thousands of atoms, which are each assigned an atomic mass, velocity, and position. The atoms are then treated as individual particles and using classical (Hamiltonian) equations, numerous properties, such as motion and kinetic energy of target atoms, are calculated as a function of time. Significantly, MD simulations use interaction potentials to take into account intermolecular interactions and chemical reactions, which are important for molecular sputtering.

The total number of neutral and ionic species removed per primary projectile is called the sputter yield, $Y$. Several factors influence the sputter yield including the topography of the sample and the primary ion beam energy, mass, and charge. Generally, the sputter yield increases as the primary ion mass and energy increase.
However, too high of a primary ion energy (> 50 keV) depresses sputter yield because the primary ion imbeds itself so deep inside the solid that little energy reaches the surface.

Sputter yield is a useful parameter for elemental analysis, but is not as informative for molecular analyses because it does not consider beam-induced damage to large molecules. Therefore, the concept of damage cross section, $\sigma$, has gained popularity in place of sputter yield. Damage cross section measures the rate of decrease of intact molecular species or fragments over time. Damage cross section can be determined from the secondary ion current of species m, $I_m$, and the primary ion flux, $I_p$, according to Equation 1-1.

$$I_m = I_{m0}e^{-\sigma I_p}$$  \hspace{1cm} \text{Equation 1-1}

**Ionization**

Secondary ion formation is the second crucial physical process in SIMS. Quasi-molecular ions can form through several routes including electron ejection, protonation, deprotonation, cationization, and loss of a functional group. Smaller fragment ions of molecules are also formed, usually because the fragmentation process results in charged species. Intact molecular ions and fragment ions most likely form at the sputter region or else just above the surface in a highly energetic region known as the plume.

Unfortunately, only a small fraction of sputtered material is ionized whilst the majority of the particles remain neutral. For this reason, methods of enhancing ionization, such as addition of an ionization-inducing matrix and postionization, are under investigation.
Because SIMS analyzes sputtered ions, greater signal and hence sensitivity are gained with a larger number of ionized species. The efficiency of secondary ion formation for an analyte, \( m \), is directly related to the secondary ion current, \( I_m \). The basic SIMS equation relates \( I_m \) to primary ion flux \( I_p \), sputter yield, \( Y_m \), ionization probability, \( \sigma^+ \), fractional concentration of the analyte \( m \), \( \theta_m \), and instrument throughput, \( \eta \), (Equation 1-2).\(^{41}\)

\[
I_m = I_p Y_m \sigma^+ \theta_m \eta
\]

**SIMS Operational Modes**

SIMS can be divided into two different modes, called dynamic SIMS and static SIMS, which are distinguished by the incident dose of primary ions. SIMS is described as static when the primary ion flux is less than 1 % of the total number of surface atoms; SIMS is dynamic when the incident dose is greater than this value.\(^{46}\) Dynamic SIMS is trademarked by extensive beam-induced damage, which destroys molecular information and limits dynamic SIMS to elemental and isotopic studies. The advantages of dynamic SIMS are high elemental sensitivity (1 ppb limit of detection) and large removal rate of surface material.\(^{41, 47}\) These characteristics make dynamic SIMS especially amenable to optimizing the elemental composition of semiconductors, which are used in a wide variety of electronics and computer technologies. Because dynamic SIMS is not limited to the near-surface region, it is often used to monitor the elemental composition of a sample from the surface through the bulk, a process known as depth profiling.\(^{48}\)
Static SIMS is inherently less damaging to the molecular integrity of a sample because of the low primary ion flux (1nA/cm² or less). There is minimal chemical damage and spatial perturbation of sample molecules. Therefore, static SIMS is favored for applications investigating large molecules or molecular fragments. Another unique aspect of the static regime, is that only the topmost sample monolayer is probed. Experiments with samples of well-defined surface chemistries have established that static SIMS is a surface sensitive technique. Because of its ability to analyze molecules at surfaces, the static mode of SIMS has been used for examining lipids on tissues and cell membranes.

Instrumentation

**Primary Ion Source**

SIMS instruments employ a variety of primary ion source configurations including electron impact ionization-based sources, duoplasmatron sources, and field evaporation-based sources, like the popular liquid metal ion sources. By far, the most commonly used primary ion sources are the liquid metal ion sources (LMIS) because of their brightness, small spot size, and simplicity. LMIS consist of two tungsten wires that are wound tightly together to create a hollow reservoir, which is filled with the liquid metal. At the top side of the reservoir, the tungsten forms a thin filament. At the base of the reservoir, the tungsten wires pass separately through an insulator cap and make electrical contacts on the opposite side. LMIS operate by applying a negative potential to
a plate, called the extraction cap, positioned in close proximity to the end of the tungsten filament. This electric field causes positively charged ions to flow up the filament towards the tip, where the liquid metal lengthens into a cone shape\textsuperscript{41}. The precise behavior of the liquid metal at the tip depends on the strength of the extraction electric field. At a certain extraction potential, the surface tension and electrostatic forces at the metal surface are in equilibrium and the liquid metal forms a stable Taylor cone, which has a 49.3° half angle.\textsuperscript{41} Above this extraction potential, primary ion emission occurs at the apex of the liquid metal cone. A variety of liquid metals have been used in LMIS, including gallium (Ga\textsuperscript{+}) and indium (In\textsuperscript{+}). Ga\textsuperscript{+} is an attractive metal for LMIS because it melts at 29.8°C and can be used at room temperature. In\textsuperscript{+} melts at a higher temperature and therefore a current must be applied to the tungsten wires in order to heat the metal to the liquid state. Although In\textsuperscript{+} is more difficult to use, it has been shown to significantly increase the secondary ion yield owing to its higher atomic weight.\textsuperscript{50}

The LMIS resides at the base of a column containing various electrostatic lenses and apertures. The entire assembly is often referred to as a liquid metal ion gun (LMIG) or more simply as the gun column. There are slight anomalies between different gun columns, and the FEI (Beaverton, OR) LMIG, designed for a Kratos (Manchester, U.K) Prism ToF-SIMS, will be described as an example (Figure 1-10).
The LMIS is located in a metal housing, called the suppressor, which surrounds the tungsten reservoir but has a machined hole that the filament can extend through. A positive bias, relative to the extraction cap, is applied to the suppressor, which serves two main purposes. First, the suppression potential shields liquid metal in the reservoir from the extraction potential, thus increasing LMIS lifetime. Additionally, the suppression potential can be adjusted for fine control of ion emission. The suppressor sits on a Macor tube which attaches to a positional stage at the rear of the gun column. The positional stage is used to align the source with the gun column’s optical axis by way of two manipulator feed-throughs.

The extractor is positioned at a close distance to the filament tip and a negative potential (0 to -15kV) is applied to the extractor to generate ions, as previously described. The emitted primary ions pass through a small hole in the extractor; stray ions impact the extractor creating an emission current, which can be monitored to assess LMIS lifetime and for alignment purposes.

Following the extractor, the ion beam passes through a beam-defining aperture. Originally, the FEI gun column included a selectable aperture that alternated the beam.

Figure 1-10: Schematic of a liquid metal ion gun (LMIG) column.
spot size between 50 nm and 200 nm. However, the selectable aperture was removed during instrument repairs and the beam spot size is now limited to 200 nm.

After the aperture, the ion beam is focused and shaped by numerous electrostatic lenses and octupoles, which contain eight electrostatic sectors. Lens 1 focuses the ion beam and the first octupole centers the beam with respect to the optical axis of Lens 2. The beam blanking plates, which pulse the ion beam for SIMS experiments, are subsequent to the first octupole. A constant potential is applied to one plate and a pulsed potential of opposite amplitude is applied to the second plate. Prior to the pulse, the ion beam is deflected to the first beam blanking plate. The voltage pulse on the second plate electrostatically attracts the ion beam, sending it down the column to the next ion optical element, Lens 2.

In order to obtain crisp SIMS images, the potential applied to Lens 1 and Lens 2 must be optimized with respect to one another so that the ion beam is centered between the beam blanking plates and the image is focused. If the ion beam is not properly centered between the plates, the beam blanking pulses will cause lateral movement in the SIMS images, and hence image blurring. Finally the ion beam travels through the second octupole, which corrects for beam astigmatism and controls beam scanning and image magnification, for SIMS imaging applications.

SIMS images are obtained by scanning the primary ion beam across the sample surface and collecting a unique mass spectrum for every image pixel (Figure 1-11). The signal sent to the computer contains the location of the primary ion impact and the spectrum collected at that position. The computer uses this information to output a spectrum, called the “total ion spectrum”, which contains the integrated intensities for all
the ions at every pixel of the image. Additionally, the computer generates a “total ion image” which is an intensity map for all of the ions in each pixel (Figure 1-12).

Computer software is then used to abstract spatial chemical-specific information from the “total ion” image and spectrum. In one method of data analysis, the user selects a mass range of interest in the total ion spectrum and the software creates a 2-D intensity map of the distribution of that signal across the sample (Figure 1-12). In an alternative way to analyze the data, the user can select a region of interest in the total ion image and the computer generates a mass spectrum with integrated intensities for the ions in only the selected pixels.
Figure 1-11: Chemical imaging with ToF-SIMS. The primary ion source is rastered across the sample surface and a mass spectrum is collected at each pixel. For every mass range of interest, an intensity plot, which maps the distribution of that signal across the sample can be generated. The intensity plots can be color coded and overlaid. In this example, an electronic device composed of titanium (m/z 48, green) and barium (m/z 138, blue) is chemically imaged.
Although the Ga\textsuperscript{+} LMIS is the most common primary ion source used in SIMS, advances in primary ion beam technology have directed the communities’ attention to the benefits of cluster ion primary sources, such as Au\textsubscript{n}\textsuperscript{+}, SF\textsubscript{5}\textsuperscript{+}, and buckminsterfullerene, C\textsubscript{60}\textsuperscript{+}.\textsuperscript{51-53} C\textsubscript{60}\textsuperscript{+} boasts enormous improvements in secondary ion yield and minimal chemical damage to sample molecules.\textsuperscript{53} Molecular dynamic simulations suggest that upon surface impact, C\textsubscript{60} cluster projectiles break into individual carbon atoms and disperse the primary projectile energy among the individual atoms, in contrast to atomic projectiles such as gallium.\textsuperscript{42} This phenomenon allows for a more shallow penetration depth and more surface-localized primary bombardment energy for cluster projectiles, in comparison to their atomic counterparts. The C\textsubscript{60}\textsuperscript{+} sputtering mechanism allows intact molecules to be gently lifted off the surface, creating higher secondary ion yield, particularly for high mass molecules, and minimal chemical damage. Therefore, C\textsubscript{60}\textsuperscript{+} and other cluster ion sources are attractive alternatives as SIMS primary ion sources for biological applications, where there is a desire to greatly reduce molecular fragmentation.

Figure 1-12: SIMS imaging of a copper London Finder grid. Grid was adhered to a copper sample block with silver paste. A. Total ion image. B. Chemical image of the distribution of copper (green) and silver (blue).
of larger-mass target analytes. Additionally, the attributes of $C_{60}^+$ suggest that molecular depth-profiling is possible, which will allow 3-D imaging of tissue and cells. The $C_{60}^+$ primary ion gun design has been described in detail elsewhere.53

**Secondary Ion Extraction**

The LMIG directs the primary ion beam onto the sample, which is mounted on a sample stage at ±2.5 kV, which may be pulsed or held constant. The stage potential repels ions of like charge, which are then collected by an extraction lens oppositely biased at 4.7 kV. For example, if the target analyte is a cation, the sample stage would be positively charged and the extraction lens would be negatively charged. This mode of operation is called “positive SIMS” because the positive ions are being analyzed. The instrumental polarities must be switched in order to generate spectra of negatively charged species.

In some experimental set-ups, the stage potential causes analysis difficulties that must be addressed. If an experiment uses a dc stage voltage, the primary ion beam will be partially deflected from its path by the strong electric field. Therefore, some instruments include electric field compensation (EFC) plates which steer the beam back to the undeflected pathway. The EFC plates align the sampling region with the ion extraction optics and correlate SIMS images with scanning ion micrographs, which will be discussed later.

The extraction lens also creates experimental challenges that must be overcome with creative instrumental design. Often, the extraction lens has a tiny physical opening,
called the optical gate, through which the secondary ions travel. Thus, the collection area for the secondary ions is severely limited, which restricts the imaging field of view. This problem is corrected by dynamic emittance matching (DEM). The position of the optical gate is electrostatically controlled by a potential applied to deflection plates (DEM plates) in the secondary ion extraction optics. When the potential is scanned, the sampling region of the extraction lens moves dynamically. In order to image large areas with the smaller optical gate, the DEM scan is synchronized with the primary ion beam scan. With this instrumental set-up, a 500 x 500 μm² area can be imaged.

**Mass Analyzers**

The extraction lens directs the secondary ions into the mass analyzer, which can be a magnetic sector, quadrupole, or time-of flight (ToF). A magnetic sector bends secondary ions along an arced trajectory, and a spectrum is generated by varying the magnetic field or the accelerating potential. Each set of variables allows ions of a particular mass range to pass through a slit to the detector; ions of other masses impact the sides of the analyzer and never reach the detector. A quadrupole mass analyzer is made up of four cylindrical rods arranged in parallel. The two pairs of opposite rods are electrically connected with a positive DC potential applied to one pair and a negative DC potential applied to the second pair. Also, each pair of rods receives a radio-frequency, ac potential that is 180° out-of-phase to the other pair of rods. The resulting electric fields cause ions to oscillate through the quadrupole, but only ions of one m/z value travel the entire distance and reach the detector. The ions of other m/z values have
unstable trajectories within the given parameters and impact one of the rods. By scanning the electric field values, it is possible to collect a full mass spectrum. Of these three mass analyzers, the ToF analyzer is the best equipped for static SIMS imaging because it detects all ions in parallel, boasts a high transmission, and has a virtually unlimited mass range. Additionally, advances in electronic timing systems have allowed ToF analyzers to provide a competitive mass resolution exceeding 10,000. Therefore, ToF analyzers will be discussed in greater detail.

The general concept of a ToF analyzer is that the secondary ions are accelerated to a specific kinetic energy and then allowed to travel a defined distance (x) before detection. Specifically, the primary ion pulse signals the start of the time measurement. After primary ion impact, the stage potential imparts the same kinetic energy to all of the sputtered secondary ions. The secondary ions are collected by the extraction optics into the ToF analyzer which has an electrically ground “drift” region of known length, x. Because kinetic energy is a function of particle mass (m) and velocity (v), the mass of each secondary ion can be deduced from the time of travel, or the flight time (t) (Equation 1-3).

\[ E = \frac{1}{2} mv^2 = \frac{1}{2} (mx^2/t^2) \]  

Equation 1-3

The molecules reach the detector sequentially because each molecule has a unique mass, allowing quasi-parallel detection.

A linear ToF analyzer contains a unidirectional drift region, with the detector positioned at the end of the flight tube, far from the sampling region. Although the linear design is relatively simple, it suffers from poor mass resolution because of
uncorrected temporal, energetic, and spatial distributions for secondary ions of the same mass. Temporal distributions occur when ions of the same mass are formed at different times.\textsuperscript{55} Without correction, the ions will maintain a constant separation in time and space throughout the flight path. Therefore the ion formed first will arrive at the detector slightly before ions of the same mass that were formed later. Additionally, secondary ions can exhibit kinetic energy spread, which also adversely affects mass resolution.\textsuperscript{55} Ions can have a distribution of kinetic energies if, for example, the ions have velocity vectors of different directionalities. An ion with a velocity vector pointed in the opposite direction of the flight path will require additional time to turn around and will reach the detector after ions of the same mass having velocity vectors pointed along the flight path. Finally, the initial spatial distribution of secondary ions of the same mass reduces the mass resolution.\textsuperscript{55} An ion formed close to the source potential field (and furthest from the detector) will have a higher kinetic energy than an ion formed further from the potential field source. Although the ion with the higher kinetic energy has further to travel, its larger velocity will allow it to overcome the other ions of the same mass in the drift region and to reach the detector first.

Reflectron ToF analyzers were developed to correct for the poor mass resolution observed in the linear ToF design.\textsuperscript{55} A dual stage reflectron consists of two electric fields: the decelerating field, which slows secondary ions, and the reflect field, which turns the ions 180° in the drift region. As in the linear design, the detector in a reflectron ToF is at the end of the drift region, however this location is now near the source. The reflectron rectifies kinetic energy and spatial spread because ions of greater energy travel further into the retard potential field before turning. The longer flight path causes ions of
higher energy to reach the detector at the same time as lower energy ions, which do not penetrate as far into the field. The energy focusing properties of the ToF reflectron have allowed mass resolution exceeding 10,000.41

**Detector**

After the secondary ions are separated in the mass analyzer, they impact a detector, such as a multi-channel plate (MCP) detector. An MCP is an array of about one hundred electron multipliers, each tens of micrometers in diameter. The channels are made of a lead silicate glass that is highly conductive and emits secondary electrons. Therefore, the impact of a secondary ion with the MCP results in a cascade of electrons that is progressively amplified down the length of a channel. Sometimes in electron multiplier devices, the high density of electrons at the output can ionize gaseous molecules in vacuum. These ions may reenter at the electron multiplier input and cause a false signal, or ion feedback. Chevron style MCPs have been developed to circumvent the problem of ion feedback. In the Chevron design, a front MCP and a rear MCP are oriented with their channels angled with respect to one another. The change in directionality at the junction of the two plates prevents the ions formed at the output from returning the channel input. The signal, thus detected, is sent to a computer processing unit.
Scanning Ion Microscopy

Scanning ion microscopy (SIM) is an *in-situ*, imaging technique, much like scanning electron microscopy. SIM provides morphological information complementary to the chemical information obtained in SIMS (Figure 1-13). An unpulsed primary ion beam is scanned over the sample and all of the emitted electrons, neutrals, and ions are collected by an adjacent channeltron electron multiplier detector. The channeltron is made of a lead silicate glass that has high electrical conductivity and secondary electron emission. Therefore, the impact of charged species from the sample generates a cascade of electrons in the detector, resulting in a high transmission of current. The signal intensity is then plotted according to the position of the primary ion source to create a morphological image with high resolution. SIM is used to study the morphology of sample and to align and focus the primary ion beam before SIMS analysis.

Figure 1-13: A scanning ion micrograph of a London Finder copper grid on silver paste. (300 x 300 µm field of view)

The high resolution of the SIM images compared to SIMS images is a result of the greater signal intensity achieved with the constant current primary ion source. An
unpulsed beam is not used in static SIMS because it causes significant physical and chemical damage to the sample. Thus, when coupling these complementary techniques to image a biological sample, SIMS should be conducted prior to SIM.

**Biological Samples: Frozen-hydrated Sample Preparation for SIMS**

ToF-SIMS is a valuable analytical method for examining membrane lipid distributions because of its molecule-specific imaging ability, high mass resolution, and quasi-parallel ion detection. However, maintaining the native chemical distribution in hydrated samples, such as cells, in the ultra-high vacuum (UHV) environment of a mass spectrometer is challenging. The low pressures cause rapid sublimation of water. In a sense, unpreserved cells explode and their membranous material and intercellular content smear across the sample surface.56 To prevent this phenomenon, proper preservation of hydrated samples for UHV analysis is of the utmost importance.

**Cryogenic Sample Preservation**

A fast-freeze, freeze-fracture sample preparation methodology has been adapted from the electron microscopy community to preserve biological samples for ToF-SIMS analysis (Figure 1-14).57, 58 A cell sample is delivered onto a clean 5x5 mm silicon wafer and a shard of silicon is placed at a diagonal on top of the cells. This assembly is referred to as the “sample sandwich”. Excess liquid is blotted from the exposed corners of the silicon wafer in order to keep the sample layer thin and to prevent ice-buildup on the
corners after freezing. The sample sandwich is then plunged into liquid ethane with reverse action tweezers to freeze the cells on a millisecond timescale. Fast-freezing with an appropriate liquid cryogen creates vitreous ice, or smooth, non-crystalline ice, which minimizes ice crystal induced sample damage.\textsuperscript{59} After freezing, the sample sandwiches are transferred to cryogenic vials which are stored under liquid nitrogen until use.

Figure 1-14: Cryogenic preservation of hydrated samples. The sample, containing cells (shown in pink) and polystyrene beads (shown in yellow), is delivered onto a silicon wafer. A silicon shard is placed on top of the sample droplet to create a sandwich assembly. The sandwich assembly is then plunged into a liquid cryogen.

\textit{Freeze-Fracture}

On the day of analysis, the top shard of the fast-frozen sample sandwich is removed by a cryogenic knife in a sample preparation chamber adjoining the SIMS analysis chamber (Figure 1-15).\textsuperscript{56} This process has been coined “freeze-fracture” and
results in the exposure of a fresh surface to the vacuum interface. The sample temperature is controlled throughout preparation by several chamber components that are cooled to liquid nitrogen temperatures by copper coil feed-throughs. Dry nitrogen gas at about 40 psi is sent through the copper coils, which are immersed into dewars of liquid nitrogen. The cold clamp, the freeze-fracture stage, the cryoshroud, and the cryogenic knife are cooled in this manner. The copper part of the horizontal transfer arm makes thermal contact with the cold clamp when the clamp is closed. Therefore any frozen sample that is introduced into the chamber can be maintained at liquid nitrogen temperatures by keeping it in the horizontal transfer arm and closing the cold clamp.

Figure 1-15: Freeze-fracture sample preparation chamber. Blue lines represent liquid nitrogen feed-throughs and the yellow parts are cooled throughout analysis. The red line represents an IR lamp used to remove residual water molecules during sample preparation.

When the horizontal transfer arm (via the cold clamp) and cryoshroud have been completely cooled, the sample is transferred into the freeze-fracture chamber. The first
step of sample transfer is to mount the sandwich under liquid nitrogen onto a copper block with two stainless steel screws and washers. The copper block is then attached to a pre-cooled, copper holder under liquid nitrogen. The sample holder can enter the preparation chamber through an opening, called the fast entry port, which is separated from the preparation chamber by a gate valve. After a slight vacuum is created with a sorption pump in the fast entry port, the gate valve is opened and the sample is lowered into the chamber. The copper block is transferred from the sample holder to a horizontal transfer arm, which has been pre-cooled with the cold clamp. The block is held in the clamp while the preparation stage and cryogenic knife are cooled.

The temperature of the preparation stage is crucial because it defines the final temperature of the sample at the time of fracture. Fracturing is performed at a pressure dependent sample temperature that equilibrates the condensation and sublimation fluxes of water.\textsuperscript{56} If the sample temperature is too low at a given pressure, water will condense on top of the fractured sample.\textsuperscript{56} In this case, the SIMS images will show a homogeneous distribution of water because no cells will be present at the surface-vacuum interface (Figure 1-16A). Alternatively if the sample temperature is too high at a given pressure, all of the water in the sample will rapidly sublime, which is called the “water vapor wind phenomenon”.\textsuperscript{56} The SIMS images will show a homogeneous distribution of lipid and other biological molecules because the molecules were redistributed during the violent sublimation process (Figure 1-16B). To avoid these two unfavorable scenarios, ideal combinations of temperature and pressure have been determined which equilibrate the fluxes of water condensation and sublimation (Figure 1-16C).\textsuperscript{56} The optimal set of freeze fracture conditions currently in use is a pressure of $2 \times 10^{-8}$ torr and a temperature of -
106°C. The desired pressure is achieved by filling the fast entry portal with dry nitrogen gas and gently bleeding the gas into the preparation chamber. The amount of gas entering the chamber is controlled by opening the gate valve between the fast entry port and the preparation chamber by small increments. The gas composition in the chamber is monitored throughout the nitrogen gas treatment procedure with a residual gas analyzer (RGA, a small quadrupole mass analyzer attached to the side of the chamber).

Figure 1-16: Freeze-fracture temperature and pressure conditions affect SIMS images. SIMS images of dipalmitoylphosphatidylcholine liposomes freeze-fractured at different temperatures. In these images, m/z 17.9-19.1, water, is represented by blue pixels and m/z 184.0-184.5, phosphocholine, is represented by green pixels. A. Sample was freeze-fractured at -110°C, resulting in condensation of water on top of the surface. B. Sample was fractured at -95°C, which caused a rapid sublimation of sample water and a redistribution of lipid across the sample surface. C. Sample was freeze-fractured at -105°C, which was an ideal temperature for a pressure of 10⁻⁷ torr. This temperature caused an equilibrium of water condensation and sublimation fluxes. Thus, the native distribution of phosphocholine was maintained.
At the time of fracture, some water gently sublimes from the sample that is evident by a peak in the gaseous water level measured by the RGA (Figure 1-17). The recently fractured sample is held in the fracture stage until the water level in the chamber returns to the original value. Moving the sample through an atmosphere full of water molecules is unfavorable because it increases the likelihood of ambient water recondensing on the sample surface. Additionally, the cryogenic knife is positioned at close proximity to the sample after the fracture but before the sample is transferred to the analysis chamber. The temperature of the cryogenic knife is much lower than the sample so any nearby water molecules will preferentially condense on the colder knife instead of the sample. Once the partial pressure of water is sufficiently low, the copper block is moved from the fracture stage to the SIMS analysis chamber using the cooled horizontal transfer arm. Throughout analysis in the SIMS chamber, the copper block is held in a clamp that has been cooled to liquid nitrogen temperature by a copper coil feedthrough.
Overview of Advances in Biological SIMS

SIMS has already demonstrated great utility in tackling complex biological questions. The major advances in the field have stemmed not only from pursuing challenging biological applications, but also from improvements in sample preparation and instrumentation.

Figure 1-17: Residual gas analyzer (RGA) traces collected in the sample preparation chamber during a freeze-fracture. The time of the freeze-fracture is indicated by the arrow. The small changes in the partial pressure of nitrogen (green) resulted from the outgasing of vacuum components during sample transfer from the cold clamp to the fracture stage. The water (blue) pressure spike results from the water sublimation that occurs during the freeze-fracture process.
**Biological Applications of SIMS**

Images of both elemental and molecular analytes, have indicated the wealth of information that promises to be learned with SIMS. For example, dynamic SIMS elemental analysis has allowed Chandra and coworkers to assess the efficacy of boron neutron capture therapy (BNCT) cancer drugs at the single cell level. Images of the subcellular distribution of boron were used to compare the accumulation of two BNCT drugs, $p$-boronophenylalanine (BPA-F) and sodium borocaptate (BSH), in normal and cancerous co-cultured cells. An ideal BNCT drug would specifically accumulate in cancerous cells and would be most lethal if the molecules aggregated in the nucleus. Although neither drug displayed these desired attributes, BPA-F accumulated in both cell types at significantly higher levels than BSH. These results indicated that BPA-F enters the cell by an active uptake mechanism and not by simple passive diffusion. This research group has been at the forefront of biological dynamic SIMS with many other notable applications including the mapping of elevated concentrations of $Ca^{2+}$ in the Golgi apparatus, which was affected by hormone and antigen treatment.

Lechene and colleagues also have an impressive repertoire of elemental imaging applications including investigations of the chemical distribution in the hair shaft, of protein turnover in fibroblast cells, and of free fatty acid transport and accumulation in adipocytes. In particular, Lechene utilized a variation of SIMS called multi-isotope imaging mass spectrometry (MIMS), in which isotopic signals can be simultaneously monitored. This is possible because the instrument has a very high mass resolution. For example, the instrument can distinguish between $^{12}C^{15}N$ (m/z = 27.0001) and $^{13}C^{14}N$ (m/z...
= 27.0064 with a mass resolution (m/∆m) of 4287.61. In one study, Lechene analyzed the isotope ratios for \(^{12}\text{C}^{15}\text{N} / ^{12}\text{C}^{14}\text{N}\) and \(^{13}\text{C} / ^{12}\text{C}\) in fibroblast cells that were untreated or treated with \(^{13}\text{C}\) glycine or \(^{15}\text{N}\) glycine.\(^{61}\) The isotope ratios for the untreated cells were equivalent to the terrestrial abundance ratios for carbon and nitrogen, emphasizing the utility of MIMS to accurately determine isotope ratios. The isotope ratios for the treated cells were significantly different from the natural abundance of \(^{13}\text{C}\) and \(^{15}\text{N}\), indicating that the isotopes had been incorporated into newly synthesized proteins within the cells. The results in this work signify that MIMS can measure \(^{13}\text{C} / ^{12}\text{C}\) and \(^{15}\text{N} / ^{14}\text{N}\) ratios on a subcellular scale and will therefore be an important method for monitoring protein turnover.

In a separate experiment, a distinct heterogeneous distribution of \(^{12}\text{C}^{15}\text{N}, ^{32}\text{S},\) and \(^{16}\text{O}\) was imaged across a human hair shaft on a subcellular scale with a 35 nm lateral resolution.\(^{62}\) Melanin granules, which are important for hair pigmentation and photo-protection, were shown to contain a low amount of sulfur containing proteins. Interestingly, the melanin granules exhibited an \(^{16}\text{O}\) gradient which implied the granules had undergone oxidation damage from UV light at the end of the granule exposed to the light. Furthermore, the isotopic ratio of \(^{34}\text{S} / ^{32}\text{S}\) was measured to be 0.0453 in the hair shaft, which was equivalent to the expected terrestrial abundance ratio for this element. Because the isotopic ratio of sulfur can be measured so accurately with MIMS, it will be possible to conduct \(^{34}\text{S}\) labeling experiments to follow the synthesis of sulfur-containing compounds in the hair shaft.

Lechene has also demonstrated that MIMS is a useful technique to image and quantitate isotope ratios in free fatty acids (FFA) on the subcellular scale.\(^{63}\) Adipocytes
are cells that sequester FFA from the blood and store them as triacylglycerol in lipid droplets, which are 1 \( \mu \)m sized features within the cells. Lechene incubated adipocytes in \(^{13}\)C oleic acid and with MIMS imaging found a higher level of \(^{13}\)C accumulation in the lipid droplets than in the extracellular space or in the remaining intracellular space (Figure 1-18). These results illustrated that FFA transport across the cell membrane is protein mediated because FFA moves up a concentration gradient. In addition to a supporting a hypothesis for the controversial FFA transport mechanism, this work was yet another example of the wide-range of subcellular, biological questions that isotope ratio quantification with MIMS imaging can answer.
The Levi-Setti research lab is a third group renowned for using the elemental imaging capabilities of SIMS to investigate biological conundrums. One study obtained

Figure 1-18: Images of unwashed adipocytes treated with $^{13}$C oleate: BSA. $^{63}$ MIMS images of A. $^{12}$C and B. $^{13}$C. C. MIMS ratio image of $^{13}$C/$^{12}$C showed the enhanced accumulation of isotopically labeled lipid in the lipid droplets. MIMS images of D. $^{12}$C$^{14}$N and E. $^{12}$C$^{15}$N. F. MIMS ratio image of $^{12}$C$^{15}$N/$^{12}$C$^{14}$N had no contrast because no exogeneous $^{15}$N was added. G. A hue saturation intensity (HIS) image of the $^{13}$C/$^{12}$C ratio. H. A reflection differential interference (DIC) image prior to MIMS analysis. I. A MIMS ratio image of $^{13}$C$^{14}$N/$^{12}$C$^{14}$N further demonstrated the accumulation of labeled lipid in the lipid droplets. Scale bar is 5 µm.
50 nm lateral resolution SIMS images of the cation distribution in mammalian interphase cells, mitotic cells, and isolated metaphase chromosomes, supporting a role for cations in the regulation of the cell cycle. The SIMS images demonstrated that the localization of Na\textsuperscript{+} and K\textsuperscript{+} remained constant during the cell cycle but Mg\textsuperscript{2+} and Ca\textsuperscript{2+} changed depending on the stage of the cell cycle: in the interphase these divalent cations were predominately in the cytosol, whereas during mitosis they were aggregated in the chromatin. Quantification of the SIMS signal revealed that the total intercellular divalent cation concentration did not change significantly during the cell cycle. From this data, the authors concluded that progression through the cell cycle resulted in a redistribution of divalent cations without a major influx or efflux of the ions. Therefore, Mg\textsuperscript{2+} and Ca\textsuperscript{2+} likely play a role in chromatin compaction only during the mitotic stage, whereas Na\textsuperscript{+} and K\textsuperscript{+} are involved in compaction throughout the cell cycle. A combination of SIMS imaging and immunofluorescence indicated that only Ca\textsuperscript{2+} localized at the chromosome axis with the nonhistone binding protein, topoisomerase II\textalpha{} (Topo II) (Figure 1-19). It follows that Ca\textsuperscript{2+} probably inhibited the catalytic activity of the protein and played an important role in stabilizing the chromosomal scaffolding proteins, such as Topo II. When the authors incubated mitotic chromosomes with chelating agents, SIMS and immunofluorescence images exhibited a loss of Topo II and a decondensed chromosome structure, indicating that Ca\textsuperscript{2+} must also be involved in maintaining chromosome structure during mitosis. This research provided the first evidence, without secondary markers, that cations have specific, functional interactions with chromatin binding proteins and that cations help control chromosome structure during the cell cycle.
Static SIMS molecular imaging has also been applied to single cell investigations. The seminal paper in this area presented static SIMS images of molecular species, as well as elemental analytes, on dopant-treated paramecia. The cocaine molecular ion, a cocaine fragment ion, dimethyl sulfoxide, and potassium all co-localized to the treated protozoa. This work demonstrated the capability of SIMS to image molecular analytes across single cells, thus paving the way for single cell studies of large molecule drugs and biological molecules, such as vitamins and membrane lipids.

Identifying mass spectral peaks from biological molecules in a complex system, such as a cell membrane, is very challenging. Therefore, experiments that associate a peak of a particular mass with a fragment of a biological molecule mark significant advances toward realizing the utility of SIMS for biological applications. One important peak identification was made by Todd and coworkers after they observed abundant high-mass peaks at m/z 184 and m/z 86 in several types of tissue. The precursor of these fragments was narrowed down to phosphatidylcholine and sphingomyelin, two prominent cell membrane lipids which are found in all types of tissue. MS/MS spectra of phosphatidylcholine and sphingomyelin standards verified that the two lipids fragment to

![Image of Ca²⁺ SIMS image and Topo II antibody immunofluorescence (IF) image of metaphase chromosomes](image)

Figure 1-19: Ca²⁺ SIMS image and Topo II antibody immunofluorescence (IF) image of metaphase chromosomes. The cation and scaffolding protein antibody colocalize, indicating that Ca²⁺ binds to Topo II and mediates its activity.
yield peaks at m/z 184 and m/z 86. Specifically, this experiment ascertained that a peak at m/z 184 results from phosphocholine and a peak at m/z 86 results from choline. The identification of mass spectral peaks for lipid fragments presented the option of using SIMS imaging to investigate the lipid distribution in tissue, model membrane systems, and cells.

Experiments with model systems have proven that SIMS imaging of lipid domains is possible and therefore the goal of studying the functionality of lipid domains in cells can be realized. The Benninghoven and Winograd labs have actively investigated lipid domains in Langmuir-Blodgett (LB) films using SIMS. An LB film is a uniform one-molecule thick layer of organic material, in this case a mixture of lipids, prepared by controllably compressing a solution of the material of interest. Benninghoven used LB films as lung surfactant model systems to assess lipid involvement in the compression/exhalation and expansion/inhalation of the lung. An LB film composed of DPPC, dipalmitoylphosphatidylglycerol (DPPG), and the lung surfactant protein (SP-C) was prepared and upon severe compression, a distinctly heterogeneous distribution of the film components was observed. Specifically, SP-C reorganized into a SP-C-rich phase following film compression, which supported the hypothesis that lung compression causes non-DPPC components to leave the monolayer.

Winograd and Ewing have also investigated LB films with static SIMS. In these experiments, LB films of varying amounts of DPPC, dipalmitoylphosphatidylethanolamine (DPPE), and cholesterol were analyzed. Micrometer-sized cholesterol domains were observed in LB films of mixtures containing DPPE; however, LB films containing the same percentage of cholesterol and DPPC were
homogeneous. The authors explained these results by the interaction strength differences between the three molecules. The DPPE headgroup is known to interact more strongly with other phospholipid headgroups than with cholesterol. Therefore, the phospholipids preferentially aggregated, which excluded cholesterol and resulted in lipid domains. The propensity of cholesterol to induce domain formation implicated a functional role for cholesterol in cell signaling and trafficking. For example, cholesterol-induced domain formation could influence the interaction of APP with cleavage enzymes, thus causing an accumulation of Aβ.

In the two LB experiments described, Benninghoven and Winograd have demonstrated the utility of LB films as model systems for cells in SIMS imaging experiments. Also, SIMS images of large-scale lipid domains in LB films have further substantiated the eventual use of this technique to investigate biomolecule distributions in cells.

Liposomes are another common artificial model for cell membranes. Ewing, Winograd, and colleagues captured molecule-specific images of dynamic membrane events during several stages of fusion between chemically distinct multilamellar liposomes. Liposomes 20 µm in size were prepared that contained cholesterol and either dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidyl-N-monomethylethanolamine (DPPNME), or dipalmitoylphosphatidyldimethylethanolamine (DPDME), the separate batches of liposomes were mixed onto a single substrate, and the samples were frozen after various mixing times. Chemical snapshots of three stages of liposome fusion were obtained: liposomes in close proximity, liposomes undergoing initial contact, and liposomes that had full fused (Figure 1-20). Interestingly, during
fusion the liposomes joined at a contact point, where lipid heterogeneity was evident. DPPC and DPPNME were only homogeneous at regions distant from the contact point, where lipid spreading and mixing had occurred. Fully fused liposomes displayed a completely uniform lipid distribution. These results showed that liposome fusion involves initial lipid domain formation followed by a homogeneous redistribution of lipids throughout the fused liposomes. This publication highlighted liposome model systems as an effective way to investigate membrane dynamics. Furthermore, this work demonstrated that SIMS can capture dynamic biological events and can image lipid domains on a subcellular sized scale.
Figure 1-20: SIMS images of freeze-fractured liposomes. Liposomes have distinct lipid compositions: DPPC/cholesterol, DPPNME/cholesterol, and DPDME/cholesterol, that were mixed together and frozen after different times. In all images, the blue pixels represent water (m/z 17.9-19.1). From left to right, the images are mapped for C$_3$ hydrocarbons (m/z 40.9-43.3, yellow), two phosphorylated headgroups (various m/z, yellow and red), and cholesterol (m/z 384.1-387.1, yellow). A. DPPC/cholesterol and DPPDME/cholesterol liposomes were mixed and frozen after 30 s of mixing. The phosphorylated headgroups mapped in the center column were phosphocholine (m/z 183.9-184.3, yellow) and phosphodimethylethanolamine headgroup (m/z 169.9-170.3, red). The two types of liposomes have not yet begun to fuse. B. and C. DPPC/cholesterol and DPPNME/cholesterol liposomes were frozen together after B. 120 s and C. 60 s of mixing time. The phosphorylated headgroups mapped in the center column were phosphocholine (m/z 183.9-184.3, yellow) and phospho-N-monomethylethanolamine (m/z 155.9-156.3, red). A homogeneous distribution of the two phospholipids was observed after full fusion in B., but a heterogeneous distribution was evident during initial fusion in C.
In addition to imaging lipids in model membrane systems, many research groups have applied SIMS imaging to single cell membranes. Ewing and Winograd were able to localize lipids to single rat pheochromocytoma (PC12) cell membranes. PC12 cells are tumor cells derived from the adrenal gland in the New England Deaconess Hospital strain rat. PC12 cells are an exciting system for neurochemistry applications because they can be differentiated to have neuronal-like properties when exposed to the nerve growth factor (NGF) protein. Therefore, PC12 cells undergo regulated exocytosis and can be used to learn if lipid composition effects the abnormalities in exocytosis observed in many neurological diseases. Additionally, this mammalian cell line is attractive because it is immortal, meaning that the cells can double an infinite number of times. Hence, PC12 cells lines are relatively easy to maintain and grow at a fast rate, offering a large population of cells for SIMS analysis. Using SIMS, Ewing, Winograd, and colleagues imaged phosphocholine and cholesterol across single PC12 cells. This study highlighted the ability of SIMS to detect multiple lipids in biological samples.

In a separate experiment, Ewing, Winograd, and co-workers found that freeze-fracture sample preparation can expose different sections of a cell. PC12 cells were dyed with the lipophilic dye, 1,1’–dioctadecyl-3,3’,3’–tetramethylindocarbocyanine perchlorate (DiI), which is known to incorporate only to the outer leaflet of the membrane. In this way, a three-dimensional chemically heterogeneous cell system was created. The outer leaflet of the cell membrane contained lipid and DiI, the inner leaflet of the cell was composed of lipid and no DiI, and the interior of the cell contained elevated amounts of potassium. Using SIMS imaging of stained PC12 cells, chemical signatures for five possible cell sections were determined. Two of these cell sections
were from exposure of the outer leaflet (top or bottom) of the cell membrane during fracturing. In this scenario, the SIMS images showed colocalization of DiI and phosphocholine, while sodium, potassium (ions present in the freezing medium), and water were depressed at the site of the cell. The top and bottom inner leaflet of the membrane were two other cell sections that were exposed during freeze-fracture. These cell sections were identified by localization of phosphocholine and potassium to the cell, an absence of DiI signal, and decreased intensities of water and sodium at the area of the cell. Finally, it was possible to fracture through a cell. Through-cell fractures were characterized by a strong localization of potassium and some phosphocholine from free lipid in the cytoplasm, lipid from organelles, and lipid from the edges of the cell membrane. This cell section contained no DiI signal and showed an inverse localization of water and sodium. Identifying chemical signatures that define the section of a cell present at the surface-vacuum interface was an important advance for the biological SIMS community. Because static SIMS is a surface sensitive technique, researchers benefit the most from analyzing properly sectioned cells for a given application. For example, inner and outer leaflet fractured cells will be most useful to study the role of lipid domains in exocytosis.

Besides the lipids and steroids discussed above, another important chemical in membrane chemistry is vitamin E because it has been implicated in lipid oxidation and therefore numerous neurodegenerative disorders and also may influence membrane-dependent enzymes. Recently, Sweedler and colleagues discovered that a peak at m/z 430 in biological samples arises from vitamin E. Along with this peak identification, the Sweedler group has reported exciting SIMS images that suggest a possible functional
role for vitamin E in neuronal membranes. Subcellular molecular images of an isolated neuron from *Aplysia californica* revealed that vitamin E (m/z 430 and m/z 165) was strongly localized to the junction between the cell soma and the neurite, compared to the rest of the cell body (Figure 1-21). The authors compared a normalized vitamin E signal for the soma-neurite junction to the cell body and found that the junction contained 165±11% more vitamin E. These results suggested a possible structural role for vitamin E, in terms of modulating the membrane radius of curvature at the junction, and a possible functional role, in terms of transport mechanisms and neuronal signaling.
Along with mammalian cells, other types of cells have been investigated with SIMS to progress towards the goal of understanding lipid distribution during membrane fusion events. For example, mating *Tetrahymena thermophila* were imaged with SIMS by Ewing, Winograd and coworkers.\(^7^0\) During mating, two *Tetrahymena* join at their anterior ends and their membranes fuse creating hundreds of nanometer-sized fusion pores within a well-defined 8-µm region, called the conjugation junction. SIMS images

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**Figure 1-21:** Molecule-specific SIMS images and corresponding line scans of an isolated single neuron.\(^6^9\)  
**A.** SIMS image of choline, m/z 86, a fragment of phosphatidylcholine and a common marker for the cell membrane.  
**B.** SIMS image of a fragment of a lipid acyl chain, m/z 69, another common cell membrane marker.  
**C.** SIMS image of vitamin E, m/z 430, which is strongly localized to the junction between the cell body and the neurite.  
**D.** Line scans for vitamin E (top) and choline (bottom) normalized to m/z 69 and taken along the line indicated in the inset, beginning at the soma. Vitamin E displays a higher signal at the soma-neurite junction than the rest of the cell body, whereas, the choline signal is relatively constant across the distance.
revealed a heterogeneous distribution of signals for specific lipids across the mating cells. This work is presented in chapter 6 as an elegant example of the capability of molecular SIMS imaging to probe subcellular, micrometer-sized membrane regions that contain structures in the nanometer regime.

**Sample Preparation and Instrumental Design Improvements in Biological SIMS**

Although all of these application-based advances have been invaluable in promoting SIMS as a bio-analytical technique, instrument and sample preparation developments have also been an important part of the picture. First, Ewing, Winograd, and colleagues determined the ideal freeze-fracture conditions to maintain the lipids in their native location in the membrane and to minimize the condensation of water on top of the membrane.\(^5^6\) Using liposomes composed of DPPC, Ewing demonstrated that a small temperature difference of only 5°C can result in a successful fracture versus an unsuccessful fracture. When the sample was 5°C too warm, phosphocholine (m/z 184) was not localized and homogeneous across the surface. Fracturing at a temperature 5°C too cold resulted in water condensation that covered the lipid membrane and prevented its analysis. Freeze-fracture at a temperature of -105°C is ideal at a pressure of 10\(^{-7}\) torr.

Ewing, Winograd, and coworkers also improved SIMS imaging ability by incorporating epifluorescence microscopy into the optical assembly of the instrument.\(^3^9\) With this design development, it was possible to use four *in-situ* imaging techniques: SIMS imaging, scanning ion microscopy, reflected bright field microscopy, and epifluorescence microscopy. Epifluorescence was a valuable instrumental addition
because it allowed easy location of small fluorescently labeled cells in ice. Ewing located 15-µm PC12 cells fluorescently labeled with DiI and subsequently imaged the cells with SIMS.39

One notable challenge in SIMS has been that information in the images is limited because of the low secondary ion yield of biomolecules. To circumvent this obstacle and improve signal, different sample preparation protocols and instrumental advances have been developed. Sjovall and colleagues developed an alternate sample preparation methodology in which cell membranes were imprinted onto Ag foil ex-situ.71 Membranous matter was transferred from cells adhered onto glass to a Ag foil by placing the foil onto the cells and applying a specific pressure to the backside of the foil with the researcher’s thumb. The foil was then transferred into the SIMS analysis chamber. This method was advantageous because silver cationization enhanced the secondary ion signal from biological fragment ions and molecular ions. In one particular experiment, the distributions of phosphatidylcholine and cholesterol were imaged, with a 500 nm lateral resolution, across blood cell imprints. The sample preparation method was also advantageous because freeze-fracture can be difficult and time consuming and therefore ill-suited for some applications.

Instrumental developments have also aided to improve secondary ion signal for biological applications. A significant instrumental advance in this respect has been the advent of cluster primary ion sources, which has revitalized biological SIMS, as previously discussed.51-53 A particularly popular cluster ion source has been C_{60}^+. Vickerman and colleagues have reported impressive secondary ion yields produced by C_{60}^+ relative to Ga+. For example, the yield enhancements for a thin film of DPPC were
158 times for m/z 100-200 and 3985 times for the molecular ion.\textsuperscript{53} This signal enhancement will make analyte signal easier to discern from noise in complex biological samples. Also, the contrast between adjacent pixels in images will be improved because resolution in ToF-SIMS images is determined by the number of ions that are collected at each pixel.

The abundance of previous SIMS research provides a wealth of examples showcasing the compatibility of this technique and biological applications. Already, SIMS has been used to study the effects of cancer drugs on single cells, to elucidate the role of cations in isolated metaphase chromosomes, to image large-molecule dopants and lipids across single cells, and to investigate large-scale lipid domains in model systems. The ambitious pursuit of challenging applications, coupled with instrumental advances, has set the groundwork for future studies of the cellular functionality of lipid domains.

**Summary and Thesis Overview**

Lipids are suspected to play an active role in membrane fusion events, such as sexual reproduction and exocytosis. Understanding this involvement will lead to a more complete picture of normal cell functionality and will help to explain abnormal cell behavior observed in some diseases. In particular, certain types of lipids are believed to promote fusion while other types of lipids may inhibit fusion. It follows that membrane regions concentrated in fusion-promoting lipids may be sites where biological fusion preferentially occurs. However, lipid domains have not yet been experimentally observed in membrane fusion events. In order to support the hypothesis that lipid
domains drive biological fusion, imaging techniques that chemically resolve molecules on a submicrometer scale will be necessary. ToF-SIMS is a technique that can image the distribution of molecules across samples with a potential spatial resolution of several hundred nanometers. In order to adapt this surface sensitive, UHV technique to the analysis of hydrated biological samples, ToF-SIMS has been coupled with a freeze-fracture sample preparation method.

In this thesis, ToF-SIMS imaging following freeze-fracture is further developed to allow the eventual investigation of the role of lipids in exocytosis. First, the effect of freeze-fracture on SIMS ionization is thoroughly studied. The water matrix is shown to greatly increase the secondary ion yield of phosphatidylcholine (PC) by providing an excess of matrix protons for ionization. It is also determined that ambient water, present in the analysis chamber for freeze-fractured samples, affects PC ionization.

The ionization of six other lipid classes is examined in chapter 3. Unique mass spectral peaks are identified for each type of lipid and these peaks are correlated with fragments of the parent ion. Knowing signature peaks for the different lipids provides a mechanism to identify these lipids in a chemically complex system like the cell membrane. The signature peaks are then used to chemically image a variety of lipids patterned onto a substrate. The secondary ion yield and imaging quality for lipids is greatly improved by using the cluster ion primary projectile, C_{60}^+, instead of the atomic projectile, In^+.

In chapter 4, the ionization of phosphatidylethanolamine (PE) is then more thoroughly investigated because this lipid is particularly difficult to detect in the presence of PC. Three characteristics of PE molecules are identified which differentiate them from
PC molecules and which cause their poor ionization. First, PE molecules are less hydrophobic and less sterically hindered which seems to inhibit their ionization. More importantly, the relative gas-phase basicities of the lipids seem to cause PC to ionize in lieu of PE. Knowing that PC abstracts protons from the surrounding matrix with greater ease than PE will enable future instrumental improvements for the detection of PE. For example, incorporating laser post-ionization into the cell imaging experiments will allow the neutral sputtered PE molecules to be ionized and detected.

In addition to fundamental lipid ionization experiments, single cell imaging applications are explored in chapters 5 and 6. Freeze-fracture conditions affect the sputter yield and ionization of analytes making it difficult to quantitatively compare cell samples fractured separately. Chapter 5 discusses the development of in-situ two-color fluorescence to allow the simultaneous analysis of two separate cell populations that have been treated under different conditions and subsequently combined into one sample and freeze-fractured under the same conditions. It is shown that the fluorescence characteristics of the labeled cells allow the treatment conditions for each cell to be discerned prior to SIMS analysis. Two populations of macrophages are labeled with the fluorophores DiI and DiD and are further incubated in serum-free media (control) and media containing β-cyclodextrin-cholesterol complex, respectively. Work described in chapter 5 demonstrates that cells in the two populations retain the original fluorophore with which they were labeled. Additionally, the cholesterol content of the separately treated cells is quantified by SIMS imaging, indicating a 99% increase in cholesterol content in the cell membranes of β-cyclodextrin-cholesterol treated cells, compared to control. This work paves the way for future comparative studies of single cells and
demonstrates the ability to quantify, on a relative basis, the chemical composition of different cells.

In a set of separate experiments, for the first time, a heterogeneous lipid distribution has been chemically imaged across cells with ToF-SIMS. In chapter 6, I show that PC is diminished and that 2-aminoethylphosphonolipid (2-AEP) is increased at the aggregated fusion pore sites in mating *Tetrahymena thermophila*. These results are exciting because they support the hypothesis that lipids mediate membrane fusion, and particularly that domains of cone-shaped lipids, like 2-AEP, catalyze biological fusion. By demonstrating the ability to image large-scale (~10 µm) lipid heterogeneity in cells, these experiments set the groundwork for future SIMS investigations into single exocytotic events of PC12 cells.

The seventh chapter in this thesis discusses preliminary SIMS imaging of several other cell systems that can be used to achieve the long term goal of better understanding the role of lipids in single membrane fusion events. Also, chapter 7 explores the possibility of molecular depth profiling through single cells with the C$_{60}^+$ primary projectile. Molecular depth profiling will allow 2D SIMS images of cell membranes to be acquired as the surface is gently sputtered away. The combination of 2D images from each new depth will provide a 3D image of a single cell with nanometer-scale depth resolution.
References


Chapter 2

Proton Transfer in ToF-SIMS Studies of Frozen-Hydrated Dipalmitoyl-Phosphatidylcholine

Introduction

Static time-of-flight secondary ion mass spectrometry (ToF-SIMS) imaging has been developed as a unique tool for imaging the location of molecules across biologically relevant surfaces, including lipids across the membranes of single cells.\textsuperscript{1-5} These cellular samples present new requirements in preparation and analysis. Specifically, the advancement of this technique has depended upon the maintenance of the structural and chemical integrity of cells when introduced to the ultrahigh vacuum environment of the instrument.\textsuperscript{2, 6-9} Freeze-fracture methods similar to those used in scanning electron microscopy have been used to analyze elements and molecules in biological samples with SIMS.\textsuperscript{2, 5, 7, 10} Methods have been developed to study phospholipids in cellular membranes which are exposed to the surface by freeze-fracture and remain in their native hydrated state throughout analysis.\textsuperscript{2, 5, 9}

One of the most common positively charged phospholipid ions observed in static SIMS, phosphocholine ion, has been shown to form from phosphatidylcholine and sphingomyelin.\textsuperscript{11-13} As a result, the phosphocholine fragment ion is important in studying these phospholipids in membranes. Since phosphatidylcholine has a large variety of tailgroups in cell membranes, detection of molecular ions is difficult. On the other hand, detection of phosphocholine is relatively easy since it results from ionization of any lipid
molecules containing the phosphatidylcholine headgroup. Although a mechanism for the ionization of phosphocholine in ToF-SIMS has not previously been described, a mechanism has been proposed for the formation of this ion in fast-atom bombardment mass spectrometry (FAB-MS). This mechanism involves a cleavage of phosphocholine from the glycerol backbone and an intramolecular abstraction of a proton from the backbone. The addition of a second proton results in a positive ion with a mass to charge ratio (m/z) of +184 (Figure 2-1).
Several groups have used phosphocholine as a marker for phosphatidylcholine-containing lipids in ToF-SIMS images of biological samples.\textsuperscript{1, 2, 10, 11, 13, 15} Todd, et al. have shown the phosphocholine ion, resulting from the ionization of phosphatidylcholine lipids and sphingomyelin, as a predominant ion in freeze-dried brain slices.\textsuperscript{11} The same ion has been imaged in freeze-dried red blood cells and varies in intensity between different samples depending upon the sample preparation.\textsuperscript{1} Cannon, et al. have imaged

Figure 2-1: Phosphocholine fragmentation and ionization mechanism proposed with FAB-MS.
the location of dipalmitoyl-phosphatidylcholine (DPPC) in freeze-fractured frozen-hydrated liposomes using ToF-SIMS and have even imaged instances of lipid exchange between liposomes of unique lipid composition. More recently, the phosphocholine ion was imaged across surfaces of single freeze-fractured rat pheochromocytoma cell membranes.

The conditions of freeze-fracture are critically important in obtaining ToF-SIMS images from frozen samples. Since static ToF-SIMS is a surface-specific technique, sublimation and condensation of water at the surface significantly alters the resultant images. Specifically, studies of phosphatidylcholine liposomes have shown that the temperature at which samples are fractured is important in balancing the flux of water at the surface. When fractures occur at relatively high temperatures, water is rapidly sublimed, resulting in homogenous images of solutes from the sublimed solution and other species that have diffused across the surface. On the other hand, when fractures occur at relatively low temperatures, water is condensed on the surface, resulting in homogenous images of condensed water instead of the underlying object of interest. Even with the optimized fracture conditions, the flux of water released during the fracture and the resulting effects in the surface spectra have not been well understood for these analyses.

In this chapter, positive ion spectra from thin films and freeze-fractured samples are compared to demonstrate a 300% enhancement of phosphocholine signal and about 2 orders of magnitude in DPPC [M+H]^+ signal in frozen water suspensions. Deuterated phosphatidylcholine standards and deuterated water matrices are used to determine that protonation of phosphocholine in ToF-SIMS occurs largely from extra-molecular proton
sources, supporting the matrix-enhancing effect of a frozen water matrix. Using residual gas analysis during freeze fracture of a deuterated sample, it is shown that both sample water and water condensed onto the outside surface of the sample are released during freeze fracture. In addition, other sources of water that emerge during sample preparation and analysis are identified and the effects this water has on the resulting ToF-SIMS spectra are discussed. Most importantly, since freeze-fracture techniques provide the ability to analyze biological samples in a frozen-hydrated state, enhanced phosphatidylcholine lipid signal is available for improved ToF-SIMS images of cell membranes.

**Experimental**

**Thin Film Sample Preparation**

Dipalmitoyl-phosphatidylcholine (DPPC) was obtained from Sigma, (St. Louis, MO) and deuterated DPPC derivatives (D-75, D-62, D-13) were obtained from Avanti Polar Lipids (Alabaster, AL). Each of the standards was dissolved in chloroform (0.1 mg/mL). 5-µL aliquots of each were delivered onto silicon substrates and allowed to dry. The samples were then transferred directly to the sample stage for ToF-SIMS analysis.

**Freeze-Fractured Sample Preparation**

DPPC suspensions were prepared by sonicating and freezing in either H₂O (Millipore (Bedford, MA) MilliQ Synthesis purified) or D₂O (Sigma-Aldrich (St. Louis,
MO) 42,345-9, >99.96% isotopic purity). H₂O samples were stored in liquid nitrogen prior to use, while D₂O samples were analyzed immediately after freezing to limit hydrogen exchange with the environment. The details of sample introduction and freeze-fracture in ultrahigh vacuum were previously described. Briefly, the sample, sandwiched between two pieces of silicon, was held in a cold stage (Kurt J. Lesker, Pittsburgh, PA) at -196°C and slowly warmed to -105°C. Next, the top piece of silicon was removed with a cold knife, exposing a fresh surface of the aqueous sample for analysis. A residual gas analyzer (RGA) using electron impact ionization, quadrupole mass analysis, and electron multiplier detection (VacCheck100, MKS Industries, Branford, CT), was used to monitor vacuum gases during the sample preparation and fracture. Water (m/z 18) and D₂O (m/z 20) were monitored over time during the freeze-fracture experiments using Spectra software, included with the RGA. Following freeze-fracture the sample was cooled to -196°C and placed into the cold sample analysis stage.

**ToF-SIMS Instrumentation**

Analyses were performed in a Kratos (Manchester, U.K.) Prism ToF-SIMS spectrometer. In this instrument, the gallium or indium primary ion beam was generated with a FEI (Beaverton, OR) liquid metal ion gun (LMIG) using either a 15 or 25-kV beam potential. The ion type, beam voltage, and primary ion dose were the same for compared spectra in the results, and the exact conditions of each experiment are described in the corresponding figure captions. The beam was pulsed for 50-ns, creating packets of ions which were focused to a spot-size 200 nm in diameter as they hit the
sample. After impact, the analysis stage, which was cooled with liquid nitrogen for frozen experiments, (Kore Tech. LTD, Cambridge, UK) was biased at +2.5 kV, accelerating positive secondary ions from the sample normal to the surface. An electrostatic lens, biased at –4.7 kV, focused and extracted secondary ions to the 4.5 m path reflectron time-of-flight mass analyzer. A microchannel plate assembly (Galileo Co., Sturbridge, MA) detected ions, separated by their mass/charge ratio, at the end of this flight path. A LeCroy time-to-digital converter collected the resulting signal which is subsequently processed on a Sun workstation using Kratos ToF-SIMS software.

Results and Discussion

Water Matrix Enhancement of DPPC Ionization

Prior to SIMS imaging experiments, it is desirable to acquire standard spectra of the substances of interest to determine unique peaks and possible fragment structures that occur during ionization. Dipalmitoyl-phosphatidylcholine (DPPC) has a molecular weight of 734 Da and a structure as shown in Figure 2-2A. A typical positive ion mass spectrum of an anhydrous thin film of this lipid from 0 to 800 m/z is shown in Figure 2-3. As expected, phosphocholine ion signal is observed at m/z 184. The headgroup-containing fragment at m/z 224 represents DPPC after loss of the palmitoyl tail groups of the molecule. A headgroup fragment at m/z 166 and the choline ion at m/z 86 are also abundant. Hydrocarbon fragments of the molecule dominate the rest of the spectrum.
Molecular or protonated DPPC ions are not observed in the thin film experiment under these experimental conditions.

Figure 2-2: Structures of DPPC and its isotopes. A. Dipalmitoyl-phosphatidylcholine (DPPC), B. D-13 DPPC, C. D-62 DPPC, and D. D-75 DPPC. The glycerol carbon numbers are denoted in A.
Figure 2-3: Spectrum of an anhydrous DPPC thin film (1.0 × 10^{12} \text{ Ga}^+ \text{ ions/cm}^2; 25-kV beam voltage). Data collected by Donald Cannon Jr. and Thomas Roddy.
To more closely represent freeze-fractured cell samples, freeze-fractured samples of DPPC in H₂O have been analyzed using the same primary ion, beam energy, and dosage conditions used in the thin film experiments. As shown in Figure 2-4, signal of higher mass ions is surprisingly high compared to that obtained from thin film DPPC, even yielding signal from the protonated ion \([\text{M+H}]^+\) of the lipid (m/z 735). In addition to the DPPC and hydrocarbon signal, water clusters are also present in lesser amounts throughout the spectrum. The presence of the molecular ion signal likely arises from matrix effects similar to those observed with other techniques like matrix-assisted laser desorption or matrix-enhanced SIMS.\textsuperscript{16-18} The matrix may increase molecular ion intensity by providing a higher abundance of available protons for ionization or by absorbing excess energy and minimizing molecular fragmentation. It is important to understand the mechanism that allows observation of higher mass ions because increased signal and a larger mass range would improve the sensitivity and spatial resolution of images obtained from biological samples.
Figure 2-4: Spectrum of frozen-hydrated DPPC ($1.0 \times 10^{12}$ Ga$^+$ ions/cm$^2$; 25-kV beam voltage). Data collected by Donald Cannon Jr. and Thomas Roddy.
Sources of Protons for DPPC Ionization in Anhydrous Films

To investigate the source of protons for DPPC ionization, different isotopes of the molecule and the matrix were investigated. First, three stable isotopic forms of DPPC were analyzed in anhydrous thin films. The D-13 form of DPPC (Figure 2-2B) has the thirteen hydrogen atoms of the headgroup substituted with deuterium. In Figure 2-2C, the D-62 form of DPPC has all of the hydrogen on the tailgroups substituted with deuterium. Finally, in Figure 2-2D, the D-75 form of DPPC has all of the headgroup and tailgroup hydrogens substituted with deuterium. It is noteworthy that five hydrogen atoms are present in the glycerol backbone of all four structures.

It is interesting to compare the ionization mechanism associated with the solid film samples reported here to the FAB ionization mechanism where the target molecule is dissolved in liquid glycerol. As shown in Figure 2-1, the hydrogen atom on the 2-carbon acts as a hydrogen source in the FAB ionization mechanism. If the same mechanism occurs during ionization from the film, the surrounding matrix should only be a source for the second hydrogen or deuterium contributing to the phosphocholine peak. Therefore, the isotopic composition of the matrix should only be relevant for one of the two protons involved in the ionization mechanism for PC. As a result, the intensity of the m/z 184 (PC) peak versus the isotope peaks at m/z 185 (PC+1) and m/z 186 (PC+2) should be representative of the natural isotopic abundances of DPPC for the intramolecular proton abstraction as well as the isotopic abundances of the surrounding matrix for the second proton addition.
As shown by the spectra in Figure 2-5, there are clear deviations from this distribution. As expected, in the spectrum of DPPC (Figure 2-5A), the majority of signal is detected at 184 m/z, representing the PC m/z and peaks present at m/z 185 and m/z 186 are present in their natural isotopic abundance. In the D-13 DPPC spectrum, the m/z 184 peak profile is shifted 13 amu to m/z 197 due to the deuterium addition on the headgroup. In addition, there is a greater abundance of signal corresponding to PC+1 m/z and PC+2 m/z due to the presence of deuterium on the phosphate group instead of hydrogen. This addition of deuterium occurs either in sample preparation or during SIMS ionization. Due to the relatively low abundance of free deuterium ions in chloroform and in dried film samples under ultrahigh vacuum, these proton exchanges most likely occur between fragments of lipid in the gas phase during ionization.
Figure 2-5: Spectra of four anhydrous thin films of stable isotopes of DPPC (1 × 10^{12} \text{Ga}^{+} \text{ions/cm}^2; 25-kV beam voltage). Structures of the headgroup ion are shown for each spectrum. Data collected by Donald Cannon Jr. and Thomas Roddy.
Additional evidence of deuterium addition is shown in the D-62 and D-75 DPPC spectra. When the palmitoyl (C16) tailgroups are completely saturated with deuterium, there is an even greater abundance of the PC+1 and PC+2 ions (m/z 185 and 186). Furthermore, saturation of the headgroup and tailgroups with deuterium in D-75 DPPC leads to the greatest amount of deuterium addition, resulting in relatively little PC ion (m/z 197) and large amounts of PC+1 and PC+2 ions. Since the degree of isotope substitution directly affects the peak profiles, it is likely that protons from the substrate or adjacent lipid molecules contribute to ionization of DPPC in SIMS of dried thin films.

Sources for Protons in DPPC Ionization in Frozen-Hydrated Samples

Static ToF-SIMS analyses of cells are often performed in a frozen water matrix which could act as a source of protons for ionization. To investigate these proton sources, spectra of frozen suspensions of DPPC have been collected using D₂O and H₂O as the matrices. The important phosphocholine region of a spectrum of freeze-fractured DPPC in H₂O is shown in Figure 2-6A. The same spectral region of freeze-fractured DPPC in D₂O is shown in Figure 2-6B. The intense PC+1 peak (m/z 185) and, more significantly, the PC+2 (m/z 186) peaks are clear evidence supporting the transfer of deuterium from the aqueous matrix during phosphocholine formation. It is noteworthy that if PC is completely surrounded by D₂O, the majority of the signal should be in the PC+1 and PC+2 peaks. Although there is a change in the peak profile that implies deuterium addition from the matrix, there is not a complete shift of the peaks. This is
likely a result of the DPPC aggregating in the aqueous solution and partially acting as its own matrix, providing hydrogen from intramolecular sources and adjacent molecules.
Figure 2-6: Spectra of freeze-fractured DPPC. A. Spectrum of freeze-fractured DPPC frozen in H₂O. B. Spectrum of freeze-fractured DPPC frozen in D₂O. (1 × 10¹¹ In⁺ ions/cm²; 15-kV beam voltage).
Conversely, the spectrum of D-75 DPPC frozen in H₂O should contain peaks corresponding to the PC ion plus 13 m/z (m/z 197) to account for the 13 deuterium atoms in the headgroup ion. Unfortunately, isobaric interference of the [H₂O]₁₁⁺ cluster ions occurs in the peaks of interest at m/z 197, 198, and 199, so another phosphocholine containing peak is required for this comparison. In Figure 2-7, a fragment that contains phosphocholine and part of the glycerol backbone (gPC), was compared for A) DPPC in H₂O, B) DPPC in D₂O, and C) D-75 DPPC in H₂O. As expected, DPPC in H₂O produces a profile of gPC (m/z 224), gPC+1 (m/z 225), and gPC+2 (m/z 226) resembling the natural isotope abundances of these peaks (Figure 2-7A). The spectrum of DPPC in D₂O (Figure 2-7B) contains more intense gPC+1 and gPC+2 peaks implying the addition of matrix deuterium to the fragment. The spectrum of D-75 DPPC in H₂O, obtained to investigate the addition of hydrogens to a deuterated headgroup ion is shown in Figure 2-7C. The spectrum contains a higher ratio of gPC to gPC+1 than what was observed in anhydrous D-75 DPPC, but it is not entirely gPC. Again, this is evidence of H₂O matrix proton addition, however gPC+1 and gPC+2 are also observed. As explained in the previous example, the D-75 DPPC aggregates in solution and is probably acting as its own matrix in addition to H₂O.
Figure 2-7: Spectra of freeze-fractured DPPC.  **A.** Spectrum of freeze-fractured DPPC frozen in H$_2$O.  **B.** Spectrum of freeze-fractured DPPC frozen in D$_2$O.  **C.** Spectrum of freeze-fractured D-75 DPPC in H$_2$O.  (1 × 10$^{11}$ In$^+$ ions/cm$^2$; 15-kV beam voltage).
Monitoring Water Flux During Sample Freeze-Fracture

The observed matrix effect of water on the ionization of phosphatidylcholine suggests that environmental water originating from either the transfer procedure or from the residual gas in the vacuum system may condense on samples to provide protons for ionization. To reveal the extent of this effect, deuterated water has been employed to distinguish whether environmental water or water from the sample itself participates in the ionization process during ToF-SIMS of freeze-fractured DPPC samples. In the experiment, DPPC samples are suspended in D_{2}O and fractured prior to analysis in the sample preparation chamber. A residual gas analyzer in the preparation chamber is used to monitor water flux from the sample during the relatively violent process of freeze-fracture. Ideally, the preparation chamber is pumped to less than 10^{-8} torr prior to freeze-fracture. After the sample is warmed to -105°C, the silicon shard covering the frozen D_{2}O sample is removed with a cold knife resulting in a large pressure spike in the chamber. Residual gas analysis is used to track the partial pressures of H_{2}O and D_{2}O during a successful freeze-fracture of DPPC in a D_{2}O matrix (Figure 2-8A). Since the sample matrix is D_{2}O, the H_{2}O pressure spike must originate from water that condenses onto the sample while freezing and transporting to the instrument. The pressure of H_{2}O increases by a factor of ten relative to baseline, whereas, the pressure of D_{2}O increases by a factor of 100. Hence, we conclude that most of the pressure burst consists of solvent molecules.
Figure 2-8: Residual gas analysis traces of the partial pressures of H$_2$O and D$_2$O in the sample preparation chamber during two freeze fractures. The time of fracture is indicated by the arrow. **A.** Successful freeze fracture at -106°C with little effects of water on the sample. **B.** Unsuccessful fracture at – 103°C demonstrating several sources of water as described in the text. For this case, the Si shard fell onto a warm part of the chamber and subsequently contributed to the background water pressure. At this point, the sample was much cooler and this adventitious source of water condensed on top of the sample. Data collected by Donald Cannon Jr. and Thomas Roddy.
Within minutes, this pressure is reduced to baseline pressures by a cryogenically cooled shroud and a turbomolecular pump (described in detail by Cannon, et al.\textsuperscript{10}). During this time, water condenses or sublimes from the surface of the sample, depending upon the pressure and the temperature. For a successful fracture, the surface remains at equilibrium and does not significantly condense or sublimes water, which would perturb the molecular structure of the surface. For unsuccessful fractures, pressures do not quickly return to baseline, as shown in Figure 2-8B, and poor ToF-SIMS spectra, which are dominated by water peaks, are obtained. For this particular example, the stage was held at a temperature above -105°C during fracture and water from the sample sublimed after fracture. As the stage was cooled toward -190°C, water condensed onto the sample and the ToF-SIMS spectra was characteristic of the water-covered surface. This example illustrates that water can either be helpful or detrimental to static SIMS experiments. By monitoring the pressure spike and its subsequent return to baseline behavior, it is possible to assess whether a fractured sample will be a good candidate for SIMS analysis.

After a successful freeze-fracture, the sample is kept at liquid nitrogen temperatures at approximately $5 \times 10^{-9}$ to $5 \times 10^{-8}$ torr throughout the subsequent ToF-SIMS analysis. Consequently, it is possible for water to condense onto the surface of the sample. To investigate the effects of possible condensation of endogenous water, spectra were collected from a freeze-fractured DPPC sample for over two hours with an approximate $1 \times 10^{-8}$ torr working pressure. The water and phosphocholine regions of spectra are shown in Figure 2-9. The water signal increases over time, whereas the phosphocholine signal decreases over time, indicating that the surface is being covered by water during the analysis.
In summary, while water enhances ionization of DPPC in frozen aqueous samples, water that condenses onto the sample after freeze fracture inhibits phosphocholine signal. Therefore, for optimal DPPC signal it is necessary to monitor pressures in the instrument throughout the experiment and to monitor the signal of water in spectra during ToF-SIMS analysis.

Figure 2-9: Detrimental effect of water condensation on SIMS results. A. Spectra of the water region of a freeze-fractured sample at the beginning of the experiment and after two hours. B. Spectra of the phosphocholine headgroup region of a freeze-fractured sample at the beginning of the experiment and after two hours. (1 × 10^{11} \text{In}^+ \text{ions/cm}^2; 15\text{-kV beam voltage}).
Summary

Freeze-fracture techniques have been used to prepare aqueous samples for ToF-SIMS analyses. More high-mass ion signal is obtained from frozen-hydrated DPPC than the corresponding dried thin film samples for m/z 184 and 734. This matrix-enhancing effect is possibly due to the abundant proton sources in the matrix of frozen aqueous samples and was supported by mass spectra of DPPC and deuterated DPPC derivatives. Specifically, ToF-SIMS analyses of thick films of DPPC isotopes and of frozen-hydrated DPPC isotopes in H\textsubscript{2}O and D\textsubscript{2}O have shown that protons leading to the formation of the phosphocholine ion can be obtained from the region surrounding the molecule in addition to the intramolecular sources previously shown in FAB-MS. The monitoring of H\textsubscript{2}O and D\textsubscript{2}O with a residual gas analyzer has allowed the optimization of conditions for freeze-fracture ToF-SIMS experiments. Also, spectral analysis has demonstrated that the condensation of water onto freeze-fractured samples during ToF-SIMS analysis affects the ionization of DPPC, so monitoring water signal is crucial during analysis. Overall, freeze-fracture preparation techniques have made it possible to analyze biological samples in their native frozen-hydrated state and to enhance the signal of DPPC for improved ToF-SIMS imaging experiments.
References


Chapter 3

SIMS Imaging of Lipids in Picoliter Vials with a Buckminsterfullerene Ion Source

Introduction

Numerous biological functions, including signal transduction, protein regulation, and secretion, are managed by the diverse array of molecules in the cell membrane. Investigation of the subtle intricacies of membrane proteins and lipids is essential to gaining an improved knowledge of cellular events. For instance, assemblies of sphingolipid and cholesterol, called lipid rafts, are believed to activate signaling proteins by controlling protein-protein interactions.\(^1\) A major limitation in the study of these lipids is the lack of a methodology with high sensitivity and spatial resolution for the lipids.

The lipids of the cell membrane are highly diverse because of the large variety of possible headgroups, backbones, fatty acid carbon chain lengths, and degrees of unsaturation. Although a certain amount of lateral fluidity exists among the membrane lipids\(^2\), domains of restricted lipid diffusion have been observed in cells and model systems.\(^3-9\) The resulting heterogeneous distribution probably mediates biological function by controlling the curvature of the cell membrane and the interaction of membrane proteins.\(^10\)

To elucidate the role of lipid heterogeneity, domains have been studied with a variety of analytical techniques. In a two-color confocal microscopy experiment, Hao
and coworkers witnessed the coalescence of nanometer-sized lipid domains into observable-sized domains in cholesterol-depleted Chinese hamster ovary cell membranes. In addition, a study with fluorescence recovery after photobleaching demonstrated the restricted diffusion of fluorescently labeled phosphatidylcholine and proteins at inter-domain regions in human fibroblast cells. While experiments with these and other fluorescence techniques have contributed a great wealth of knowledge about lipid domains, they suffer from indirect chemical localization, and the fluorescent tags have been shown to interfere with the native chemical distribution of the membrane. Other analytical techniques, such as atomic force microscopy and electron microscopy, give valuable topographical and morphological information, but no chemical specificity. Spatially resolved chemical images of the lipid distribution in single-cell membranes will expand the current understanding of the role of lipid heterogeneity in biological function and disease.

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) can be used for chemical imaging of lipid domains on cell membranes. This technique has been well-established for bioanalytical applications involving tissues, liposomes, and cells. For example, Chandra and co-workers have used dynamic SIMS to determine the efficiency of boron neutron cancer therapy drugs by monitoring the subcellular elemental distribution of boron in treated human glioblastoma cells. In addition to the elemental imaging allowed by dynamic SIMS, static SIMS is capable of molecular imaging because the low dose of incident ions (less than 1 % of the total number of surface atoms) minimizes analyte fragmentation. Static SIMS imaging of single cells has been challenging, however, because it is unfavorable to place hydrated cellular samples into
the ultra high vacuum environment of a mass spectrometer. Therefore, a freeze-fracture technique has been adapted from the electron microscopy community to maintain the native distribution of biomolecules in cell samples. This freeze-fracture sample preparation enabled Ewing, Winograd and colleagues to spatially resolve hydrocarbon, cocaine, and DMSO in dopant-treated, freeze-fractured paramecia. An exciting advance towards the chemical imaging of lipids was made when Todd and coworkers found a prominent SIMS signal at m/z 184 in dog adrenal tissue and established that it was phosphocholine, the headgroup fragment of phosphatidylcholine (PC). Further progress toward imaging the distribution of lipids on cell membranes was achieved when phosphocholine was localized to single freeze-fractured rat pheochromocytoma (PC12) cells. Additionally, there have been numerous examples of ToF-SIMS imaging of lipid domains in Langmuir Blodgett model film systems, establishing that this technique can be similarly applied to image domains on cell membranes. Molecular images of cell membrane domains were achieved recently when a heterogeneous distribution of phosphatidylcholine and 2-aminoethylphosphonolipid was observed at the junction of mating Tetrahymena. More recently, a heterogeneous distribution of vitamin A was observed on Aplysia californica neuronal membranes, demonstrating that SIMS can also image non-lipid membrane components.

The use of cluster ion primary sources in SIMS is an important advance in the instrumentation that will aid in examining the low concentrations of specialized cell membrane lipids. These cluster sources, notably SF$_5^+$, gold cluster ions Au$_n^+$, and buckminsterfullerene, C$_{60}^+$, boast a greatly improved secondary ion signal which will be
essential for future cell membrane investigations.\textsuperscript{27-29} In particular, using C\textsubscript{60},
Vickerman and colleagues have demonstrated over a 100-fold ion yield enhancement for
some organic samples.\textsuperscript{29} In this chapter, I characterize the SIMS spectra of PE, PG, PS, PI, cholesterol, and sulfatide and identify unique mass spectral peaks for each membrane component. Using the molecule-specific imaging abilities of static ToF-SIMS, I further
demonstrate that the signature peaks enable these lipids to be spatially resolved in
picoliter vial arrays. A 40 to 1000-fold improvement in signal is observed with C\textsubscript{60} for
PE, PG, PS, PI, cholesterol, sulfatide, and PC compared to the Ga\textsuperscript{+} ion source. This yield
enhancement results in better contrast in SIMS images providing greater promise for
future studies of the lipid distribution in single cell membranes.

\textbf{Experimental}

\textbf{Thick Film Sample Preparation}

Dipalmitoylphosphatidylethanolamine (DPPE), dipalmitoylphosphatidylglycerol
(DPPG), dipalmitoylphosphatidylserine (DPPS), soybean phosphatidylinositol (PI),
cholesterol, sulfatide, and dipalmitoylphosphatidylcholine (DPPC), (Avanti Polar Lipids,
Alabaster, AL) were dissolved in 9:1 chloroform:methanol at a 1 mg/mL concentration.
The lipid solutions were spin-coated onto pirhana-etched silicon wafers (Ted Pella,
Redding, CA) for standard analyses and mixture experiments.
Picoliter Vial Array Solution Delivery

The imaging experiments utilized picoliter vial arrays nanofabricated at the Cornell University National Nanofabrication Facility, as described elsewhere. Samples were delivered into the picoliter vials using micropipets made from borosilicate capillaries (Sutter Instrument Co., Novato, CA P/N B120-69-10, 1.2 mm o.d., 0.69 mm i.d.) that were pulled to a fine tip using a Sutter Instrument Co. (Novato, CA) pipet puller. For each lipid solution, a virgin micropipette was backfilled using a microliter syringe (Hamilton, Reno, NV); the syringe was thoroughly washed with chloroform before administering the next solution. The nontapered end of each micropipet was attached to a General Valve Picospritzer (Fairfax, NJ), which controlled the delivery volume by regulating the pressure (3 psi) and pulse length (5 ms) applied to the pipet. The tip of the micropipette was positioned to directly contact the bottom of each picoliter vial with the aid of a micromanipulator stage and a stereomicroscope (Leica Stereozoom5, Bannockburn, IL).

Secondary Ion Mass Spectrometry

Unless otherwise noted, experiments were performed on a Kratos Prism ToF-SIMS spectrometer (Manchester, U.K.) equipped with an indium liquid metal ion source (FEI, Beaverton, OR). The pulsed primary ion source was angled at 45° to the sample and was operated at 15-kV beam energy, with a 500-pA beam current, a 200-nm beam diameter at the surface, and a 50-ns pulse width. Secondary ions were electrostatically directed to the time-of-flight tube by a sample stage biased at ±2.5 kV and by an
extraction lens oppositely biased at 4.5 kV. Prior to detection by a microchannel plate assembly (Galileo Co., Sturbridge, MA), the secondary ions were mass-separated in a 4.5-m horizontal reflectron time of flight path. Spectra for all thick-film lipid analyses were taken from a sample area of 300 x 300 µm² and with one million primary ion pulses.

Mass spectrometry images were acquired by rastering the primary ion beam across the sample region and collecting a mass spectrum for each pixel in the image. Using computer software written in-house, a peak of interest was then selected in the total ion mass spectrum. The intensity of this mass was plotted in micrometer-scale pixels to generate an image showing the location of the analyte. The color of the pixels could be converted to red, green, or blue using the ToF-SIMS software to distinguish between different chemical maps. Additional colors were possible by using Spot Advanced imaging software (Diagnostic Instruments, Sterling Heights, MI) to alter the hue of the SIMS images. The color-coded chemical maps were overlaid using either the ToF-SIMS software or the Spot Advanced software.

Additional imaging and ion yield comparison experiments were executed using a BioToF ToF-SIMS spectrometer, which features a C₆₀⁺ primary ion source and a gallium primary ion source (Ionoptika Ltd., Southampton, U.K.). The instrument and ion sources have been described in detail elsewhere.²⁹, ³¹ For the C₆₀⁺ picoliter imaging experiments, a 300-µm beam-defining aperture in the gun column was used to create a 30-µm probe size and 0.25 nA of primary ion current. In the ion yield comparison experiments, the C₆₀⁺ beam was focused to 50-µm with a 1-mm beam-defining aperture and 1.1 nA of primary ion current; the Ga⁺ primary ion beam was focused to approximately 200 nm and
3 nA of primary ion current. The smaller probe size was chosen for imaging experiments to allow finer image resolution but for the ion yield comparison experiments, image resolution was not as important a factor. For all ion yield comparison experiments, a baseline was determined for each lipid peak by finding a nearby flat region of the spectrum and determining the average signal in this region of the spectrum. This value was subtracted from the intensity in every bin for the peak of interest. The ion yields were then determined by calculating the peak area from the baseline-subtracted intensities and normalizing to the primary ion current. Finally, for all peaks of interest, ratios were taken of the resulting ion yields obtained using the two primary ion sources.

Results and Discussion

Overview of SIMS of Lipids

The SIMS fragmentation and ionization of PC has been well characterized \(^\text{12, 32}\); however, the abundance of diverse and functionally important lipids in the cell membrane necessitates the investigation of the SIMS spectra for other lipids (Figure 3-1). The non-lamellar phospholipids, phosphatidylethanolamine (PE, Figure 3-1A), phosphatidylglycerol (PG, Figure 3-1B), phosphatidylinerine (PS, Figure 3-1C), and phosphatidylinositol (PI, Figure 3-1D), are interesting membrane species. It is hypothesized that a heterogeneous distribution of non-lamellar lipids drives or restricts membrane fusion events, such as exocytosis, by reducing or increasing the packing
constraints associated with highly curved fusion structures. Cholesterol (C, Figure 3-1E) is also an intriguing membrane component because it holds lipid rafts together and because abnormalities in membrane cholesterol levels have been observed in atherosclerosis and bacterial, viral, and pathogenic diseases, such as HIV and Alzheimer disease. Another notable membrane component is the glycosphingolipid, sulfatide (S, Figure 3-1F), which is found in many organ tissues. For example, sulfatide is present in the islets of Langerhans in the pancreas, where it is suspected to have a role in insulin trafficking, and is found in myelin, where it may be involved in demyelination diseases, such as multiple sclerosis.
The ionization of six biologically relevant lipids was studied with SIMS in order to provide a basis for future experiments that will assess the involvement of these lipids in cellular events. Standard spectra were generated by analyzing anhydrous films of the individual lipids of interest with an $\text{In}^+$ ion source (Figure 3-2 and 3-3). These spectra were compared, and unique positive and negative fragment ion peaks were identified for each of the lipids (Table 3-1). For the phospholipids, these characteristic peaks were phosphate headgroup fragments because, in general, the samples examined were synthetic dipalmitoyl phospholipids, which all have the same fatty acid side-chains. This
experimental design was chosen to focus the analysis on the headgroups because the large variety of fatty acid tailgroup structures in a biological membrane makes it difficult to detect molecular ion or subtle tailgroup variations with current SIMS technology.

Common positive ion peaks were found at m/z 125, 143, 165, 313, and 552 (C_{35}H_{68}O_4\textsuperscript{+}) for most lipids and therefore are either fatty acid fragment peaks or common contaminants introduced during synthesis or sample preparation. Similarly, common negative ion peaks were 79 (PO_3\textsuperscript{−}), 97 (HPO_4\textsuperscript{−}), 137, 153, 181, and 255 (C_{16}H_{31}O_2\textsuperscript{−}, palmitic acid).

### Table 3-1: Characteristic SIMS Fragment Peaks for Several Lipid Classes

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Fragment</th>
<th>Calculated Mass (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylcholine</td>
<td>C_{5}H_{12}N\textsuperscript{+}</td>
<td>86.0970</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>C_{5}H_{15}NPO_4\textsuperscript{+}</td>
<td>184.0739</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>C_{8}H_{19}NPO_4\textsuperscript{+}</td>
<td>224.1052</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>C_{2}H_{7}NPO_3\textsuperscript{+}</td>
<td>124.0164</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>C_{2}H_{7}NPO_4\textsuperscript{+}</td>
<td>142.0269</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>C_{2}H_{5}NPO_3\textsuperscript{−}</td>
<td>122.0007</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>C_{2}H_{3}NPO_4\textsuperscript{−}</td>
<td>140.0113</td>
</tr>
<tr>
<td>Phosphatidylglycerol</td>
<td>C_{3}H_{9}PO_6Na\textsuperscript{+}</td>
<td>195.0035</td>
</tr>
<tr>
<td>Phosphatidylglycerol</td>
<td>C_{3}H_{9}PO_6\textsuperscript{−}</td>
<td>171.0059</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>C_{6}H_{10}PO_8\textsuperscript{+}</td>
<td>241.0114</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>C_{6}H_{12}PO_9\textsuperscript{−}</td>
<td>259.0219</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>C_{3}H_{16}PO_9\textsuperscript{−}</td>
<td>299.0533</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>C_{3}H_{8}NPO_6Na\textsuperscript{+}</td>
<td>207.9988</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>C_{27}H_{45}\textsuperscript{+}</td>
<td>369.3521</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>C_{27}H_{45}O\textsuperscript{+}</td>
<td>385.3470</td>
</tr>
<tr>
<td>Sulfatide</td>
<td>C_{6}H_{9}SO_8Na\textsuperscript{+}</td>
<td>263.9916</td>
</tr>
</tbody>
</table>
Signature peaks were identified for PE, PG, PS, PI, cholesterol, and sulfatide. The ionization of PC has been described in detail. Phosphatidylethanolamine ionized to form intense positive ion peaks at m/z 142 and 124 corresponding to the phosphate headgroup and the headgroup minus water, respectively, (Figure 3-2A). The peak observed at m/z 282 probably resulted from C_{18}H_{36}NO^+, a plastic extrusion lubricant and common lab contaminant, and not from PE. The characteristic positive ion peaks for several phospholipids were sodium adducts of the phosphate headgroups (PG, m/z 195, (Figure 3-2B); PS, m/z 208 (Figure 3-2C)) and of the molecular ion (PG, m/z 767, (Figure 3-2B)). These lipids were Na salts and the formation of matrix adduct ions was expected because there was a high concentration of matrix Na available for ionization and because this ionization behavior has been observed in fast atom bombardment mass spectrometry (FAB-MS) for phosphatidylserine. Phosphatidylinositol did not exhibit any unique peaks in the positive mode (Figure 3-2D). For the cholesterol sample, the molecular ion was detected at m/z 385 (M-H)^+ and an additional intense positive fragment at m/z 369 was identified to be the molecular ion minus a hydroxyl group (Figure 3-2E). Similar to the Na salts of PG and PS, the characteristic positive ion peak for the sulfatide salt was a Na adduct of the sulfate headgroup minus water (Figure 3-2F).
The negative ion peaks for PE were detected at m/z 122 and 140, which were analogous to the headgroup and headgroup minus water peaks observed in the positive mode (Figure 3-3A). Ionization of PG also resulted in a unique negative fragment ion at m/z 171 which was the deprotonated phosphate headgroup (Figure 3-3B). The negative ion spectrum of PS did not show any characteristic peaks unique to this lipid (Figure 3-3C). Phosphatidylinositol exhibited unique fragment peaks only in the negative ion
mode of operation at m/z 241, 259, and 299, consistent with reports in the literature for FAB-MS (Figure 3-3D). Cholesterol (Figure 3-3E) and sulfatide (Figure 3-3F) spectra indicated a few unique negative ion peaks which have not yet been identified and may result from contaminants (C, m/z 123, 195, 465, 499) (S, m/z 217, 261).

Figure 3-3: Negative SIMS spectra for six lipids. A. DPPE  B. DPPG  C. DPPS  D. PI (soybean)  E. Cholesterol  F. Sulfatide.
Chemical Imaging in Picoliter Vials

To determine whether the signature peaks discussed in this chapter could be used to chemically map the lipids in spatially different environments, an array of lithographically fabricated picoliter vials was used. Four different lipids were delivered one by one into adjacent silicon picoliter vials (Figure 3-4). The imaging area of the instrument has a maximum field of view of 400 x 400 μm, which is slightly larger than four vials. Figure 3-4A shows an image of the four lipids in picoliter vials obtained with an In\(^+\) ion source. Signals from PC (m/z 184), PG (m/z 195), and sulfatide (m/z 264) headgroups and from cholesterol (m/z 369) were localized to the vials into which they were delivered. In this image, the blue pixels represent regions of high silicon signal and black pixels represent low silicon signal due to substrate coverage by the lipid film. The void in signal also indicates that the lipid signal is quite low under these experimental conditions, emphasizing the importance of improving secondary ion yield for biological species.
Use of the C$_{60}^+$ Ion Source for Lipids

One method of increasing the signal of large organic species, like the lipids in question, is to increase the sputter yield by using cluster ion primary sources, like C$_{60}^+$. Molecular dynamic simulations suggest that, in contrast to atomic projectiles such as In$^+$ and Ga$^+$, C$_{60}^+$ cluster projectiles break into and disperse the primary projectile energy among individual carbon atoms upon surface impact. This phenomenon allows for a more shallow penetration depth and more surface-localized primary bombardment energy for cluster projectiles in comparison to their atomic counterparts. An additional benefit is that large, intact molecules are gently lifted off the surface by this “softer” C$_{60}^+$ sputtering process. Therefore, C$_{60}^+$ and other cluster ion sources are attractive alternatives as
SIMS primary ion sources for biological applications, where there is a desire to greatly reduce molecular fragmentation of larger-mass target analytes. To evaluate the magnitude of signal improvement for lipids with $C_{60}^+$, the picoliter vial array was also imaged in a separate SIMS instrument equipped with the cluster ion source (Figure 3-4B). Clearly, the $C_{60}^+$ bombardment resulted in a dramatic increase in signal from the lipids. Interestingly, while the positive ion lipid signals using $C_{60}^+$ were increased between 80- and 700-fold compared to the yield when In$^+$ was used, the signal from nearby silicon was changed by only 4-fold. This result is not surprising because the real advantage of the $C_{60}^+$ source over a liquid metal ion source is the ability to remove large, organic, molecular fragments from the surface. Thus, the relative amounts of lipids sputtered with $C_{60}^+$ versus In$^+$ were comparatively much greater than the amount of atomic species sputtered.

The data reported in Figures 3-4A and B were collected on two different instruments. In a more thorough investigation of lipid signal enhancement the secondary ion signals resulting from $C_{60}^+$ bombardment were compared to the signals resulting from Ga$^+$ bombardment on the same instrument. This eliminated variations in signal resulting from differences in instrument throughput and detector gain. The increased secondary ion signal from the selected lipids was remarkable, ranging from 40 to 1000 (Table 3-2, Figure 3-5). The high mass peaks (above m/z 700) were enhanced the most with the cluster ion source, demonstrating the ability of $C_{60}^+$ bombardment to gently lift-off intact molecules from the surface. I have generally observed two times the signal obtained with Ga$^+$ when using an In$^+$ primary projectile. The difference in yield between these two atomic projectiles probably stems from the difference in atomic weight. Thus the signal
enhancement when using a C_{60}^{+} ion source versus In^{+} ion source should be half as much but still highly significant, ranging from 20 to 500 improvement, which is consistent with the data in Figure 3-4.

Table 3-2: A comparison of the secondary ion yields of various lipid fragments as obtained with a Ga^{+} and a C_{60}^{+} primary ion source

<table>
<thead>
<tr>
<th>Lipid Fragment</th>
<th>Ga^{+} Yield/ nC</th>
<th>C_{60}^{+} Yield/ nC</th>
<th>C_{60}/ Ga</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC (m/z 86)</td>
<td>2.1 x 10^9</td>
<td>8.5 x 10^{11}</td>
<td>410</td>
</tr>
<tr>
<td>PC (m/z 184)</td>
<td>4.5 x 10^9</td>
<td>1.2 x 10^{12}</td>
<td>280</td>
</tr>
<tr>
<td>PC (m/z 224)</td>
<td>3.3 x 10^8</td>
<td>8.1 x 10^{10}</td>
<td>240</td>
</tr>
<tr>
<td>PC (m/z 734)</td>
<td>4.5 x 10^7</td>
<td>3.6 x 10^{10}</td>
<td>800</td>
</tr>
<tr>
<td>PE (m/z 124)</td>
<td>9.6 x 10^8</td>
<td>8.7 x 10^{10}</td>
<td>90</td>
</tr>
<tr>
<td>PE (m/z 142)</td>
<td>7.4 x 10^8</td>
<td>4.9 x 10^{10}</td>
<td>70</td>
</tr>
<tr>
<td>PE (m/z 691)</td>
<td>1.2 x 10^8</td>
<td>2.9 x 10^{10}</td>
<td>240</td>
</tr>
<tr>
<td>PE (m/z 122)</td>
<td>1.4 x 10^9</td>
<td>4.7 x 10^{11}</td>
<td>320</td>
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<tr>
<td>PE (m/z 140)</td>
<td>2.1 x 10^9</td>
<td>4.9 x 10^{11}</td>
<td>240</td>
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<tr>
<td>PG (m/z 195)</td>
<td>7.4 x 10^8</td>
<td>1.6 x 10^{11}</td>
<td>220</td>
</tr>
<tr>
<td>PG (m/z 767)</td>
<td>1.0 x 10^8</td>
<td>7.2 x 10^{10}</td>
<td>720</td>
</tr>
<tr>
<td>PI (m/z 241)</td>
<td>2.6 x 10^8</td>
<td>1.6 x 10^{11}</td>
<td>640</td>
</tr>
<tr>
<td>PI (m/z 259)</td>
<td>2.1 x 10^8</td>
<td>1.4 x 10^{11}</td>
<td>670</td>
</tr>
<tr>
<td>PI (m/z 299)</td>
<td>7.0 x 10^7</td>
<td>6.8 x 10^{10}</td>
<td>970</td>
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<td>PS (m/z 208)</td>
<td>1.8 x 10^8</td>
<td>4.7 x 10^{10}</td>
<td>260</td>
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<td>PS (m/z 757)</td>
<td>3.9 x 10^7</td>
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<td>750</td>
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<tr>
<td>Cholesterol (m/z 369)</td>
<td>2.3 x 10^6</td>
<td>1.6 x 10^{10}</td>
<td>70</td>
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<tr>
<td>Cholesterol (m/z 385)</td>
<td>1.2 x 10^8</td>
<td>6.1 x 10^{9}</td>
<td>50</td>
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<td>Sulfatide (m/z 906)</td>
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<td>Silicon (m/z 28)</td>
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<td>1.2 x 10^{11}</td>
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Identification of Lipids in a Mixture by SIMS

The absence of isobaric interferences between the different lipids of interest should allow them to be distinguished in a complex lipid mixture. As a rough mimic of...
the lipid complexity of a cell membrane, an equimolar mixture of seven lipids (PC, PE, PG, PS, PI, cholesterol, sulfatide) was examined with C$_{60}^+$ (Figure 3-6). Both positive ion (Figure 3-6A) and negative ion (Figure 3-6B) spectra were necessary to identify all seven lipids. PC was readily ionized and dominated the positive ion mixture spectrum. It was easily possible to identify the presence of PG, PS, PI, cholesterol, and sulfatide, demonstrating the ability of this technique for analyzing these lipids in complex mixtures. In contrast, the positive ions of PE did not give strong signal, apparently for two reasons. First, there are many low mass cholesterol fragments in the same mass range, thus masking the signal. Second, the m/z 124 and 142 peak areas are diminished by 10-fold when mixed with PC. The reason for the PE signal suppression by PC, observed in five separate experiments, probably arises from charge exchange processes within the matrix and is the subject of further investigation.
Figure 3-6: SIMS spectra of lipid mixtures. A. +SIMS spectrum of an equimolar mixture of PC, PE, PG, PS, PI, cholesterol (C), and sulfatide (S) with a C$_{60}^+$ source. B. –SIMS spectrum of the same mixture.
Summary

Using model systems and picoliter vials, I have shown that the signature peaks for phosphatidylcholine, phosphatidylglycerol, phosphatidylycerine, phosphatidylinositol, cholesterol, and sulfatide in static SIMS can be used to distinguish several membrane lipids both spatially and in a mixture. The signature peaks were used to generate chemical maps of the lipids in a picoliter vial array and should be similarly valuable for molecular imaging the distribution of lipids on cell membranes. A 40- to 1000-fold improvement in signal from the lipids has been shown using a C$_{60}^+$ cluster ion source versus a Ga$^+$ ion source. The signal enhancement of lipids with cluster ion sources and SIMS is especially significant because it will allow for improved contrast between adjacent image pixels. Ultimately, this advancement in instrumentation will enable subtle differences in lipid concentration across a cell membrane to be probed.
References


Chapter 4

Effects of Hydrogen Bonding, Steric Hindrance, and Hydrophobicity in Phosphatidylethanolamine Ionization by ToF-SIMS

Introduction

The biological membrane is a compelling aspect of cell biology because a vast array of cellular functions involves membrane components, making the membrane the site of numerous interesting molecular interactions. For instance, the cell membrane plays an active role in signal transduction, membrane trafficking, and intra- and inter-cellular communication.¹ The cell membrane is comprised of a diverse assembly of lipids, proteins, and carbohydrates, which each contribute to the complexity of the membrane. One major class of lipid, called phospholipids, is composed of a hydrophilic phosphate headgroup attached to a hydrophobic tailgroup, which is made up of a glycerol with two fatty acid side chains (see Figure 1-2).¹ Thermodynamics dictates that the phospholipids in a cell membrane adopt a bilayer structure. A lipid bilayer is two adjacent layers of lipid arranged so that the hydrophilic headgroups face outward and the hydrophobic tailgroups are sequestered from the hydrophilic, aqueous environment (see Figure 1-3). Phospholipids introduce a great amount of complexity to membranes because of the numerous alcohols, such as choline and ethanolamine, which can be incorporated into the phosphate headgroup and because of the variations carbon chain length and degree of saturation that exists in the tailgroup. This large diversity of
phospholipids suggests that these molecules are not simply a structural background of the membrane but that they may play important functional roles as well.

One possible functional role for phospholipids is to facilitate membrane fusion events, such as exocytosis and sexual reproduction. Membrane fusion allows two aqueous volumes, which were initially separated by the two bilayers, to join and thus enable, for example, the release of neurotransmitter into the extra-cellular space or the transmission of genetic material between cells. When two membranes fuse they are first positioned in propinquity (<2-3 nm) and then merge by way of various high curvature structural rearrangements of the bilayer, determined theoretically and observed experimentally (see Figure 1-5). Various proteins have been linked with biological membrane fusion and may either direct the necessary bilayer structural rearrangement or define the fusion site and supply a thermodynamic driving force for fusion to occur. The idea that lipids may control biological fusion to some degree has been supported by experimental observations that protein-free liposomes can fuse under favorable conditions.

The intrinsic curvature hypothesis suggests that lipid heterogeneity in cellular membranes is important for membrane fusion. This hypothesis is based on the observation that different classes of phospholipids have different dynamic shapes. The shape of a lipid is determined by the physical cross-sectional areas of the headgroup and tailgroup regions, the degree of headgroup hydration, and the effects of counter-ions, charge, and hydrogen-bonding. Some type of lipids may aid biological fusion because they have a shape that readily adopts the high curvature intermediate membrane structures involved in fusion. For example, phosphatidylethanolamine (PE) is a cone-
shaped lipid that fits well into structures with a high radius of curvature and its presence in a membrane may ease the bilayer through the fusion process.\textsuperscript{12} Other lipids may hinder fusion by their inability to fit into high curvature structures. Cylindrical lipids, such as phosphatidylcholine (PC), do not promote biological fusion.\textsuperscript{12} In support of this concept, it was recently experimentally shown that the amount of the cone-shaped lipid, 2-aminoethylphosphonolipid (2-AEP), is elevated at membrane fusion sites of mating \textit{Tetrahymena}, whereas PC is depleted in those regions.\textsuperscript{13}

Development of our understanding of the structure and function of lipids in membranes is continual; however, the chemical complexity of biological membranes presents enormous analytical chemistry challenges. Various techniques have been implemented to investigate the lipid distribution in biomembranes including fluorescence recovery after photobleaching (FRAP),\textsuperscript{14} two-photon fluorescence microscopy,\textsuperscript{15, 16} near field scanning optical microscopy (NSOM),\textsuperscript{17} electron microscopy,\textsuperscript{18} and time-of-flight secondary ion mass spectrometry (ToF-SIMS) imaging.\textsuperscript{13, 19} ToF-SIMS imaging is an attractive technique because it can determine the molecular composition and distribution across biological membranes without making inferences from the indirect chemical localization of fluorescent labels or macromolecular markers.\textsuperscript{13, 15, 16, 18-21} ToF-SIMS images are typically comprised of over 10,000 mass spectra plotted in a 2-D array of pixels. Snap-shots of the fusion of two chemically distinct liposomes have been captured with ToF-SIMS which revealed an initial heterogeneous lipid distribution followed by a homogeneous redistribution upon full fusion.\textsuperscript{19} ToF-SIMS images have also exposed a strong localization of vitamin E to the junction between the cell body and neurite of a
single neuron. Segregation of 2-AEP and PC in mating *Tetrahymena* was also observed using the imaging capability of ToF-SIMS.

From these examples, it is evident that TOF-SIMS imaging will be an invaluable tool to further examine the functional role of membrane lipids in cell biology. In order to realize the full potential of this technique for biological applications, the SIMS ionization of several lipids and cholesterol was investigated in chapters 2 and 3 of this thesis. One interesting observation reported in chapter 3 was that although characteristic mass spectral peaks could be used to identify PE, the peaks became strongly masked in complex lipid mixtures.

This chapter explores the SIMS ionization mechanism of PE to understand the signal suppression of PE and to devise analytical approaches to circumvent this obstacle. Specifically, thick films of deuterated PE have been analyzed to pinpoint the proton sources involved in PE ionization by SIMS. From the resulting data, it is concluded that the PE headgroup and headgroup minus water fragments form by intra- and intermolecular proton exchange, similar to the PC headgroup fragment ionization discussed in chapter 2. Additionally, mass spectra of structural analogues of PE have been used to correlate signal suppression with molecular structure. A trend is observed in which the signal suppression of PE and its analogues worsens with an increased number of headgroup hydrogen bond donation sites, decreased steric hindrance on the headgroup amine, and increased hydrophobicity. In a cell membrane, the propensity of PE to donate protons to adjacent molecules is most likely the largest factor that would contribute to the signal suppression. PE appears to sacrifice protons to other molecules, and possible analytical solutions to improve the SIMS detection of PE are therefore explored, such as
increasing sputter yield with cluster projectiles and enhancing ionization with laser postionization.

**Experimental**

**Thick Film Sample Preparation**

Dipalmitoylphosphatidylethanolamine (DPPE), D-62 DPPE, dipalmitoylphosphatidylmonomethylethanolamine (PMME), dipalmitoylphosphatidyltrimethylethanolamine (PDME), dicaproylphosphatidylethanolamine (DCPE), distearoylphosphatidylethanolamine (DSPE), and dipalmitoylphosphatidylcholine (DPPC) (Avanti Polar Lipids, Alabaster, AL) were dissolved in 9:1 chloroform:methanol at a 1.4 mM concentration. Thick films were prepared by spin-coating 50 µL of the lipid solutions onto pirhana-etched silicon wafers (Ted Pella, Redding, CA). The spin coating was performed at 0.5 rpm for approximately 1 min followed by faster spin rates of 1 rpm and 2 rpm for 30 s each. Thick films of mixtures were prepared the same way, using solutions containing 1.4 mM of each lipid.

**Secondary Ion Mass Spectrometry**

All experiments used a Kratos Prism ToF-SIMS spectrometer (Manchester, U.K.) equipped with an indium liquid metal ion source (FEI, Beaverton, OR), angled at 45° to
the sample, operated at 15-kV beam energy, 500-pA beam current, a 200-nm beam diameter at the surface, and a 50-ns pulse width. Secondary ions were electrostatically directed to the time-of-flight tube by a sample stage biased at +2.5 kV and by an extraction lens biased at -4.5 kV. Prior to detection by a microchannel plate assembly (Galileo Co., Sturbridge, MA), the secondary ions were mass-separated in a 4.5-m horizontal reflectron time of flight path. Spectra for all thick-film lipid analyses were collected using a sampling area of 300 x 300 µm² and one million primary ion pulses.

**Results and Discussion**

**PE Ionization Mechanism**

The ionization of PE by ToF-SIMS involves the formation of positively charged fragment ions at m/z 142 and 124, corresponding to the protonated phosphate headgroup and the protonated phosphate headgroup minus water (Figure 4-1A). Throughout this discussion, these two peaks will be referred to collectively as PE headgroup peaks. The PE headgroup peaks are very difficult to detect in mixtures, as shown in chapter 3. Specifically, the presence of PC in the mixture thwarts the detection of PE (Figure 4-1B). To quantitatively assess the PE signal in a mixture, the peak areas for m/z 142 and 184 (PC) were normalized to the total ion peak area for neat samples and mixtures. A comparison of the normalized peak areas for PE and PC in neat samples showed that in neat samples, m/z 142 has less intense signal than m/z 184. With these results, it was
unclear whether the difficulty in detecting PE in a mixture stemmed from the effects of the presence of PC or from simply a difference in signal that occurs even in neat samples. Therefore, to be confident that the presence of PC influences the detection of PE, it was important to determine whether the signal difference between PE and PC was greater in a mixture than in neat samples.

The normalized signal for m/z 142 in the mixture was further corrected by a factor of 9.1 because in the neat samples, the m/z 184 normalized peak area was on average 9.1 ± 0.5 times greater (n=3) than the m/z 142 normalized peak area. The resulting normalized, corrected peak area for PE (m/z 142) in the mixture was 0.0030, while the normalized peak area for PC (m/z 184) was 0.039. Even after taking into account the differences in signal observed between PE and PC in neat samples, the signal for PE in a mixture was decreased by 13-fold, compared to PC. These results indicate that the signal for the PE headgroup is smaller than that for the PC headgroup in ToF SIMS, but is further suppressed dramatically in the presence of PC. Cell membranes are highly concentrated in PC, thus, the suppression of the PE signal in the presence of PC makes detecting and imaging PE in cell membranes with SIMS analytically challenging. The difficulty in detecting the PE headgroup ion is indeed unfortunate as PE is perhaps the most prevalent cone-shaped lipid, and is thus a target analyte for testing the hypothesis that lipids of certain shapes can facilitate membrane fusion. In order to learn why the PE headgroup ion is suppressed in the presence of PC, it is important to first understand the mechanism of PE ionization SIMS.
Figure 4-1: Phosphatidylethanolamine spectra exemplify the difference in detecting PE in a neat sample versus a mixture containing PC. **A.** Positive SIMS mass spectrum of a clean neat thick film of PE on Si. **B.** Positive SIMS mass spectrum of a 1:1 mixture of PE and PC on Si. The y axis was adjusted to a suitable scale for the PE headgroup peaks. The intense peaks at m/z 184, 166, and 150 result from PC.

Determination of differences in the mechanism of ionization between PE and PC may be used to explain the ease at which PC forms positive ions compared to PE. The ionization mechanism for the protonated PC headgroup was discussed in detail in chapter
2. Briefly, the mechanism for PC headgroup ion formation was shown to proceed by PC fragmentation and two proton transfer reactions, which involved significant contributions from intermolecular sources, and did not necessarily involve intramolecular exchange. The mechanism of PC ionization was elucidated by altering the isotopic abundance of deuterium within the molecule and the surrounding matrix and monitoring the effect on the isotopic profiles of PC in the mass spectra. In one specific experiment a stable isotopic form of dipalmitoylphosphatidylcholine (DPPC), which contained fully deuterated palmitic fatty acid side chains (D-62 PC), was analyzed. The D-62 PC mass spectrum contained a strong isotopic peak for a PC headgroup fragment containing two deuteriums (m/z 186). This peak was more intense than would be expected based on the natural abundance of deuterium suggesting that both deuteriums can be seized from the surrounding matrix (in this case, other D-62 PC molecules). Therefore, the matrix appears to be an important source of protons for the formation of the positively charged PC headgroup ion during SIMS analysis.

Similarly, experiments to compare the isotopic profile of the PE headgroup fragment ion have been carried out with thin films of dipalmitoyl-PE (DPPE) and D-62 DPPE, which contains fully deuterated palmitic acid side chains. It was not possible to examine the D-13 and D-75 isotopes of PE because they are not commercially available. The mass spectrum for D62-PE contains isotopic headgroup ion peaks at m/z 143, 144, 125, and 126 which are all larger than expected based on the natural abundance of deuterium (Figure 4-2). Therefore, it appears that formation of the PE headgroup fragment ions takes place via a similar mechanism to PC and involves proton exchange with the surrounding matrix. Because the mechanism for formation of the PE headgroup
ion appears to be similar to that for PC, it is unlikely that mechanistic differences can account for the poor detection of PE in a mixture containing these two phospholipids.

Figure 4-2: Comparison of DPPE and D62-PE positive ion mass spectra indicate the PE ionization mechanism includes protons from intra- and inter-molecular exchange A. Positive SIMS mass spectrum of a neat sample of DPPE B. Positive SIMS mass spectrum of a neat sample of D62-PE. Intense peaks from the deuterated (PE+1) and doubly deuterated (PE+2) headgroup are apparent.
Because PE appears to ionize with protons from the surrounding matrix, it is significant that the matrix molecules in these thin film mixtures and also in cell membranes are other lipids. Therefore the significance of the similarity between the PE and PC ionization mechanisms is that PE and PC can accept protons from adjacent lipids or can act as a source of protons for the ionization of other lipids. In order to explain the data in Figure 4-1 in concert with a similar ionization mechanism for both phospholipids when isolated, I hypothesize that in the mixture PE preferentially donates protons to PC rather than accepting protons from other molecules in the matrix. This then leads to lower ionization efficiency for the PE in the presence of PC.

**Hydrogen-Bonding Effects in PE Ionization**

The proposition that PE readily donates protons to PC during SIMS ionization can be supported by examining the structural differences between the molecules Figure 4-3. The PE and PC headgroups both contain an amine functional group; however the PE amine has three hydrogen atoms whereas the PC amine is trimethylated. Thus, the PC nitrogen is more basic than the PE nitrogen. This structural difference suggests that intermolecular interactions and/or steric effects could contribute to the different ionization probability of the two lipids. The lipids both have the same number of hydrogen-bond accepting sites. However, the PE amine has three hydrogens available for hydrogen-bond donation while the PC amine can only undergo weaker dipole-dipole intermolecular interactions. Therefore when compared to the amine hydrogens of PC, the amine hydrogens of PE can associate more strongly with other phospholipids.
Additionally, the PE amine should experience little or no steric hindrance and thus its hydrogen atoms should be readily available for hydrogen bonding.

![Phospholipid chemical structures. A. Phosphatidylethanolamine. B. Phosphatidylcholine.](image)

In fact, the experimentally determined orientation of phospholipids in a bilayer suggests an ease of proton transfer between headgroups. Phosphatidylcholine and PE were found to be positioned with their fatty acid side chains perpendicular to the bilayer surface and their phosphate headgroups approximately parallel with the bilayer surface. Interestingly, the methyl hydrogen atoms on the PC headgroup were shown to interact intermolecularly with the phosphates of adjacent phospholipids. Phosphatidylethanolamine amine hydrogen atoms underwent stronger intermolecular interactions with adjacent phosphates, and the authors explained this result as the effect of the comparatively stronger hydrogen-bonds.

In mass spectrometry, ionization mechanisms that involve intermolecular proton exchange may be facilitated by hydrogen-bonding interactions that are not sterically hindered. This applies to the preferential ionization of PC versus PE. It is highly likely that an amine hydrogen on PE will transfer, by way of a hydrogen bond, to a PC phosphate because of the strong association of the hydrogen with PC and because of the
large amount of PC present (Figure 4-4). This idea may explain why the PE SIMS signal is significantly decreased when PC is present in equimolar amounts.

The effect of intermolecular interactions and steric hindrance on the signal suppression of PE by PC has been experimentally evaluated using PE, PC, and two analogues of PE. The PE analogues used contain structural modifications at the headgroup amine that alters the hydrogen bonding ability and steric hindrance of the molecule. As shown in Figure 4-5, phosphatidylmonomethylethanolamine (PMME) has one amine methyl and therefore two hydrogens available for donation (Figure 4-5A) and phosphatidyldimethylethanolamine (PDME) has two amine methyl groups and one hydrogen atom that can be donated (Figure 4-5B). Mass spectra of PC, PE, a PE/PC mixture, PMME, a PMME/PC mixture, PDME, and a PDME/PC mixture are compared. Mass spectra for PDME and a PDME/PC mixture are shown as an example in Figure 4-6. The PC headgroup signal is unchanged in the neat sample versus any of the mixtures. The mass spectra of the mixtures of the PE analogues have noticeably more intense headgroup peaks than those for the PE mixtures. The PE analogues have fewer hydrogens available for donation, and thus are less likely to sacrifice protons to PC. For

Figure 4-4: Hydrogen-bonding interactions between PE and PC.
example, the PDME positive ion peaks include m/z 170, corresponding to the headgroup, and m/z 152, the headgroup minus water (Figure 4-6A). Although the intensities of m/z 170 and 152 are still lower in a PDME/PC mixture than in a neat sample, the two peaks can be distinguished from the background in the mixture (Figure 4-6B).

Figure 4-5: PE analogue chemical structures. A. PMME  B. PDME. With each additional methyl group, the PE analogue nitrogen atoms become increasingly more basic. The additional methyl groups also may create steric hindrance at the amine which lessens the accessibility of the hydrogen atom(s) for intermolecular bonding with adjacent lipid molecules.
The ratio of the PE headgroup signal intensity in a neat sample to the PE headgroup signal intensity in a PE/PC mixture reflects the degree to which PC suppresses the headgroup signal of PE. Before calculating this ratio for PE, PMME, PDME, and PC, all signal intensities have been normalized to the total ion signal for that spectrum. These ratios were then plotted as a function of the number of hydrogen bonding sites (Figure 4-
Interestingly, the trend in the graph depicts an increase in headgroup signal suppression with each additional hydrogen bond donating site. Alternatively, these data can be interpreted to show increasing headgroup signal suppression with decreasing steric hindrance.

Figure 4-7: The signal for the PE or PE analogue headgroup is suppressed more in a mixture as the number of amine hydrogens is increased in the phospholipid. Signal suppression is reflected by an increase in the peak area ratio, the ratio of the headgroup peak area in a neat sample to the headgroup peak area in a mixture. Headgroup peak areas were normalized to the total peak area before the ratios were calculated. Phosphatidylcholine is represented by the point at 0 amine hydrogens and PE is represented by the point at 3 amine hydrogens. Each point is an average of n=3 trials. Errors bars are the standard deviation.

It is interesting that gas-phase basicity (GB) has been shown to be an important property in mass spectrometry when the formation of an analyte ion involves a proton transfer reaction.\textsuperscript{22-24} The GB is the negative free energy change of the protonation reaction of a molecule and can be used in a similar fashion to Gibbs free energy to determine the thermodynamic feasibility of a reaction in the gas phase. Proton transfer is
thermodynamically favored to occur from an analyte with a lower GB to an analyte with higher GB. For example, GBs have been used to predict whether or not a MALDI matrix will suppress the signal of various analytes through proton exchange.\textsuperscript{24} Other investigators have published work that appears to mirror the signal suppression of the PE headgroup observed in this chapter.\textsuperscript{24} In one example, a spectrum of the matrix 2,4,6-trihydroxyacetophenone (THAP) presented a strong signal from [THAP+H]\(^+\). When this matrix was mixed in equimolar amounts with the analyte glycyl-glycyl-histidine (GGH), the [THAP+H]\(^+\) signal disappeared and only a peak from [GGH+H]\(^+\) was evident. The authors argued that the signal suppression of [THAP+H]\(^+\) results from the thermodynamically favored proton transfer from [THAP+H]\(^+\) to GGH (\(\Delta G = -130 \text{ kJ/mol}\)).

GB values for phospholipids have not yet been determined; however, the GB for ethanolamine is 896.8 kJ/mol, and the GB for choline is unavailable.\textsuperscript{25} Thus, a \(\Delta G\) for proton transfer from PE to PC can only be estimated based on existing values for molecules of similar structure to the phospholipid headgroups. One general observation is that secondary amines are weaker bases than tertiary amines.\textsuperscript{22, 26} For instance, GB values for ammonia, methylamine, dimethylamine, and trimethylamine have been calculated to be 864.4, 869.0, 877.8, and 886.2 kJ/mol, respectively.\textsuperscript{22} Based on these values, proton transfer from ammonia to trimethylamine would be energetically feasible (\(\Delta G = -21.8 \text{ kJ/mol}\)). As the degree of methyl substitution increases, proton transfer to the trimethylamine would have an increasingly less negative \(\Delta G\). Assuming that GB values for phospholipids follow this trend, it can be theorized that proton transfer from
the PE headgroup (PEHG) to the PC headgroup (PCHG) would be most energetically favorable (Equation 4-1) and that proton transfer from PMME and PDME would be increasingly less favorable.

\[
[\text{PEHG} + \text{H}]^+ + [\text{PCHG}] \rightarrow [\text{PEHG}] + [\text{PCHG}+\text{H}]^+ \hspace{1cm} \Delta G = - \text{kJ/mol}
\]

Correlating the final ion distribution in SIMS with thermodynamics is a difficult proposition because SIMS is believed to not be an equilibrium process. Complicated energetics occur in the sample upon primary ion bombardment and in the energy-rich plume of sputtered material above the sample surface. However, it is possible that the ion distributions reflect the equilibrium of preformed ions on the surface and not the nonequilibrium events that occur after primary ion impact. In fact, there is evidence that polar molecules ionized by Brönstead acid-base reactions may form on the surface prior to bombardment. With this fore-warning of the numerous non-equilibrium events implicit in the SIMS process, it appears that thermodynamics at least influences the final relative ion yield of phospholipids in SIMS.

The relative amount of lipid present influences the likelihood that PE will donate an amine hydrogen to adjacent PC. All the previously examined mixtures of PE and PC contained equimolar amounts of the lipids resulting in a 50% chance that a PE molecule was hydrogen-bonded to a PC, as opposed to a PE. At higher relative concentrations, a given PE will be more likely to interact with other PE molecules than PC molecules. The signal suppression of PE should therefore diminish as the number of PC molecules decreases.
The suppression of the signal for the PE headgroup in a mixture of 10:1 DPPE: DPPC has been investigated to test this notion. The PE headgroup peaks are much more intense than the background and result in a peak profile that is more reflective of the DPPE standard mass spectrum (Figure 4-8). The PE signal suppression in the 10:1 DPPE:DPPC mixture has been quantitatively compared to the PE signal suppression in the equimolar mixture by comparing the peak area ratio values for the two mixtures. The average peak area ratio for m/z 142 in the equimolar mixture versus the neat DPPE sample, as shown in Figure 4-7, was 11.8±0.5. The average peak area ratio for m/z 142 in the 10:1 DPPE:DPPC mixture versus the neat DPPE sample was calculated to be 1.6±0.5 (n=3). This peak area ratio is drastically smaller than the ratio calculated in the equimolar mixture. In fact, the PE headgroup peak area ratio in the 10:1 DPPE:DPPC mixture versus the neat sample is only slightly more than the PC headgroup peak area ratio of 0.93±0.05. It can be concluded that the PE headgroup signal is considerably less suppressed in mixtures engorged in PE, which may result from the greatly reduced probability for a PE molecule to interact with PC in mixtures highly concentrated in PE.
Hydrophobicity Effects in PE Ionization

Along with effecting the ability to donate hydrogen and steric hindrance of the phosphate headgroup, addition of methyl groups to the PE amine changes the hydrophobicity of the molecule. Therefore, it is important to determine whether the trend observed in Figure 4-7 results from the hydrophobic differences between PC, PE, and the PE analogues. Phosphatidylethanolamine is available with different fatty acid carbon chain lengths which alters the hydrophobicity of PE. If the suppression of the PE headgroup does not change as a function of fatty acid carbon chain length, the hydrophobic effect can be eliminated as a cause of the signal suppression. Conversely, if

Figure 4-8: Mass spectrum of a 10:1 DPPE: DPPC mixture. Note that the y axes in the previous mixture spectra were adjusted to a range for the PE headgroup peak profiles, which caused m/z 184 (phosphocholine) to be off-scale. The PE signals were so intense in this concentrated mixture, that they can be seen on the same y axis scale as m/z 184.
the signal suppression of PE headgroup increases with hydrophobicity, then hydrophobic effects may influence the SIMS ionization of PE and PC.

The suppression of the PE headgroup signal has been monitored for dicaproylphosphatidylethanolamine (DCPE), dipalmitoylphosphatidylethanolamine (DPPE), and distearoylphosphatidylethanolamine (DSPE). These lipids have saturated fatty acids that are six, sixteen, and eighteen carbons in length, respectively. The PE headgroup signal suppression increases dramatically with decreasing carbon chain length and hydrophobicity (Figure 4-9). The effect of hydrophobicity on signal suppression observed in thin films may not be applicable to lipids arranged in a bilayer structure, as in a cell. The experiments reported here have been carried out on disordered, thin films prepared by spin-coating lipid solutions onto a silicon substrate. Lipids in the resulting sample are oriented haphazardly across the surface. It is possible that phospholipid headgroups can interact in this sample through dipole-dipole and hydrogen-bonding intermolecular interactions. However, tailgroups may be interdigitated between some of the headgroups, thus shielding headgroup-headgroup intermolecular interactions. It is possible that in disordered films, lipids with longer tailgroups block headgroup-headgroup interactions more effectively than do lipids with short tailgroups. It follows that proton exchange reactions will be less likely in disordered films of lipids with long tailgroups. Therefore, the trend observed in Figure 4-9 may not arise from hydrophobic nature of the lipids but rather from a different type of steric effect involving the tailgroup of the lipids. Importantly, a steric effect stemming from tailgroup length will only be applicable to disordered films and should not be observed in samples with an ordered lipid orientation, such as a cell membrane.
However, it is possible that the signal suppression trend observed for thin films in Figure 4-7 may reflect the hydrophobic differences between the PE analogues and this hydrophobic effect should not be disregarded until it can be tested for lipids in bilayer or monolayer structures, such as LB films. It is difficult to pinpoint the degree of PE signal suppression to just one factor and the observed ionization characteristics may be an interplay of hydrogen donation, steric effects, and hydrophobicity.

![Peak Area Ratio vs. Total Number of Carbons](image)

**Figure 4-9:** Suppression of the signal for the PE headgroup decreases as the hydrophobicity of the PE molecule is increased. Signal suppression is reflected by an increase in the peak area ratio and relative hydrophobicity is reflected by the total number of carbons in the PE molecule. Error bars are the standard deviation.

**Analytical Solutions to Address PE Signal Suppression in SIMS**

Clever sample preparation and analytical chemistry will overcome PE signal suppression and allow the cone-shaped lipid to be imaged across cell membranes. One possible way to do this is to incorporate synthetically labeled lipids, similar in shape to
PE, into cell membranes. These PE-like synthetic lipids would be detected by the mass spectral peaks of the labels. Several possible lipids for these experiments are commercially available, such as phosphothioethanol, PE with an NBD labeled headgroup or tailgroup, and PE with a brominated tailgroup.

A second possible approach is to increase the PE headgroup signal above the background. Chapter 3 of this thesis described the enormous signal enhancement afforded by cluster primary ion sources, such as buckminsterfullerene, C\textsubscript{60}\textsuperscript{+}. Phosphatidylethanolamine headgroup signal increased by almost 100-fold using C\textsubscript{60}\textsuperscript{+} versus an atomic source. This large increase in PE headgroup signal can be attributed to an increase in sputter yield from the “softer” C\textsubscript{60}\textsuperscript{+} sputtering process.\textsuperscript{28} Using a cluster ion source instead of an atomic source may increase the PE signal sufficiently above the background for detection and imaging.

Finally, ionization of the desorbed molecules with a laser source (laser postionization) is an attractive solution for PE signal suppression. The majority of the sputtered material during SIMS has a neutral charge whereas only a small fraction of the material is comprised of charged ions that can be directly mass analyzed.\textsuperscript{27} Laser postionization enables analysis of neutral species by photoionizing the desorbed molecules with a femtosecond laser.\textsuperscript{29} For example, SIMS with laser postionization has been used for the analyses of phenylalanine, dopamine, and phosphatidylcholine.\textsuperscript{29-31} Probing the neutral portion of desorbed PE will circumvent the poor ionization characteristics of PE and allow the lipid to be imaged in cells.
Summary

SIMS images of PE across single-cell membranes will lead to an improved understanding of what function cone-shaped lipids may serve in biological events. The detection and identification of many membrane components by SIMS in thin film mixtures and membranes is possible. However, PE signal is virtually undetectable in the presence of PC at equimolar quantities. In order to resolve the PE detection difficulty, the ionization of PE in a neat sample and in a mixture with PC has been investigated. Phosphatidylethanolamine and PC headgroups appear to ionize by similar mechanisms that involve proton exchange with the surrounding matrix. The signal suppression of PE in a mixture can be explained if PE preferentially transfers protons to PC. This concept is supported by the observation that suppression of the PE signal increases with increasing number of hydrogen-bond donation sites and with decreasing steric hindrance. These observations correlate with the relative GB of the lipids which suggests that the final ion distribution in SIMS is partly influenced by the thermodynamics of preformed ions. The non-equilibrium behavior of the SIMS process and the inverse relationship between signal suppression and hydrophobicity complicates conclusions about hydrogen-bonding and steric hindrance. However it is possible that the hydrophobic effect may only be prevalent in thick, disordered films because extremely long fatty acid side chains appear to shield headgroup-headgroup interactions. Knowledge of the PE ionization mechanism and of the PE signal suppression by PC can be used to develop creative biological sample preparation methods, such as incorporating lipid labels into cell membranes, and novel
instrumentation advances, such as cluster ion sources and laser postionization, to ultimately image PE on single-cell membranes.
References


Chapter 5
SIMS Imaging of Cholesterol in Membranes of Fluorescently Identified Single-Cells

Introduction

Tackling investigations at the single-cell level introduces unique analytical challenges because of the small size, volume, and amount of material that is being probed. However, single-cell experiments promise an enhanced understanding of fundamental biological events, such as intra- and intercellular communication (exocytosis, endocytosis, and signal trafficking), the physiological effect of exogenous agents (drug treatments and the environment), and abnormalities in normal biological function associated with disease. Numerous analytical techniques, including electrochemical detection, separation-based methods, fluorescence microscopy, and mass spectrometry have been used to surmount the inherent difficulties of measurements down to the µm-, pL-, and zmole-scale to study the intricate chemistry that exists within single cells.

Secondary ion mass spectrometry (SIMS) is one emerging technique for single-cell experiments with the ability to image the molecular distribution of chemicals across individual cells. SIMS operates on the principle that sample bombardment by a pulsed, electrostatically focused primary ion beam will result in the ejection of secondary species (electrons, neutrals, and ions) from the sample. The secondary ions can be electrostatically collected and mass analyzed by their time of flight (ToF). Imaging with
SIMS involves scanning the primary ion beam across the sample and collecting an entire mass spectrum for each pixel.

SIMS instruments are held under ultra-high vacuum and this requires strict sample preparation considerations for hydrated samples, such as cells. Freeze-fracture is a popular SIMS sample preparation method which cryogenically preserves biological samples.\textsuperscript{18, 21, 25-27} Freeze-fracture is an attractive protocol because it has been shown to maintain the native distribution of molecules in liposomes and cells.\textsuperscript{15, 18-22, 27} However, freeze-fracture involves many experimental variables, such as the temperature and pressure conditions during the fracture,\textsuperscript{27} the section of the cell exposed to the sample-vacuum interface,\textsuperscript{20} and the thickness of ice remaining on the sample substrate, that contribute to experiment-to-experiment variations in secondary ion yield and SIMS images. This issue has been insignificant for many SIMS imaging experiments such as the analysis of individual cells,\textsuperscript{18-21} the comparison of morphologically distinct cells,\textsuperscript{15} and the comparison of morphologically-defined areas of model systems and cells.\textsuperscript{22, 28} These types of experiments were easily accessible with freeze-fracture SIMS because of morphology, however, the application of comparing morphologically similar cells is challenging due to the inherent variability introduced by freeze-fracture. Evaluating the molecular content and distribution within treated cells versus control cells is a valuable potential application of SIMS imaging because it may help elucidate cellular function and the effects of exogenously added agents on these cells. One often manipulated compound in cell membrane experiments is cholesterol.
Cholesterol is an intriguing membrane component because it controls physical membrane properties, such as fluidity and permeability. Cholesterol also is highly concentrated in lipid rafts, and therefore may be a crucial molecule in normal cell function, such as signal transduction. In addition, better understanding of the role of cholesterol is important because abnormalities in cholesterol content have been observed in numerous diseases, including HIV, Alzheimer’s disease, and atherosclerosis, a major cause of heart disease. In particular, advanced lesions in atherosclerosis develop from early lesions which consist of aggregated, cholesterol-engorged macrophages.

One traditional method for manipulating the amount of cholesterol in cells is to incubate the cells in β-cyclodextrin (BCD), a seven-member glucose ring with a cavity for cholesterol binding. This treatment depletes cell membranes of cholesterol and has been used to study lipid rafts. Alternatively, pre-formed complexes of cholesterol and BCD (chol-BCD) have been used to elevate the native amount of cholesterol in a cell.

In this chapter, SIMS imaging with in-situ two-color fluorescence is demonstrated in order to permit the simultaneous analysis and comparison of single cells. Expanding upon a previous work, which incorporated fluorescence microscopy into the SIMS analysis chamber, an in-situ two color fluorescence methodology has been developed to address variations that result from freeze-fracture for comparative single-cell studies. This technique allows cells originating from separate populations that were incubated under different treatments, to be combined into the same sample, freeze-fractured under the same conditions, and identified prior to SIMS analysis. The cholesterol content of macrophage (J774) cells treated to contain elevated levels of cholesterol has been compared to the cholesterol content of control J774 cells using SIMS imaging and in-situ
two color fluorescence. Separate populations of J774 cells have been incubated in serum-
free media containing chol-BCD (treated) and in blank serum-free media (control) and
further labeled with the fluorophores 1,1’-dioctadecyl-3,3,3’,3’-
tetramethylindodicarbocyanine (DiD) and 1,1’-dioctadecyl-3,3,3’,3’-
tetramethylindocarbocyanine (DiI), respectively. The disparate cell populations have
then been mixed together in the same sample and freeze-fractured under identical
conditions. As expected, the mass spectra obtained from treated and control cells are
distinctly different. Fluorescence images are presented which identify treated and control
J774 cells and which correlate with SIMS images for DiD, DiI, hydrocarbon, and
cholesterol. The cholesterol SIMS images show a higher signal from cholesterol in the
outer membrane of the treated J774 cells versus control. Line scans through the
cholesterol SIMS images enable relative quantification of the cholesterol content for
treated and control cells. The membranes of chol-BCD treated J774 cells are found to
contain twice the level of cholesterol, compared to control, for n = 6 cell pairs.

Experimental

Secondary Ion Mass Spectrometry

SIMS experiments were performed on a Kratos (Manchester, U.K.) Prism ToF-
SIMS spectrometer equipped with a 15-kV FEI (Beaverton, OR) indium liquid metal ion
source (LMIS) oriented 45° to the sample. The LMIS was focused to a beam size of 200
nm and delivered 0.5 nA of current to the sample with a 50-ns pulse width. The sample
was mounted onto a liquid nitrogen (LN$_2$) cooled analysis stage (Kore Tech. Ltd, Cambridge, U.K.) and biased at +2.5 kV. An extraction lens, biased at -4.7 kV, collected the secondary ions which then traveled along a 4.5-m flight path before being detected at a microchannel plate (MCP) detector (Galileo Co., Sturbridge, MA). SIMS imaging was performed by electrostatically rastering the LMIG across the sample and collecting a mass spectrum at every pixel in the 128 x 128 pixel image. All images acquired for this experiment had a field of view of 100 x 100 µm$^2$. From the LMIG scan, software written in-house generated both a “total ion” mass spectrum, which was the summation of the individual spectra for every image pixel, and a “total ion” image, in which the signal of each pixel represented the integrated intensity of all mass spectral peaks for that pixel.

Using these data, three different types of analyses were performed. First, regions of the total ion image were selected and a mass spectrum, representative of the summation of the mass spectra in the selected pixels, was produced. In order to compare two of these mass spectra, each spectrum was normalized to the total signal to account for the different number of selected pixels for each region. In a second type of analysis, mass ranges of interest were chosen from the total ion mass spectrum to create 2D intensity plots of the location of a given analyte in the imaging area. The intensity plots were also called molecule-specific images. The molecule-specific images were then color-coded and overlaid using the SIMS software. Additional colors were generated with Adobe Photoshop. The third type of analysis was a line scan, which used molecule-specific images to graph the summed intensity of a given analyte across the thickness of a line as a function of the lateral distance of the line.
Line Scans and Quantification Methods

The line scans in this experiment used a line width of 15 pixels in order to sample a large percentage of each cell. For a given molecule-specific image, a single line was drawn through each pair of cells. Line scans for both m/z 69 and m/z 366-370 were created for every pair of cells and the same lateral distance was analyzed for both masses for a given cell pair. The raw line scans were then treated with 25% Savitsky-Golay smoothing in PeakFit software. The smoothed line scan was then used to quantify the average signal intensity for the cells for the two mass ranges. Unlike the raw data, the smoothed line scans allowed plot sections to be selected for quantification based on a change in the slope of the line. The same length range along the line scan was used for every evaluation. Using this approach, raw, average signal intensities were determined for n=6 cell pairs. To calculate the normalized percent increase in cholesterol, the average signal intensity for m/z 366-370 on a cell was normalized to the intensity for m/z 69 of that cell. Only outer leaflet cell sections were compared in this experiment. Cell pairs were excluded from analysis if their m/z 69 signals were different by greater than 10%.

Microscopy

A vertical illuminator microscope (Olympus, Melville, NY) was previously incorporated into the SIMS instrumental design for brightfield and epifluorescence imaging of samples on the SIMS analysis stage. The microscope includes two illuminator columns equipped with a 50-W halogen and a 100-W Hg light source. A Spot
RT CCD camera (Diagnostic Instruments, Sterling Heights, MI) was used in monochrome mode to capture fluorescence images. For fluorescence images of DiD, a XF110 filter set (Omega, Battleboro, VT) was used to select excitation wavelengths of 644 nm and emission wavelengths of 663 nm. As in previous experiments, fluorescence images of DiI were possible using a XF101 filter set (Omega, Brattleboro, VT) which selects 549 nm and 565 nm excitation and emission wavelengths, respectively. The monochrome DiD and DiI images were colored and combined using the Spot software. The fluorescence images were then rotated, flipped, and cropped in Adobe Photoshop to align them with the SIMS images, as described elsewhere.

Standards and J774 Cell Preparation

Stock solutions of 10 mg/mL DiD and DiI (Molecular Probes, Eugene, OR) were prepared in ethanol. To obtain a standard spectrum for DiD, 100 µL of the stock solution was drop-dried onto a pirhana etched silicon substrate (Ted Pella, Redding, CA). The standard spectrum was taken using a 300 x 300-µm² field of view and a dose of 1 million primary ions. J774 cells were the generous gift of R.A. Schlegel and were cultured to confluence in sterile, polystyrene cell culture flasks at 5% CO₂ and 37°C. When confluent, the J774 cell flasks were treated for 1 hr with 25 µg of DiD or DiI /mL of serum-free RPMI media (Invitrogen, Carlsbad, CA). Next, 100 µL of a 350 mg/mL aqueous stock solution of chol-BCD (CDT, Inc., High Springs, FL), which corresponded to a 260 µM loading dose of cholesterol, was added to the DiD labeled flask. Both the DiI and chol-BCD-containing DiD flasks were further incubated for 1 hr. After the
incubation, both flasks were rinsed 5 times with serum-free RPMI to remove the dye and chol-BCD solution. The cells then were dislodged from the bottom of the flask with gentle tapping and the two cell populations were combined into a culture dish containing sterilized 5 x 5 mm silicon wafers (Ted Pella, Redding, CA). After a short settling period, a top silicon shard was placed on every wafer and the assemblies were cryogenically frozen by plunging them into liquid ethane. Before SIMS analysis, all frozen sample assemblies were stored under LN$_2$. The freeze-fracture sample preparation method used has been described in detail and has been slightly modified.$^{27}$ Briefly, the sample stub was held in the sample preparation chamber in a cold clamp cooled to -196°C with LN$_2$. Meanwhile, a freeze-fracture sample stage was cooled to -106°C and the pressure inside the vacuum was maintained at 2 x 10$^{-8}$ torr. Once the fracture stage had reached the desired temperature, the sample was transferred onto it and the top shard was removed with a cryogenically cooled knife. The sample was then cooled back to -196°C and transferred onto a stage in the SIMS analysis chamber that was also cryogenically cooled to -196°C.

Results and Discussion

Use of DiI and DiD to Classify Cells

Two-color fluorescence experiments require fluorophores with excitation and emission profiles that have suitably different energies. DiI and DiD have been chosen here because they fit this criterion and are commonly used in two-color fluorescence
studies. Additionally, these particular dyes adhere only to the outer membrane of a cell, which is advantageous for analysis with a surface-sensitive technique, like SIMS. Earlier SIMS experiments using DiI–stained cells determined chemical signatures for various cellular sections that can be exposed to the vacuum-surface interface during freeze-fracture. Detecting signal from DiI on a freeze-fractured cell indicated that freeze fracture can be used to uncover the outer leaflet of the cell membrane. In terms of single-cell comparative studies, chemically labeling the outer membrane of the cells helps classify the exposed cellular plane to ensure that the same cell sections are compared.

Because DiI and DiD are present in the cell membranes during SIMS imaging, it is important to collect standard mass spectra in order to identify characteristic dye peaks. The mass spectrum for DiI has formerly been described to contain the molecular ion peak (m/z 834), fragment peaks (m/z 410 and 424), and a hydrolysis product peak (m/z 414). The DiD ionization pattern has not been explored with SIMS and its positive ion spectrum is shown in Figure 5-1. DiD ionizes to form an intense molecular ion peak at m/z 860 (Figure 5-1). The two conjugated ring systems in the DiD molecule are joined by an unsaturated linker that is 5 carbons in length, allowing it to be symmetrically fragmented across the linker (Figure 5-2A) to yield a fragment peak at m/z 424 (Figure 5-1). The symmetrical cleavage accounts for the absence of a peak at m/z 410, which had been identified in the DiI spectrum to result from an asymmetrical fragmentation across the DiI linker which is only 3 carbon atoms in length. Similar to the DiI spectrum, a DiD hydrolysis peak is observed at m/z 414 (Figure 5-1 and Figure 5-2B). Because m/z 414 and m/z 424 are mass spectral peaks for both dye molecules, only m/z 860 can be
used to image DiD in the presence of DiI. Similarly, m/z 410 and 834 can be used to
distinguish DiI from DiD.

Figure 5-1: Positive SIMS mass spectrum for a DiD standard on a Si substrate. The peak
at m/z 860 corresponds to the DiD molecular ion. The peak at m/z 424 is a fragment of
the molecule which cleaved symmetrically about the unsaturated linker. The peak at m/z
414 is a hydrolysis product of the DiD.
A proof-of-concept experiment to demonstrate *in-situ* two-color fluorescence and SIMS imaging is the measurement of elevated cholesterol content in treated cells versus control cells where the two cell populations are fluorescently labeled. One population of J774 cells (treated) is incubated with a solution of chol-BCD and labeled with DiD; a

**Figure 5-2:** Chemical structures for DiD and DiD fragments. **A.** The structure for DiD with a dotted line indicating the position of cleavage for the m/z 424 fragment observed in the standard mass spectrum. **B.** Chemical structure for the DiD hydrolysis product observed at m/z 414.

**SIMS Spectra and Images Comparing Cholesterol in Cell Membranes**

A proof-of-concept experiment to demonstrate *in-situ* two-color fluorescence and SIMS imaging is the measurement of elevated cholesterol content in treated cells versus control cells where the two cell populations are fluorescently labeled. One population of J774 cells (treated) is incubated with a solution of chol-BCD and labeled with DiD; a
second J774 population (control) is incubated in media and labeled with DiI. Therefore, the treated, DiD-labeled cells should exhibit a higher SIMS cholesterol signal than the Dil-labeled control cells. Because cholesterol is a major analyte of interest for these experiments, its SIMS ionization warrants discussion here. Ionization of cholesterol yields arrays of peaks in the mass range of the molecular ion (m/z 385) and in the mass range of a fragment ion (m/z 369). The array of peaks in the mass range of the fragment ion (m/z 366-370) is used for all cholesterol molecule-specific images and quantification in this chapter.

From a SIMS image of a fluorescently characterized mixed population of treated and untreated J774 cells, two mass spectra are generated: one from the integrated intensity across a treated cell (Figure 5-3A) and the second from the integrated intensity across a control cell (Figure 5-3B). As expected, the two spectra are distinctly different. The normalized mass spectrum of the treated cell contains higher signal from cholesterol than the control cell. Additionally, the treated cell mass spectrum contains a peak for the molecular ion of the identifying marker, DiD, and the control cell mass spectrum contains signal corresponding to the molecular ion of the identifier, DiI. Along with supporting the identification of the two cells, the presence of the dye signals indicates that the outer leaflet is uncovered for both cells. If the membrane inner leaflet or the cytoplasm had instead been exposed, then the SIMS dye signals would not be detected from the cells and SIMS alone would be unable to classify the cells.
A two-color fluorescence image demonstrates that the excitation and emission profiles of the two dyes do not interfere with one another and that mixing the two populations of cells does not cross-contaminate the dyes (Figure 5-4A). A composite SIMS image of m/z 834 (DiI; green) and m/z 860 (DiD; red) correlates with the fluorescence image and further shows, by mass spectrometry, that the two dyes have not cross-contaminated on differing cells during sample preparation (Figure 5-4B). The fluorescence and SIMS images also identify the two cells at the bottom of the image as DiD-treated cells and the two cells at the top as DiI-control cells. Furthermore, the SIMS image indicates again that the outer membranes of the left-most DiD-treated cell and of both DiI-control cells are exposed to the vacuum interface because the signal from the
lipophilic dyes localize to the cells (Figure 5-4B). It is important that the same cellular section is compared between treated and control cells because different amounts of cholesterol will likely incorporate into the cytoplasm and into the outer leaflet and the inner leaflet of the plasma membrane.\textsuperscript{29} Since the DiD-treated cell on the right does not appear to be fractured to expose the outer-leaflet, it has not been considered in further analyses. This cell exhibits less intense fluorescence because the DiD-enriched outer membrane was removed during freeze-fracture, resulting in significantly less fluorophore molecules associated with the cell, compared to the outer-leaflet exposed DiD-cell. The brightness and contrast settings in the imaging software that are appropriate for the outer-leaflet exposed cells cause the DiD-cell with less fluorescence to appear smaller than actuality. The control cell located in the upper right of the images was optically determined to have been damaged during the freeze-fracture process and also has not been further analyzed. Therefore, the remaining left-most treated and control cells are the subject for the rest of the discussion of Figures 5-4 and 5-5.
Relative quantification in SIMS is possible if the signal intensity is normalized to an internal standard. In this experiment, the C$_5$H$_9^+$ hydrocarbon (m/z 69) serves as an internal standard because it is a generic fragment from lipid fatty acid side chains and should remain constant between the treated and control J774s. The SIMS image for m/z
69 (Figure 5-4C) shows that both cells contain a similar concentration of signal-containing pixels that are of a similar intensity for m/z 69. Therefore, any differences in signal observed for other mass ranges should represent a true dissimilarity in the membrane chemistry between the two cells, and not an artifact of sample topography. In the SIMS image of the cholesterol fragments (m/z 366-370), there are visibly more signal-containing pixels localized to the treated J774 cell, compared to the control cell (Figure 5-4D). It follows that this chol-BCD treated J774 cell displays higher SIMS cholesterol signal and hence contains elevated amounts of cholesterol, compared to the control J774 cell.

Relative Quantification of Cholesterol Content in Treated versus Control J774 Cells

Line scans have been created for the SIMS images of m/z 69 (Figure 5-5A) and m/z 366-370 (Figure 5-5B) in order to obtain a more thorough comparison of the cholesterol content in the J774 cell membranes. A line scan is a plot of the summation of pixel intensities for a given chemical map as a function of the lateral distance of the line. For the line scans in Figure 5-5, the line is indicated in the graph insets and was drawn from the treated cell located in the lower left through the control cell on the top left of the image. As suggested from Figure 5-4C, the signal obtained from m/z 69 is very similar for both cells (Figure 5-5A). In contrast, the cholesterol line scan shows a large discrepancy in cholesterol signal intensity between the treated and control cells (Figure 5-5B). In this example, there is approximately 180% more cholesterol signal detected from the treated cell, compared to the control, supporting the conclusion from the SIMS
images that the chol-BCD J774 cells contain increased amounts of cholesterol in the outer leaflet of their membranes.

Figure 5-5: Line scans comparing a chol-BCD treated J774 cell and a control J774 cell. The insets depict the distance selected for the line scan, which was drawn from the lower left to the upper left of the each respective chemical map. A. Line scan for cholesterol (m/z 366-370). B. Line scan for $\text{C}_5\text{H}_9^+$ hydrocarbon (m/z 69).
SIMS images have been acquired for numerous pairs of treated and control J774 cells to calculate the average increase in cholesterol in the chol-BCD cells. Three criteria have determined whether a given pair of cells is included in the subsequent analysis. First, both cells in a pair must be in the same SIMS image and cannot be damaged. Second, only cell pairs that have the outer leaflet of their membranes uncovered are analyzed. The exposed cellular sections are verified by molecule-specific images of DiD and DiI. As previously discussed, cholesterol from the chol-BCD treatment will incorporate into different regions of the cell by different amounts, making the identification of the exposed cellular section crucial. The third factor for analysis is that only cell pairs that yield similar m/z 69 signal are considered. When the m/z 69 signal from the cells changed by greater than 10%, the cell pair has been excluded because any changes in cholesterol signal could be attributed to an overall signal change due to differences in sample height or salt effects.

Six cell pairs met the selection criteria and have been quantified. Use of a single line scan, drawn for each pair of cells for a given mass of interest (m/z 366-370 or m/z 69) resulted in unnormalized signal intensities detected from every cell that are reported in Table 5-1. The raw cholesterol signals for m/z 366-370 for treated versus control cells are statistically different according to the student’s t-test (p < 0.02). As discussed before, quantification in SIMS requires normalization of the signal of interest to an internal standard, in this case m/z 69. Therefore, the m/z 366-370 raw signal intensity on a given cell has been normalized to the m/z 69 intensity from the same cell, prior to calculating the percent increase in cholesterol. The normalized percent increase in m/z 366-370 for n=6 cell pairs ranged from 184% to 74%, with an average increase of 99% (Table 5-1).
Therefore, the SIMS data demonstrates that chol-BCD treatment increases the amount of cholesterol in the outer leaflet of J774 cell membranes by an average of 99% or two-fold, compared to the native cholesterol level. The variation in cholesterol percent increase may result from the intrinsic variability in biological systems and from environmental factors during the chol-BCD treatment. Both populations of J774 cells were not evenly confluent in their respective culture flasks. A small percentage of chol-BCD cells may have originated from sparsely populated regions of the treated flask, allowing a greater amount of the membrane surface area to interact with the cholesterol complex for a higher amount of cholesterol loading.

Table 5-1: Comparison of signal intensities for two J774 cell populations (n=6).

<table>
<thead>
<tr>
<th>Trial</th>
<th>Treated Cells m/z 366-370*</th>
<th>Control Cells m/z 366-370*</th>
<th>Treated Cells m/z 69</th>
<th>Control Cells m/z 69</th>
<th>Normalized % Change Relative to Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>34.4 ± 0.2</td>
<td>12.5 ± 0.2</td>
<td>651 ± 1</td>
<td>674 ± 1</td>
<td>184% ± 4</td>
</tr>
<tr>
<td>2</td>
<td>33.2 ± 0.2</td>
<td>17.4 ± 0.2</td>
<td>931 ± 3</td>
<td>852 ± 6</td>
<td>74% ± 2</td>
</tr>
<tr>
<td>3</td>
<td>40.0 ± 0.3</td>
<td>21.1 ± 0.2</td>
<td>604 ± 1</td>
<td>570 ± 1</td>
<td>79% ± 2</td>
</tr>
<tr>
<td>4</td>
<td>40.3 ± 0.4</td>
<td>21.7 ± 0.1</td>
<td>1150 ± 5</td>
<td>1207 ± 4</td>
<td>95% ± 1</td>
</tr>
<tr>
<td>5</td>
<td>42.1 ± 0.4</td>
<td>21.9 ± 0.2</td>
<td>610 ± 3</td>
<td>557 ± 2</td>
<td>75% ± 2</td>
</tr>
<tr>
<td>6</td>
<td>126 ± 1</td>
<td>62.2 ± 0.3</td>
<td>1860 ± 5</td>
<td>1720 ± 4</td>
<td>87% ± 1</td>
</tr>
<tr>
<td>AVG</td>
<td>52.7 ± 1</td>
<td>26.1 ± 0.5</td>
<td>968 ± 8</td>
<td>930 ± 9</td>
<td>99% ± 5</td>
</tr>
</tbody>
</table>

* p < 0.02 (t test).
All errors are standard deviations.
Summary

Integration of two-color fluorescence into the SIMS experimental design presents the opportunity for numerous single-cell applications previously difficult with SIMS. The ability to identify cells from two disparate populations with fluorescent labels eliminates the necessity to analyze morphologically similar cells in separate samples, under variable freeze-fracture conditions. DiD and DiI have been selected to fluorescently label two populations of cells. Fluorescence images have proven that the absorption and emission profiles for the two dyes do not overlap. Additionally, fluorescence and molecule-specific SIMS images have demonstrated that mixing the two populations of labeled cells does not cause cross-contamination of the dyes. As an example application for this experiment, one population of DiD-labeled J774 cells has been chemically altered with chol-BCD and compared to untreated, control cells. Prior to SIMS analysis, in-situ two color fluorescence images have been used to distinguish the J774 cells cultured under these separate conditions. Mass spectra and SIMS images have been correlated with the fluorescence images and have been used to demonstrate the expected chemical differences between the two cell populations. Cholesterol increases by two-fold in the membranes of the treated J774s versus control cells for n=6 trials.

SIMS coupled with in-situ fluorescence has many future prospects and should be useful to gain insight into the process of cholesterol accumulation in macrophage cells in atherosclerosis, as well as aid in the discovery of biomarkers and metabolites caused by cholesterol-lowering drugs. Furthermore, this new methodology will be a valuable tool
to learn about the subcellular distribution of drugs and to gauge drug efficacy and drug resistance at the single-cell level.
References


Chapter 6

Mass Spectrometric Imaging of Highly Curved Membranes During Tetrahymena Mating

Introduction

During membrane fusion, two adjacent lipid bilayers merge and a channel (fusion pore) forms which joins the aqueous volumes initially enclosed within the membranes. The protozoan, Tetrahymena thermophila (Figure 6-1A), is an attractive cell system for membrane fusion studies because it is possible to induce the simultaneous formation of hundreds of fusion pores within a well-defined membrane region of about 8 µm.\(^1,2\)

During mating, or conjugation (Figure 6-1B), the membranes of two complementary Tetrahymena join at the anterior end and 100 to 200-nm sized fusion pores form (Figure 6-1C) to allow the migration of nuclei between the cells. Interestingly, conjugation depends on de novo lipid synthesis \(^3\) and Tetrahymena can readily modify the lipid composition of surface membranes via intracellular lipid exchange.\(^4\) Thus, it seems likely that certain types of lipid are required to allow the mass formation of fusion pores and that these biophysically relevant lipids may be directed to the fusion site, called the conjugation junction. Additionally, in preparation for conjugation, these cells actively modify their pattern of protein synthesis and the anterior ends of the cells transform from pointed to blunt in shape and from ciliated and ridged to smooth in texture.\(^2\) The dependence of conjugation on lipid synthesis, the membrane morphological changes, and the excess of fusion pores might suggest that there are
substantial spatial alterations in the chemistry of the membrane bilayer in the conjugation junction.

Figure 6-1: Hundreds of fusion pores observed during *Tetrahymena* conjugation result in lipid heterogeneity at membrane fusion sites. Brightfield images of *Tetrahymena* A. prior to mating and B. during full conjugation (see arrows). Scale bars are 50 µm. C. A TEM of a 70-nm cross section through mating *Tetrahymena* showing eight fusion pores along the conjugation junction in this plane of view. Therefore, an estimated pore density for the entire junction is about 200 fusion pores, in agreement with the literature.\(^2\) Scale bar is 2 µm.

The cellular machinery and thermodynamic driving forces behind biological fusion are a bit of an enigma, although it is widely believed that the machinery involve an
intricate cooperation between membrane proteins, the cytoskeletal framework, and lipids. Specifically, the interaction of complementary membrane proteins dictates, in part, the fusion location and likely regulates the events by bringing the bilayers into close proximity. The cell cytoskeleton also is believed to have a key role by confining fusogenic proteins to fusion regions and by providing the needed force to merge the electrostatically repelled bilayers. Along with these elements, the local lipid composition may mediate membrane fusion. The existence of biophysically functional lipid domains, or rafts, which are membrane regions concentrated in a particular type of lipid, is well documented. Lipid movement through the membrane can be restricted, which creates a heterogeneous distribution of lipids. These structures appear to involve longer term or semi-permanent formations and may drive biological fusion.

During fusion, the lipid bilayer undergoes considerable changes in shape and most likely adopts several intermediate structures with high radius of curvature. One such proposed intermediate structure, the stalk intermediate, has been experimentally observed by x-ray diffraction (Figure 6-2). Thus, if present, lipid domains involved in membrane fusion would form temporarily during the fusion process and would require a driving force and cell-to-cell signaling of some form. According to the intrinsic curvature hypothesis, cone-shaped, non-lamellar lipids, such as phosphatidylethanolamine (PE), are particularly apt at forming highly contoured structures, such as the stalk intermediate, because they contain hydrophilic headgroups and acyl chain tailgroups of greatly different cross-sectional areas (Figure 6-2). This hypothesis suggests that highly fusogenic membrane regions may adjust to contain small amounts of lamellar lipids, including PC, in comparison to the rest of the cell membrane, and fusion events may
preferentially occur at these depleted sites. In *Tetrahymena*, the formation of the plethora of highly curved fusion pores would require that the entire length of the conjugation junction be concentrated in non-lamellar lipids.

![Image of membrane fusion intermediate structure](image)

Figure 6-2: A schematic of the membrane fusion intermediate structure, the stalk. The wavy lines depict the acyl tailgroups for the membrane phospholipids. The white circles represent the headgroup of phosphatidylcholine, a cylindrical-shaped, lamellar lipid. The shaded circles symbolize the headgroup of phosphatidylethanolamine, a cone-shaped, non-lamellar lipid. Membrane fusion sites probably contain a high quantity of cone-shaped lipids because those lipids fit well into contoured intermediate structures.

The *Tetrahymena* membrane is unique in that it contains 2-aminoethylphosphonolipid (2-AEP), which is a cone-shaped, non-lamellar lipid, like PE, that should be adept at forming highly curved membrane fusion structures. Importantly, 2-AEP is very abundant in *Tetrahymena*, making up about 25% of the *Tetrahymena* whole cell membrane phosphorous lipid content, whereas PC represents 26% of the phosphorous lipids. It is probable that the conjugation junction is concentrated in 2-AEP to allow the formation of many fusion pores in this region.

The molecule-specific imaging capabilities of time of flight secondary ion mass spectrometry (ToF-SIMS) make it uniquely well-suited for investigations of lipid heterogeneity. ToF-SIMS is a well-established technique for biological applications with
a repertoire including elemental imaging of the distribution of cancer therapy drugs in single cells and molecular imaging of tissue, liposomes, paramecia, and rat pheochromocytoma (PC12) cells. ToF-SIMS experiments are performed under ultra high vacuum (UHV) conditions which make special sample handling considerations necessary to maintain the native chemical spatial distribution of hydrated biological samples. A sandwich style freeze-fracture sample preparation technique has been adapted from the electron microscopy community for this purpose.

In this chapter, I use imaging ToF-SIMS to investigate the distribution of PC and 2-AEP across the membranes of frozen-hydrated, mating *Tetrahymena thermophila*. Molecule-specific images show that C₅H₉⁺ is homogeneous across mating cells indicating that the total lipid content is evenly distributed. In contrast, images of m/z 184 (phosphocholine) reveal that PC is depleted at the conjugation junction compared to the rest of the cell body. Line scans through the molecule-specific images graphically demonstrate the homogeneous total lipid distribution and the heterogeneous PC distribution observed in the SIMS images. Similar results are observed for the majority of mating *Tetrahymena*, with an average of 67±8% signal decrease at the conjugation junction, compared to the rest of the cell body. In order to determine which lipid is present at the conjugation junction, I examine the mass spectra arising from the pixels on the cell body versus the pixels at the conjugation junction and I use principal component analysis to verify which peaks contribute most to the variances between the two spectra. From these data, the conjugation junction exhibits elevated signal from m/z 126, which is likely the phosphate headgroup of 2-AEP. Therefore, regions of aggregated membrane fusion sites in *Tetrahymena* contain decreased amounts of cylindrical lipids (PC) and
increased amounts of non-lamellar, high curvature lipids (2-AEP). Significantly, these results indicate that cells can either direct newly synthesized lipids to specific membrane regions or can rearrange the lipid distribution of their cell membranes in preparation for membrane fusion.

**Experimental**

**Secondary Ion Mass Spectrometry**

Secondary ion mass spectrometry (SIMS) images of mating *Tetrahymena* were acquired on a Kratos (Manchester, U.K.) Prism ToF-SIMS spectrometer with a FEI (Beaverton, OR) indium liquid metal ion beam (15 kV, 500 pA, 200-nm diameter). The ion beam was positioned at 45° to the sample and was pulsed with a 50-ns pulse width. A + 2.5-kV potential was applied to a liquid nitrogen (LN₂) cooled sample stage and secondary ions were collected by an extraction lens biased at - 4.5 kV. The secondary ions traveled a 4.5-m time of flight path before being detected with a microchannel plate assembly (Galileo Co., Sturbridge, MA). SIMS images were obtained by rastering the primary ion beam across the sample region and collecting a total mass spectrum. The m/z ranges of interest were selected from the total spectrum and intensities were plotted for each pixel to generate an image. SIMS images were acquired using a 200-nm beam spot size, resulting in an approximately 250-nm lateral resolution, as in previous work.¹⁸ In order to obtain morphological images of the samples, *in-situ* scanning ion micrographs were acquired with a channeltron detector (Burle, Lancaster, PA) located ~0.5 cm from
the sample. Brightfield images were taken using a Leica (Stereozoom5, Bannockburn, IL) stereoscope and a Kodak (DC290 Zoom, Rochester, NY) digital camera.

Line scans, which graphed the variation in signal intensity for a particular mass range across the image, were achieved using software written in-house. Lines were drawn through the conjugation junction for each mass and graphs displaying the signal intensity as a function of the lateral distance along the line were obtained. The lines were drawn along the same pixels for both the m/z 69 and the m/z 184 chemical maps to allow direct comparison of the resulting graphs. For each graph, the intensity was normalized to one, and the x axis was converted from pixel number to lateral distance. A conversion factor for lateral distance was determined by imaging a fine mesh grid (Electron Microscopy Sciences, T1000-Cu, Hatfield, PA) on the SIMS instrument and determining the micron distance that corresponded to 128 pixels.

**Determination of Phosphocholine Signal Decrease at the Conjugation Junction**

The average PC signal intensity was calculated for three regions across mating *Tetrahymena* membranes: the first cell body (cell body I), the conjugation junction, and the second cell body (cell body II). First, m/z 184 line scans were taken for each of the mating cells, as described above. The line scans were further treated with 15.5% Savitzky-Golay smoothing and then fitted with a chromatography/HVL PeakFit function, resulting in $R^2 \geq 0.98$. The signal intensities in the line scan were averaged for each of the three regions indicated in the corresponding columns of Table 6-1. The regions were selected based on a change of slope in the line scan. The average signal decrease
between the two cell bodies and the conjugation junction was then determined. For the first cell body, the difference between the cell body I intensity and the junction intensity was divided by the cell body I intensity. This was repeated for the second cell and the two percentages were averaged. Standard deviations were determined using propagation of errors. One sample yielded too low a signal to provide conclusive data due to sample charging and a second sample had extremely high signal variability. These samples were not used.

**Mating *Tetrahymena Thermophila***

Complementary mating types B2086 (II) and Cu428.1 (VII) of *Tetrahymena thermophila* (kindly provided by N.E. Williams) were grown overnight in PPYGFe medium (2% proteose peptone, 01.% yeast extract, 0.2% glucose, and 0.003% ferric EDTA) at 30°C with shaking. The cells were washed twice by centrifugation (400g x 5 min), resuspended, and shaken overnight in 10 mM Tris-HCl, pH 7.4 at 30°C. To initiate conjugation, the starved, complementary strains were mixed and incubated at 30°C without shaking. The mating cells were cryogenically frozen after a 4 hour mating period.

**Freeze-Fracture Sample Preparation**

Small aliquots of the cell-containing solution were placed between 5 x 5 silicon wafers (Ted Pella, Redding, CA) and silicon top shards and the assemblies were
cryogenically frozen in liquid ethane. Then, the frozen samples were individually freeze-fractured under ultrahigh vacuum and directly transferred into the analysis chamber. Freeze-fracture has been described in detail but was slightly modified. Briefly, the sample assembly was fastened to a sample stub under LN\(_2\) and transferred into the sample preparation chamber. In the preparation chamber, the sample stub was secured in a cold clamp cooled to -196°C with LN\(_2\). Meanwhile, a freeze-fracture sample stage was cooled to a goal temperature of -106°C and the vacuum pressure was kept at 2 \( \times 10^{-8} \) torr. Once the fracture stage had reached -99°C, the sample was transferred onto it, the temperature was allowed to equilibrate, and the top shard was removed with a cryogenically cooled knife at a final temperature of -106°C. The sample was then cooled back to -196°C and transferred onto a stage in the SIMS analysis chamber that was also cryogenically cooled to -196°C.

**Transmission Electron Microscopy**

Mated cells were prepared for TEM by microwave assisted tissue processing. The cells were pelleted and fixed for 30 minutes in 1.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) and 2.5% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) buffered with 0.1 M sodium cacodylate (pH 7.4, Electron Microscopy Sciences, Hatfield, PA). The fixed cells were washed with 0.1 M sodium cacodylate, post-fixed in 1% osmium tetroxide (Electron Microscopy Sciences, Hatfield, PA) buffered with 0.1 M sodium cacodylate, and washed again in cacodylate. The sample was then dehydrated using ethanol, acetone, and propylene oxide, and embedded
in Spurrs resin. A Leica ultramicrotome (LKBIII-8800, Ultracut E, Deerfield, IL) was used to slice 70 nm thick sections of the embedded cells and the sections were analyzed in a JEOL transmission electron microscope (1200 EXII, Peabody, MA) equipped with a TIEZT camera (F224, Gaiting, Germany).

Results and Discussion

Imaging time-of-flight secondary-ion mass spectrometry (ToF-SIMS) after freeze-fracture was used to examine the lipid distribution along the conjugation junction of mating Tetrahymena. SIMS has been applied to elemental imaging of the distribution of cancer therapy drugs in single cells\textsuperscript{15} as well as molecular imaging of tissue\textsuperscript{17}, liposomes\textsuperscript{18,19}, paramecia\textsuperscript{20}, and rat pheochromocytoma (PC12) cells\textsuperscript{21,22}, but not yet demonstrated for subcellular regions. Tetrahymena were stimulated to conjugate, incubated for 4 hours to guarantee full conjugation, cryogenically frozen, freeze-fractured, and then directly transferred into the analysis chamber for SIMS imaging. After ToF-SIMS imaging, the samples were further analyzed by scanning ion microscopy and brightfield microscopy to ensure that no substantial sample damage occurred during sample preparation and SIMS analysis. In-situ scanning ion micrographs provide morphological images of the cell samples and show no evidence of physical damage to the mating Tetrahymena (Figure 6-3A). The brightfield image verifies this conclusion (Figure 6-3B).
In SIMS studies of cells, mass information of m/z 69 corresponds to C$_5$H$_9^+$ which is most likely an acyl chain fragment from a phospholipid. Because all of the membrane phospholipids contain this species, the distribution of C$_5$H$_9^+$ in SIMS images is representative of the total phospholipid distribution. A molecule-specific image of m/z 69 for mating Tetrahymena shows that the phospholipids are homogeneous across the joined cells (Figure 6-4A). The uniformity of the entire phospholipid signal over the fusing region of the cells is expected as the current spatial resolution for this technique is above the size of a fusion pore. Additionally, the homogeneous distribution of m/z 69 indicates that sample topology is not influencing the SIMS images. Any signal variations of other m/z values should be representative of the chemistry of the cell membrane.

The SIMS fragmentation behavior of PC has been well characterized, and through tandem mass spectrometry, m/z 184 has been attributed to the phosphocholine headgroup. A substantial decrease in the m/z 184 signal seen at the junction region (Figure 6-4B) shows the phospholipids composition varies considerably in the mating junction because of the abundance of highly curved membrane structures in that region.
Line scans were taken through the molecule-specific images to quantitatively compare the distribution of m/z 184 and of m/z 69 across the conjugation junction (Figure 6-5). Although there are slight fluctuations that are expected with molecular measurements on the micrometer scale, the m/z 69 signal is constant across the junction (Figure 6-5A), whereas the m/z 184 signal clearly drops at the junction region (Figure 6-5B). This heterogeneity was reproduced for the majority of mating *Tetrahymena* examined (Table 6-1), with an average decrease in signal of 67 ± 8 %. The hydrocarbon signal remained relatively constant across all mating cells with a slight average increase in signal of 7 ± 11 % (data not shown). Principal component analysis further verified that PC is decreased in the junction relative to the cell body and also identified a peak at m/z 126 that increased in the junction (Figure 6-6).²⁷ Compared to the cell body, the membrane junction region of mating *Tetrahymena* is composed of significantly less PC.
Figure 6-5: Line scans of the molecule-specific images graphically support the decrease of phosphocholine at the conjugation junction. Data points were collected every 120 nm. 

A. Line scan for m/z 69 through the conjugation junction illustrating that the total lipid content is relatively constant across the mating cells. The inset shows the SIMS image for m/z 69 highlighting the pixels used for the line scan. 

B. Line scan for m/z 184 through the junction demonstrating a sharp decrease in signal at the conjugation junction. The inset shows the SIMS image for m/z 184 highlighting the pixels used for the line scan.
Figure 6-6: Loadings plot from principal component analysis comparing the mass spectra of the cell bodies and the conjugation junction. Plot prepared using n=3 mating *Tetrahymena*. Data analysis by Dr. Matthew Wagner.

Table 6-1: Summary of PC signal intensities (m/z 184) across mating *Tetrahymena*

<table>
<thead>
<tr>
<th>Trial</th>
<th>Cell Body I *</th>
<th>Conjugation Junction</th>
<th>Cell Body II *</th>
<th>Signal decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.591 ± 0.025</td>
<td>0.321 ± 0.053</td>
<td>0.881 ± 0.057</td>
<td>55% ± 10</td>
</tr>
<tr>
<td>2</td>
<td>0.926 ± 0.029</td>
<td>0.361 ± 0.093</td>
<td>0.732 ± 0.041</td>
<td>56% ± 12</td>
</tr>
<tr>
<td>3</td>
<td>0.619 ± 0.0077</td>
<td>0.0751 ± 0.016</td>
<td>0.734 ± 0.021</td>
<td>89% ± 4</td>
</tr>
<tr>
<td>4</td>
<td>0.488 ± 0.020</td>
<td>0.240 ± 0.019</td>
<td>0.384 ± 0.011</td>
<td>44% ± 6</td>
</tr>
<tr>
<td>5</td>
<td>0.656 ± 0.0065</td>
<td>0.179 ± 0.010</td>
<td>0.437 ± 0.0076</td>
<td>66% ± 3</td>
</tr>
<tr>
<td>6</td>
<td>0.693 ± 0.012</td>
<td>0.325 ± 0.021</td>
<td>0.806 ± 0.013</td>
<td>56% ± 3</td>
</tr>
<tr>
<td>7</td>
<td>0.852 ± 0.014</td>
<td>0.189 ± 0.045</td>
<td>0.560 ± 0.020</td>
<td>72% ± 8</td>
</tr>
<tr>
<td>8</td>
<td>0.895 ± 0.011</td>
<td>0.0622 ± 0.032</td>
<td>0.343 ± 0.019</td>
<td>87% ± 9</td>
</tr>
<tr>
<td>Average</td>
<td>0.711 ± 0.017</td>
<td>0.219 ± 0.044</td>
<td>0.610 ± 0.028</td>
<td>67% ± 8</td>
</tr>
</tbody>
</table>

* p < 0.001, versus the conjugation junction (t test).
All errors are standard deviations.
It is likely that the decreased amount of PC in this junction region correlates with an elevated concentration of 2-AEP, the phosphonolipid analog of PE. Like PE, 2-AEP is a cone-shaped, non-lamellar lipid and should form highly curved membrane fusion structures. Importantly, 2-AEP makes up about 34% of the *Tetrahymena* whole cell membrane phosphorus lipid content, whereas PC represents 26% of the phosphorus lipids. 14 2-AEP represents a class of lipids that contain a variety of fatty acid tailgroups that cannot be detected via the molecular ion with ToF-SIMS. Instead, 2-AEP can be identified with the headgroup fragment ion, m/z 126 (Figure 6-7A), similar to detecting PC with the m/z 184 headgroup ion.

![Chemical structure of 2-AEP](image)

**Figure 6-7:** SIMS images do not reveal increased 2-AEP at the conjugation junction. **A.** 2-AEP headgroup fragment corresponding to m/z 126. **B.** SIMS image for m/z 126. Scale bar is 50 µm.

The molecule-specific image of m/z 126 for the mating *Tetrahymena* appears homogeneous across the cells, probably because the entire surface of the *Tetrahymena* membrane is concentrated in this lipid (Figure 6-7B). However, a comparison of the mass spectra from the pixels of the cell body region to the conjugation junction suggests an
increase in 2-AEP at the conjugation junction. (Figure 6-8A and B) The normalized intensity of m/z 126 and m/z 184 can be directly compared between the two spectra and not only does the intensity ratio between the two peaks change, but the relative amount of m/z 126 increases in the junction region as m/z 184 decreases. Principal component analysis showed a clear correlation of m/z 126 with the conjugation junction, in comparison to the cell body supporting the presence of increased amounts of 2-AEP at the conjugation junction (Figure 6-6). Mammalian cells are not known to contain any substantial amount of 2-AEP.28 Frozen-hydrated rat pheochromocytoma cells and beige mouse mast cells do not localize m/z 126 to the cell structure, thus its localized increase in the *Tetrahymena* junction is clearly significant.

These results indicate that *Tetrahymena* can either reorganize or manufacture the necessary lipids and adjust its membrane structure for pore formation during the time necessary for full conjugation. The extent to which lipids in the membrane are involved in these changes and the exact cellular machinery that drives these alterations are unknown, although the changes probably occur during the required period of cell-cell contact prior to membrane fusion. It is likely that these interactions initiate a biological response that prepares the cells for conjugation by directing a higher concentration of high-curvature lipids, which are adept at fusion pore formation, to the membrane fusion sites.
Figure 6-8: Mass spectra from image pixels indicate that the conjugation junction contains elevated amounts of 2-AEP. Mass spectra were generated by selecting the pixels of interest using software written in-house. The insets are images of the summed signal intensity at each pixel, on a thermal-scale, and were artificially colored to indicate pixels selected for analysis. **A.** Mass spectrum from the pixels along the conjugation junction, as indicated in the inset. **B.** Mass spectrum from the pixels in the cell bodies, as indicated in the inset.
Summary

The junction between mating *Tetrahymena thermophila* contains hundreds of fusion pores making it an elegant system to study the role of lipids in membrane fusion events. Using ToF-SIMS and principal component analysis, I have evaluated the distribution of lipids across frozen-hydrated, mating *Tetrahymena*. A molecule-specific image of m/z 69 depicted a homogeneous distribution of the total lipid content of mating *Tetrahymena*. This result indicated that sample artifacts were not causing signal deviations and therefore molecule-specific images would be representative of the chemistry of the cell membrane. A molecule-specific image of m/z 184 showed a decrease in signal between the cells, which correlated with PC depletion at the conjugation junction. Line-scans of m/z 69 and 184 graphically supported the visual observations from the images. Principal component analysis and mass spectra from image pixels revealed that the cone-shaped lipid, 2-AEP, is concentrated at the conjugation junction, compared to the rest of the cell body.

The unique aspect of these experiments is the spatial selectivity and chemical specificity of ToF-SIMS, which allows this analysis of regional changes in phospholipid. The results present the first evidence that it is possible to image specific lipid domains on single cells with ToF-SIMS. Significantly, the decrease in PC and increase in 2-AEP in the junction region of mating *Tetrahymena* provides a direct physical view of heterogeneous membrane structure and clearly suggests that membrane fusion events, such as conjugation, are mediated by this lipid heterogeneity.
References

(13) Gruner, S. M. *Proc Natl Acad Sci USA* 1985, 82, 3665-3669.
Thesis Summary and Future Directions

Thesis Summary

This thesis describes the use of imaging time of flight secondary ion mass spectrometry (ToF-SIMS) to probe the functional role of lipids and other membrane components on the single-cell level. The enormous variety of lipids that exist in cells suggests that cells may synthesize different types of lipids and distribute them to specific locations in the cell in order to assist in various important cellular functions.\(^1\) For example, high curvature lipids may be concentrated in regions of the lipid bilayer where membrane fusion preferentially occurs because these lipids allow the bilayer to bend into the high curvature structures that form throughout the membrane fusion process.\(^2,3\) Therefore, lipids may be key molecules in membrane fusion events, such as exocytosis and sexual reproduction.\(^4\) Cholesterol, another important lipid, controls physical membrane properties and is highly concentrated in lipid rafts, which implies that it has a crucial role in normal cellular function.\(^1,5\) Cholesterol is also interesting because abnormalities in the cellular amount of cholesterol have been observed in various diseases, such as HIV, Alzheimer’s disease, and atherosclerosis.\(^6-8\) The ToF-SIMS spectra and images presented in this thesis demonstrate the ability of this analytical technique to study the spatial distribution of lipids and cholesterol in single cells and provide the groundwork for many exciting future applications.
The Ionization Behavior of Lipids in SIMS

Because of the diversity of lipids and membrane components, it was imperative to evaluate the ionization behavior of standards prior to ToF-SIMS imaging of cells. Phosphatidylcholine (PC) was a very prevalent analyte in these experiments because it is the most highly concentrated phospholipid in many cell membranes and therefore its ionization was thoroughly investigated. SIMS spectra of deuterated PC thin films and of PC suspended in deuterated water matrices indicate that protonation of phosphocholine occurs largely from extra-molecular proton sources. This finding is advantageous for ToF-SIMS imaging of cells, because cells are often analyzed in a frozen-water matrix which provides abundant extra-molecular protons for enhanced PC signal and improved ToF-SIMS images. Additionally, SIMS spectra of a frozen-hydrated lipid suspension demonstrate that an over-layer of water gradually becomes the predominant species at the surface and causes lipid signal to diminish. These results indicate that residual water in the vacuum system can condense onto frozen-hydrated samples over time, which makes addition of water a negative factor for SIMS experiments. These results establish an appropriate time window for future freeze-fracture experiments so that the beneficial proton donation attributes of water can be exploited while the negative effects of water condensation are minimized. Later in this chapter, preliminary data will show that a buckminsterfullerene (C_{60}^+) primary ion source can be used to etch away water that condenses onto the sample surface, thus minimizing the negative aspects of water.

It was necessary to expand the repertoire of lipids that can be identified in order to take full advantage of the capability of SIMS to image lipids in biomembranes. A mass
spectral library was created for six biologically relevant lipids (phosphatidylethanolamine, phosphatidylglycerol, phosphatidylserine, phosphatidylinositol, sulfatide, cholesterol). By comparing these spectra, characteristic positive and negative ion peaks were identified for every analyte. The characteristic peaks allowed the lipids to be identified in a complex mixture and to be spatially resolved. The results signified that these membrane components could be detected, identified, and imaged with ToF-SIMS. Furthermore, the secondary ion yield for these lipids was compared using gallium (Ga⁺) and C₆₀⁺ primary ion sources. The signal achieved with C₆₀⁺ was between 40-1000 times greater than with Ga⁺ and, in general, the best signal enhancement was observed for the higher mass fragments. The improvement in bio-molecule signal with cluster ion sources, like C₆₀⁺, will undoubtedly provide greater contrast in SIMS images of single cells.

The use of SIMS to image lipids faced a significant challenge in chapter 4 when it was discovered that the phosphatidylethanolamine (PE) headgroup is difficult to detect in the presence of PC. This property is problematic when mapping the distribution of PE in PC-rich cell membranes. In order to develop analytical solutions to circumvent this obstacle, the SIMS ionization mechanism of PE has been studied in detail. Experiments with deuterated PE indicate that matrix protons are large contributors to the ionization mechanism of PE headgroup fragments. Because the ionization mechanisms of both phosphocholine and PE headgroup ions rely heavily on extra-molecular protons, differences in the ionization mechanisms are not a likely cause for the dramatic differences in PE and PC ion yield. It is hypothesized that fundamental difference between the molecules cause PC to be preferentially protonated by other lipids in the
matrix. The hydrogen-bond donation ability, stearic hindrance, and hydrophobic
differences between PE and PC have been compared systematically using PE, PC, and PE
analogues with varying degrees of headgroup methylation. All three factors seem to
contribute to the signal suppression of the PE headgroups, although it is likely that
hydrogen bonding is the most important contributor. It is crucial to resolve the signal
suppression of PE because it is a high-curvature lipid and therefore will be a target
analyte in single-cell experiments. Further work is necessary to establish effective
solutions.

SIMS Imaging of Single Cells

The experiments described in the remainder of this thesis applied the knowledge
from the lipid ionization experiments to SIMS imaging of single cells. In one study, the
cholesterol content in macrophage (J774) cells that were treated to contain elevated
amounts of cholesterol was compared to the cholesterol content in untreated J774 cells.
A challenge in this experiment was that unreliable comparisons were found between cells
that are freeze-fractured under separate conditions owing to the large number of variables
in freeze-fracture sample preparation. Therefore, it was necessary to adapt the
experimental design to allow two populations of morphologically similar cells to be
freeze-fractured in the same sample and simultaneously analyzed with SIMS. To
accomplish this goal, an in-situ two-color fluorescence methodology has been developed
in which the two cell populations are labeled with fluorophores having different energetic
properties. Prior to SIMS analysis, it is then possible to identify the origin of a given cell
by its fluorescence characteristics and to identify the section of the cell exposed to the sample-vacuum interface. After J774 cells are classified with fluorescence images, corresponding SIMS images reveal that the cholesterol content of cholesterol-β-cyclodextrin treated J774 cells is twice that of untreated J774 cells. These data are significant because they demonstrate the ability of SIMS imaging to quantify membrane components, on a relative basis. Furthermore, development of the protocol for this experiment is an important advance because it extends the types of biological applications possible with ToF-SIMS. For example, in-situ two-color fluorescence and SIMS imaging will be useful to study the cholesterol accumulation in macrophage cells that contributes to atherosclerosis and to assess cholesterol-lowering drug treatments for heart disease.8

In a separate cell experiment, the role of lipids in membrane fusion events was directly investigated with molecule-specific images of the distribution of lipids across mating Tetrahymena thermophila. During Tetrahymena mating, two complementary cells bind at their anterior ends and hundreds of fusion pores form in this 8–μm region, called the conjugation junction.9, 10 The hypothesis tested in this experiment was that certain biophysically relevant lipids may be concentrated in this well-defined membrane region to allow the aggregation of high curvature membrane structures. SIMS images of mating Tetrahymena demonstrate that PC, a low-curvature lipid, is diminished in the conjugation junction while the total lipid distribution is constant across the region. Additionally, mass spectra and principal component analysis indicate that the fusion region contains elevated amounts of 2-aminoethylphosphonolipid (2-AEP), a high curvature lipid. These results have enormous biological impact because they indicate that
Tetrahymena can synthesize lipids or redistribute lipids to adjust the physical properties of their membrane at the conjugation junction for fusion pore formation. It can be extrapolated that single membrane fusion events, such as exocytosis, may occur preferentially at membrane regions that are enriched in high-curvature lipids and that the large variety of lipids that exist in cells are manufactured for a functional purposes.

This thesis makes significant contributions to biological SIMS imaging and demonstrates the impact that SIMS can have on cell biology. Specifically, the fundamental ionization studies, the instrumental developments, and the single-cell imaging applications set the groundwork for many exciting future experiments.

**Future Directions of ToF-SIMS Imaging of Biological Samples**

**Prospects for Single-Cell Investigations with C\textsubscript{60}\textsuperscript{+}**

The developments in biological SIMS presented in this thesis pave the way for the pursuit of the underlying goals of this project, which are to understand the structural aspects of exocytosis, particularly the storage and release of chemical messengers and the role of lipid domains in exocytosis. Addition of a C\textsubscript{60}\textsuperscript{+} primary ion source to the SIMS instrument, described in chapter 3, was one important instrumental contribution to the attainment of these goals.
Single-Cell Imaging with $C_{60}^+$

Future SIMS imaging of single-cells will be greatly aided by the higher secondary ion yield of lipids with the $C_{60}^+$ source. Preliminary work has demonstrated the possibility of resolving single-cells with the $C_{60}^+$ source and the resulting enrichment in secondary ion yield, image contrast, and information quality. *Spirostomum* were chosen for the initial studies because these protozoa can grow up to 3 mm in length, which makes them easy to optically locate for alignment with the SIMS imaging area. The cells were freeze-dried onto a copper substrate and imaged with $C_{60}^+$ and $\text{In}^+$ sources. The molecule-specific image of phosphocholine obtained with the $\text{In}^+$ source showed low signal across the cell membrane and many of the pixels exhibited no signal (Figure 7-1A). In contrast, the $C_{60}^+$ phosphocholine image displayed pixels with very intense phosphocholine signal and all of the pixels along the cell membrane contained signal (Figure 7-1B). Therefore, there was better contrast between adjacent pixels in the $C_{60}^+$ image, compared with the $\text{In}^+$ image. The mass spectra from pixels for these images indicated that the *Spirostomum* image taken with $C_{60}^+$ has 70 times more phosphocholine signal, than the $\text{In}^+$ image (Figure 7-2).
Figure 7-1: SIMS images of a *Spirostomum* captured with different primary ion sources (blue, copper, m/z 63; green, phosphocholine, m/z 184).  

**A.** SIMS image taken with an In⁺ atomic primary projectile (71 shots/pixel; 400 x 400 µm² field of view).  

**B.** SIMS image of the same *Spirostomum* taken with a C₆₀⁺ cluster primary projectile (60 shots/pixel; 700 x 700 µm² field of view). The C₆₀⁺ image was collected by Christopher Szakal.
There are two additional interesting aspects of these images that should be noted.

First, the freeze-dried *Spirostomum* were oval in shape whereas viable *Spirostomum* are long and worm-like. This discrepancy in cell morphology was likely because stress induced by the freezing process caused the cells to contract. *Spirostomum* are renowned for their ability to contract to up to half their length on a millisecond time-scale (similar
to the rate of freezing with liquid ethane) when exposed to an electrical, chemical, or mechanical stimulation.\textsuperscript{11} Interestingly, contraction is linked with the release of large intracellular calcium stores.\textsuperscript{11} The cells shown in Figure 7-1 were prepared in spring water which is highly concentrated in calcium and therefore calcium was homogeneously distributed across the imaging area. Therefore, future experiments could be performed to image calcium release from \textit{Spirostomum} undergoing contraction by rinsing the samples with deionized water prior to freezing.

A second important observation is that on the left of the $C_{60}^+$ image there were three small regions of phosphocholine localization, which were paramecia that were co-incubated with the \textit{Spirostomum}. This finding exemplified that $C_{60}^+$ images could spatially resolve smaller cells. The size limitation for cells that can be imaged with $C_{60}^+$ is the subject of future work and will be improved by a new $C_{60}^+$ source with 40-kV beam energy.

\textit{Molecular Depth Profiling with $C_{60}^+$}

The large increase in secondary ion yield with $C_{60}^+$ will be clearly beneficial for cell imaging experiments; however, a more significant attribute of $C_{60}^+$ is the minimal subsurface damage that it creates during sample bombardment.\textsuperscript{12} The absence of extensive physical damage has been demonstrated theoretically with molecular dynamic simulations and experimentally with atomic force microscopy measurements of craters created by $C_{60}^+.\textsuperscript{13}$ Chemical damage has been assessed experimentally by a parameter called the disappearance (or damage) cross section, which is the slope of the line in a plot
of signal intensity versus primary ion fluence. The damage cross section for a multilayer polymer film was measured to be much smaller for $C_{60}^+$ than for Ga$^+$, indicating that much less chemical damage is created by $C_{60}^+$. In fact, many types of analytes, such as polymers, peptides, and fatty acids, undergo minimal chemical damage by $C_{60}^+$, which has been determined by a gradual signal decay with increasing $C_{60}^+$ fluence. These results also may reflect that any chemical damage that is created is immediately removed by the large $C_{60}^+$ sputter rate, before it can accumulate on the surface. Significantly, it is possible that the low amount of chemical and physical damage induced by $C_{60}^+$ bombardment will allow the removal and analysis of surface molecules layer-by-layer, which is called molecular depth profiling.

Molecular depth profiling is a very exciting prospect for single-cell experiments for many reasons. First, molecular depth profiling may be used to etch away up to several microns of surface water in frozen-hydrated biological samples, thus eliminating the need for freeze-fracture. Additionally, $C_{60}^+$ bombardment may enable cellular material to be controllably removed to expose a desired cell section to the sample-vacuum interface. Because membrane lipids are composed of fatty acids, it is very promising that monolayers of fatty acids have been shown to undergo minimal chemical damage under $C_{60}^+$ bombardment. Another future endeavor is to use depth profiling to create 3-D images of cells with nm-scale depth resolution. There are countless applications for 3-D cell imaging, such as determining the distribution of histamine in mast cells and the accumulation of drugs in target cells.

Preliminary experiments with an artificial cell-like model have been used to gauge the possibility of molecular depth profiling through a cell (Figure 7-3). An infinite
“cell” was initially used to obviate the need to finely align the SIMS imaging area to a particular region on the sample. The “cell” was created by first dropping an aqueous solution of sucrose onto a disordered lipid film. The lipid under-layer provided favorable surface tension properties so the aqueous solution aggregated into a sphere, similar to the geometry of a cell. Next, a small volume of a supersaturated lipid-chloroform solution was delivered onto the droplet and the chloroform rapidly evaporated, leaving behind a thin PC residue at the surface to mimic a cell membrane. The assembly was then frozen in liquid nitrogen and transferred into the SIMS analysis chamber, thus providing ample opportunity for water condensation onto the “cell” surface.

![Bulk artificial “cell” model used in initial molecular depth profiling experiments.](image)

Depth profiling experiments revealed the 3-D chemical heterogeneity of the system and substantiated the use of this method to analyze real cells. The intensities of several analytes of interest were plotted as a function of C$_{60}^+$ fluence (Figure 7-4). The graph shows that water (m/z 18) was initially detected at the surface. Bombardment by C$_{60}^+$ then removed the surface water, as is evidenced by the dramatic decrease in the m/z 18 signal intensity, and fully exposed the lipid layer, which was identified by an increase in DPPC (m/z 734). Next, the thin lipid layer was removed because the signals decreased
for DPPC and its fragment ions at m/z 184 and 86 at equal rates. The similar rate of signal decrease for m/z 184 and 86 signified that phosphocholine was not being severely chemically damaged by C₆₀⁺. Appreciable chemical damage would have been manifested by increased fragmentation of phosphocholine and thus a more rapid signal decrease of m/z 184 signal compared to the m/z 86 signal. As molecular-depth profiling continued, the signal intensities for m/z 365 and 18 increased as the sucrose and water region was uncovered. Finally, the m/z 365 and 18 signals steadied out as the depth profiling experiment continued partly through the frozen aqueous droplet. This initial experiment indicated that molecular-depth profiling through a cell is feasible. Further experiments should examine molecular depth profiling through more intricate cell models and real cells to determine if the spatial distribution of biological samples is maintained after C₆₀⁺ depth profiling.
Single-Cell Applications for Probing the Role of Lipids in Membrane Fusion

**Lipid Involvement in Tetrahymena Conjugation and Exocytosis**

Other future plans for this project might involve ToF-SIMS imaging of a variety of cell systems, including *Tetrahymena* and beige mouse mast cells, to examine the role of lipids in biological fusion. Experiments are already underway to continue to explore the membrane chemistry of *Tetrahymena* during various membrane fusion events (Figure 7-5A). First, the time-scale and signaling mechanism for lipid domain formation in mating *Tetrahymena thermophila* will be investigated. Prior to full conjugation, *Tetrahymena* experience a lengthy pre-mating process, called co-stimulation, when...
complementary cells briefly interact (Figure 7-5B). It is possible that contact between
cells initiates a signaling cascade to prepare each cell for conjugation. This signaling
cascade may catalyze the experimentally observed cellular changes including the
modification of protein synthesis and the physical transformation of the mating region
(Figure 7-5B). The signaling cascade may likewise induce de novo lipid synthesis or the
redistribution of particular lipids to the conjugation junction to allow fusion pore
formation. To test these possibilities, SIMS images of mating Tetrahymena
thermophila could be captured at different time periods during co-stimulation and
conjugation to provide insight into the magnitude and time-scale of lipid domain
formation. Once normal lipid domain formation is established, it would be possible to
systematically block Tetrahymena membrane receptors and monitor how lipid domain
formation is effected. This experiment would pinpoint which proteins are responsible for
signaling the cellular machinery to prepare the membrane for conjugation.

Figure 7-5: Brightfield microscopy images of Tetrahymena thermophila. A. Tetrahymena in the resting stage prior to the induction of mating. Note that the anterior ends of the cells are rounded. B. Tetrahymena during the co-stimulation process prior to full conjugation. During this time, complementary cells readily interact and the tip morphology of the cells changes from rounded to pointed. Field of view is approximately 150 x 150 µm².
A second future SIMS project with *Tetrahymena thermophila* could examine the functional role of lipids during exocytosis. At any time, the inner leaflet of the *Tetrahymena* membrane contains several hundred docked secretory granules that are interdigitated between rows of cilia, in a vertical pattern.\(^{21}\) Synchronous release of these granules could be stimulated with the polycyclic polycation, Alcian Blue.\(^{21}\) *Tetrahymena* therefore would be an elegant system for SIMS exocytosis studies because any lipid domains that form would be aggregated into a distinct pattern of large vertical regions of lipid heterogeneity. It is anticipated that SIMS images of stimulated, frozen-hydrated *Tetrahymena* would reveal a heterogeneous distribution of PC. The experiment would examine whether PC is depleted in the fusion regions and if a cone-shaped lipid, such as 2-AEP, is elevated in those regions. This work would be valuable because it would support or negate a correlation between the membrane effects observed in sexual reproduction with exocytosis. Similar lipid heterogeneity in these two fusion processes would allow a generalized conclusion that all membrane fusion events necessitate the presence of high curvature lipids.

**Role of Lipids During Individual Release Events in Mast Cells**

The *Tetrahymena* studies would probe membrane regions containing aggregated fusion sites and the corresponding SIMS images would reflect gross changes in lipid composition for many individual fusion events. It would be interesting to establish whether the lipid compositional changes observed for many concentrated fusion events could be related to changes occurring during single fusion events. Furthermore, major
long-term goals of this project are to understand cell-to-cell chemical communication in mammalian cells and to push the spatial resolution of the technique. For these reasons, upcoming research could be geared towards SIMS imaging of individual exocytosis events in mutant beige mouse mast cells. Beige mouse mast cells are an attractive model system because they contain vesicles that are as large as 2 µm in diameter (Figure 7-6A). Fusion of these large vesicles with the cell membrane would affect an area up to 4 µm in diameter. Features 2-4 µm in size are well within the spatial resolution currently attainable with the ToF-SIMS instrumentation in the Winograd lab. The aims of imaging exocytosis in beige mouse mast cells would be to examine the distribution of lipids during single release events in mammalian cells and to gauge the possible functional role of lipids in individual membrane fusion events.

Preliminary SIMS images of frozen-hydrated mast cells have been obtained in preparation for tackling these long-term goals (Figure 7-6B). Phosphatidylcholine was localized and sodium was anti-localized to the mast cells, which was not surprising based on the high concentration of sodium in the extracellular solution. The cells in these images were not stimulated to undergo exocytosis prior to freezing and no spontaneous release events were captured for these particular cells. However, these images demonstrate the feasibility of imaging the distribution of PC across mast cells.
Forthcoming experiments could be carried out to image the distribution of PC and high curvature lipids across releasing mast cells. To accomplish this, mast cells could be sequestered from the abdominal cavity of beige mice, fluorescently labeled with Dil, and rinsed in the standard extracellular solution. A droplet of the cell solution could be delivered onto a silicon wafer and the cells would then be stimulated to release with dinitrophenyl human serum albumin the instant before freezing. After freeze-fracture, the stimulated mast cells would be imaged with SIMS. If these experiments are successful, the SIMS images obtained would be the first example of spatially resolved biological molecules across features in this size range and more importantly would reveal the distribution of lipids during single exocytosis events.

Figure 7-6: Brightfield and SIMS images of beige mouse mast cells. A. Brightfield image illustrating the enormous vesicles inside mast cells. The object on the left is a stimulation pipette and a mast cell is approximately 5 µm from the pipette tip. Image courtesy of Julie Lapos. B. SIMS image of frozen-hydrated mast cells taken with an In⁺ primary ion source, a dose of 0.8 million primary ion shots, and a 60 x 60 µm² field of view (blue, sodium, m/z 23; green, phosphocholine, m/z 184).
Investigations of Intracellular Chemicals in Single Cells and Tissue

This thesis has focused on SIMS imaging of lipids and cholesterol and the role of these molecules in membrane biochemistry. However, there are many other chemicals important in cell biology, such as biogenic amines and drug agents, which are excellent candidates for molecule-specific imaging with SIMS. The remainder of this chapter will introduce exciting potential applications for SIMS involving biogenic amine release, biogenic amine localization in the brain, and the assessment of chemotherapy drug efficacy.

Histamine Distribution in Mast Cell Vesicles

As previously discussed, the beige mouse mast cell is a well-designed cell system for SIMS imaging because of the unusually large size of the vesicles. An additional benefit to this cell line is that beige mouse mast cell vesicles contain abnormally high concentrations of histamine (estimated to be 0.5 M), a biogenic amine involved in immune response. SIMS imaging should have the sensitivity to detect and image this concentration of histamine in individual vesicles.

Mast cells and many other cell types have vesicles composed of a polyanionic “core” surrounded by a neutral volume (the “halo”), which is hypothesized to contain the majority of the secretory products. To test this hypothesis, potential future work could use SIMS imaging to map the vesicular distribution of histamine in a frozen-hydrated mast cell. The molecule-specific images of histamine would display whether the amount of histamine is elevated in the halo versus the core. The protocol for this experiment
would be identical to that described for imaging lipids at release sites in mast cells, although stimulation should not be necessary. This study will be challenging because SIMS is a surface sensitive technique which will require that the inside of a vesicle is exposed to the surface-vacuum interface by freeze-fracture. Alternatively, this could be the perfect application for C$_{60}^+$ molecular depth profiling. Instead of using freeze-fracture, depth profiling could be used to etch through mast cells and expose the inside of a vesicle. Understanding the distribution of histamine in mast cells will have implications for the mechanism of exocytosis and, in particular, release events involved in immune response.

*Neurotransmitter Distribution in the Brain of Drosophila Melongaster*

Another prevalent biogenic amine is dopamine, which is intimately involved in many aspects of animal physiology, including emotion, cognition, motor control, addiction, and learning and memory.\textsuperscript{25} Abnormalities in dopamine have been observed in diseases such as Parkinson’s Disease and schizophrenia. Like histamine, dopamine is a small mass biological molecule (m/z 153) and will be an excellent analyte in SIMS imaging analyses.

*\textit{Drosophila melanogaster*}, the common fruit fly, has been frequently used to investigate neuronal processes and behaviors, including those involving dopamine, and will be used in upcoming dopamine-related SIMS experiments. *Drosophila* is an attractive system to study because of its small size, short life cycle, and sequenced genome, which allows easy genetic and molecular manipulation.\textsuperscript{26} Importantly, the key
molecules involved in many neurological processes are markedly similar between *Drosophila* and mammals, allowing neurological studies of mammals to be converted to the simpler fruit fly.\textsuperscript{26}

Through immunohistochemistry experiments, dopamine has been shown to be highly concentrated in a region of the *Drosophila* brain called the mushroom bodies. This region is often related to the cerebral cortex in vertebrates and thought to be associated with intelligence. The response of the mushroom bodies to visual and tactile stimuli has suggested these structures may act as sites for sensory integration and therefore may have a crucial role in learning and memory.\textsuperscript{27-29} The mushroom bodies contain hundreds of compact, parallel neurons that are sectioned into three morphologically distinct structures known as the calyces, the peduncles, and the lobes.\textsuperscript{30} Despite the importance of the mushroom bodies in the *Drosophila* brain, there currently is no information on how its three structures interact to mediate learning and memory. Furthermore, dopaminergic catabolism and anabolism is not well understood in the fly and it is likely that the mushroom bodies contain large amounts of dopamine metabolites. SIMS should have the needed sensitivity to image dopamine and its metabolites in the mushroom bodies because of the large number of analyte molecules present in the area. Therefore, the goal of this particular project would be to collect SIMS images of the distribution of dopamine and its metabolites in the *Drosophila* mushroom bodies to gain insight into the localized metabolism of dopamine and the function of the mushroom bodies in learning and memory.

Preliminary experiments have been performed in preparation for SIMS imaging of biogenic amines and metabolites in the *Drosophila* brain. The complexity and diversity
of molecules in the brain requires standard analyses of potential analytes prior to SIMS imaging experiments. Mass spectra of both positively and negatively charged ions have been collected for neurotransmitters and metabolites that may be present in the Drosophila brain (Figure 7-7). Unique mass spectral peaks have been identified for ten compounds and are reported here in tabular form (Table 7-1). Significantly, all analytes thought to be involved in the dopaminergic pathway exhibited signature mass spectral peaks that will allow them to be distinguished in the mushroom bodies with SIMS imaging (Figure 7-8).
Figure 7-7: Biogenic amines possibly present in the *Drosophila* brain. A. 3-methoxytyramine (3-MT) B. 3,4-dihydroxyphenylacetic acid (DOPAC) C. Dopamine D. Epinephrine E. Homovanillic acid (HVA) F. L-3,4-dihydroxyphenylalanine (L-DOPA) G. N-acetyl dopamine (naDa) H. N-acetyl tyramine (naTa) I. Serotonin J. Tyramine.
### Table 7-1: Characteristic SIMS peaks for several neurotransmitters and metabolites

<table>
<thead>
<tr>
<th>Neurotransmitter/Metabolite</th>
<th>Fragment</th>
<th>Mass (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Methoxytyramine (3-MT)</td>
<td>C$<em>9$H$</em>{13}$NO$^+$</td>
<td>151</td>
</tr>
<tr>
<td>3-Methoxytyramine (3-MT)</td>
<td>C$<em>9$H$</em>{14}$NO$_2$$^+$</td>
<td>168</td>
</tr>
<tr>
<td>DOPAC</td>
<td>C$_8$H$_8$O$_4$$^+$</td>
<td>168</td>
</tr>
<tr>
<td>DOPAC</td>
<td>C$_8$H$_7$O$_4$</td>
<td>167</td>
</tr>
<tr>
<td>Dopamine (DA)</td>
<td>C$<em>8$H$</em>{11}$NO$^+$</td>
<td>137</td>
</tr>
<tr>
<td>Dopamine (DA)</td>
<td>C$<em>8$H$</em>{12}$NO$_2$$^+$</td>
<td>154</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>C$<em>9$H$</em>{12}$NO$_2$$^+$</td>
<td>166</td>
</tr>
<tr>
<td>Homovanillic Acid (HVA)</td>
<td>C$<em>9$H$</em>{10}$O$_4$$^+$</td>
<td>182</td>
</tr>
<tr>
<td>Homovanillic Acid (HVA)</td>
<td>C$_9$H$_9$O$_4$</td>
<td>181</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>C$<em>9$H$</em>{10}$NO$_3$$^+$</td>
<td>180</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>C$<em>9$H$</em>{12}$NO$_4$$^+$</td>
<td>198</td>
</tr>
<tr>
<td>N-acetyl dopamine (naDa)</td>
<td>C$<em>{10}$H$</em>{14}$NO$_3$$^+$</td>
<td>196</td>
</tr>
<tr>
<td>N-acetyl tyramine</td>
<td>C$<em>{10}$H$</em>{14}$NO$_2$$^+$</td>
<td>180</td>
</tr>
<tr>
<td>Serotonin</td>
<td>C$<em>{10}$H$</em>{12}$N$_2$O$^-$</td>
<td>176</td>
</tr>
<tr>
<td>Tyramine</td>
<td>C$<em>8$H$</em>{12}$NO$^+$</td>
<td>138</td>
</tr>
</tbody>
</table>
The next step in this study should be to image dopamine and its metabolites in the *Drosophila* mushroom bodies. In collaboration with the Han lab, the *Drosophila* will be bred to contain a green fluorescent protein tag that is specific for the mushroom bodies. The brains of these flies will be sectioned into 5-20 μm thick slices, transferred onto a substrate, and fast frozen with liquid ethane. Prior to SIMS analysis, it will be possible to locate the mushroom bodies with *in-situ* fluorescence using an XF101 filter set (Omega, Brattleboro, VT). Once the mushroom bodies are aligned with the SIMS imaging area, molecule-specific images of dopamine and the metabolites could be generated. A

Figure 7-8: Positive ion SIMS spectra for six analytes potentially involved in the *Drosophila melanogaster* dopaminergic pathway.
challenge in this experiment will be exposing either extracellular or intravesicular biogenic amines and metabolites to the surface-vacuum interface. Molecular depth profiling through the *Drosophila* brain with C$_{60}^+$ may solve this difficulty and imaging with C$_{60}^+$ and Au$_{n}^+$, instead of In$^+$, will greatly improve the sensitivity for the biogenic amines.

The significance of these experiments will be two-fold. First, images of dopamine in the *Drosophila* brain will be the first example of biogenic amine SIMS imaging and will open the door to numerous new and exciting applications. Secondly, identifying and distinguishing the enzymatic degradation products of dopamine localized in the mushroom bodies would be a novel achievement and would further support or refute the presence of monoamine oxidase metabolic products versus N-acetylation products that has been suggested from micellar electrokinetic capillary chromatography (MEKC) and high performance liquid chromatography (HPLC) experiments.$^{31-33}$

If initial dopamine imaging experiments are successful, other biogenic amines could be tackled with SIMS imaging of the *Drosophila* brain. For example, tyramine is an intriguing fly neuromodulator because it is believed to be the fly equivalent of norepinephrine, which is involved in adrenergic signaling in mammals.$^{34}$ However, the precise physiological role of tyramine remains evasive.$^{34}$ SIMS imaging would be a unique approach to examine the role of tyramine in *Drosophila*. Systematically collecting SIMS images of different sections of the *Drosophila* brain might reveal locations of tyramine that are functionally significant and may help elucidate its possible correlation with norepinephrine in mammals.
Chemotherapy Drug Efficacy Studies at the Single-Cell Level

In addition to small bio-molecules that are native to cells and tissue, SIMS imaging is well-suited as a probe of non-native chemical components, such as low molecular weight chemotherapy drugs, in cells and tissue. SIMS images of the distribution of chemotherapy drugs in single cells would be valuable because the efficacy of many drugs is influenced by subcellular localization. For example, platinum (II) drug therapies act by damaging DNA through covalent bond formation between platinum and the purine bases of DNA. Therefore, the effectiveness of platinum (II) drugs depends on the localization of the drug molecules to the cell nucleus. Single-cell SIMS images of new platinum (II) drug candidates would reveal the relative amount of the drug in the cell nucleus versus the cytoplasm and would help in the selection of the best drug candidates for further screening and development. Furthermore, in-situ two color fluorescence and SIMS imaging would allow the simultaneous analysis of drug-treated cancer cells and drug-treated healthy cells to gauge the specificity of platinum (II) drugs for cancer cells.

Chandra and colleagues have used SIMS to image anticancer drugs, specifically to assess boron neutron capture therapy (BNCT) drugs for the treatment of brain cancer glioblastoma multiforme. This work promoted the use of SIMS imaging for drug analysis. However, the experiments operated in the dynamic mode of SIMS, which uses a high fluence of primary ions, creates extensive molecular damage, and therefore restricts analysis to elemental species. Additionally, in order to classify co-cultured cancerous and normal cells prior to SIMS imaging, the authors chose morphologically dissimilar cells (glioblastoma cells from the human brain, healthy human skin fibroblast
and healthy porcine kidney cells) that originated from different organs and even different organisms. In contrast, SIMS imaging in the static mode would result in minimal molecular fragmentation to chemotherapy drugs and would expand the potential drug analytes beyond those containing specific elemental markers, like isotopic $^{10}$B. Thus, the novel aspect of the experiments proposed here are the ability to generate molecule-specific images of antitumor drugs, the possibility to assess drug specificity for cancerous cells versus healthy cells, and the potential to understand the mechanisms of drug resistance in morphologically similar cancerous cells.

Cisplatin, a platinum (II) chemotherapy drug, was chosen for preliminary experiments because it is a low molecular weight compound that targets DNA and should be heterogeneously distributed inside a cell (Figure 7-9A). A standard positive ion SIMS spectrum of cisplatin shows the expected isotopic profile for platinum at m/z 195 and a second clustering of peaks at m/z 212 corresponding to PtNH$_3^+$ (Figure 7-9B). A third intense cluster of peaks are present around m/z 342. These peaks may correlate with the products of cisplatin exchange reactions between a chlorine atom and DMSO, the solvent. This reaction has also been observed in fast atom bombardment mass spectra of cisplatin. The atomic and molecular masses identified here could be used in future SIMS imaging experiments of cisplatin-treated cells.
Subsequent experiments should involve incubating DiI-labeled NIH: OVCAR-3 cells (human ovary epithelial cells afflicted with adenocarcinoma) with 100 µM cisplatin, rinsing the cells once with drug-free buffer, transferring the cell solution onto a silicon substrate, and immediately fast-freezing in liquid ethane. After freeze-fracture, the cells can be located with in-situ fluorescence microscopy and the cell sections that are exposed to the surface can be classified with the chemical signatures from SIMS imaging. Chemical maps for platinum and cisplatin molecular fragments can be generated for

Figure 7-9: SIMS analysis of cisplatin. A. Chemical structure of cisplatin. B. Positive SIMS mass spectrum of cisplatin with asterisks labeling the three clusters of peaks described in the text. Spectrum was collected with an In⁺ primary ion source scanned across a 300 x 300 µm² with 1.0 million primary ion shots.
cross-fractured cells to determine the distribution of the drug within the cell. Alternatively, using the $C_{60}^+$ and $Au_{69}^+$ primary ion sources, instead of $In^+$, would greatly increase the secondary ion yield of cisplatin for improved SIMS imaging and $C_{60}^+$ molecular depth profiling would enable 3D imaging of the drug distribution within the cell.

These experiments should illustrate the molecule-specific imaging capabilities of static SIMS for chemotherapy drug analysis and will introduce molecular depth profiling as a novel approach to study the efficacy of antitumor drugs. If the initial experiment is successful, the accumulation of platinum (II) chemotherapy drugs, which are known to have different uptake characteristics (ex. cisplatin versus $trans$-$[Pt((Cl)_{2}(pyridine)_{2})]$, could be compared to further validate SIMS imaging. SIMS imaging could also be used to test other classes of chemotherapy drugs, such as the phosphate-based molecule, cyclophosphamide, in the phosphate-rich cellular environment, for which molecular imaging instead of elemental imaging is of the utmost importance. Once the methodology has been fully established, SIMS images could aid in evaluating the specificity and distribution effectiveness of antitumor drugs and could help understand the mechanisms of drug resistance in certain cells in order to design improved drugs.

Clearly, there are a multitude of possibilities for future SIMS imaging experiments of cells and tissue and many of these challenging biological endeavors will be realized with further development of $C_{60}^+$ imaging and molecular depth profiling. The progress described in this thesis, coupled with thrilling new applications and continued instrumental advances, create a very bright outlook for biological SIMS imaging.
References

(3) Gruner, S. M. Proc Natl Acad Sci USA 1985, 82, 3665-3669.
(26) Ream, P. J.; Suljak, S. W.; Ewing, A. G.; Han, K. A. Anal Chem 2003, 75, 3972-3978.


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Affiliations

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