SECONDARY ION MASS SPECTROMETRY AS A TOOL TO EVALUATE CHEMICAL COMPOSITION WITHIN MODEL AND CELLULAR MEMBRANES

BY

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DISSERTATION

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ABSTRACT

Developing tools to elucidate the chemical distribution of lipid components within the eukaryotic cellular membrane is critical to understanding their role in many cell processes. Secondary ion mass spectrometry (SIMS) is a technique that offers both chemical and spatial specificity, and has become popularized over the last decade for analyzing model and native cellular membranes. Herein, this thesis describes the use and development of SIMS for such samples. By employing high-resolution SIMS, performed on a Cameca NanoSIMS 50, and atomic force microscopy (AFM) the influence of cholesterol on the phase behavior of supported lipid membranes containing saturated phosphatidylcholine lipid species was studied. While the NanoSIMS 50 afforded unprecedented lateral resolution on the chemical distribution of these model membranes, it was achieved at the cost of employing stable-isotope labels for component identification. Time-of-flight SIMS (TOF-SIMS), on the other hand, is a molecular imaging technique that does not require the use of labeled species. However, the ability to image characteristic lipid fragments (i.e. lipid headgroups, etc.) at lateral resolutions comparable to the NanoSIMS 50 is challenging. Furthermore, many of the characteristic fragments are common between structurally similar lipids, such as different phosphatidylcholine species, making discrimination between these species difficult. This challenge was overcome by developing a multivariate analysis (MVA) method, called principal component analysis (PCA), for evaluating the TOF-SIMS spectra of these samples. As a result, the ability to image and identify saturated phosphatidylcholine lipids that differ only in chain length within phase-separated
membranes was achieved and could be registered to the corresponding AFM image. By performing PCA to compare TOF-SIMS spectra of labeled and unlabeled species, the molecular ion peaks that are associated with these phosphatidylcholine lipids were identified. These known ion peaks were then used to optimize PCA for TOF-SIMS imaging of phase-separated supported lipid membranes to attain a greater lateral characterization of these samples. The ability to gain quantitative information from TOF-SIMS analysis of homogenous supported lipid membranes was made possible by performing partial least squares regression (PLSR) on the resulting mass spectrum. Here, calibration samples were modeled, and then used to quantitatively predict the content of unknown membrane samples. Lastly, a TOF-SIMS MVA approach was utilized to evaluate native cellular membranes with the goals of differentiating between cell types, and in a separate project, identify the binding of vascular endothelial growth factors to human endothelial cells.
To my family
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“If I have seen further it is by standing on the shoulders of giants.” -Isaac Newton
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Chapter 1.
INTRODUCTION

Cellular Membranes

The eukaryotic cellular membrane is a highly dynamic and complex structure. Classically, the membrane was thought to be a homogenous sea of lipids, where integral proteins could freely diffuse laterally across the membrane, and displayed no semblance of an ordered structure. However, mounting evidence over the last few decades has suggested that nonrandom distribution of components exists laterally across the cellular membrane.\textsuperscript{1-4} This has been proven conclusively for protein clustering in the plasma membrane, which can be observed using affinity labels (\textit{i.e.} labeled antibodies) or fluorescent protein constructs.\textsuperscript{3,5} Lipid components, on the other hand, were not generally thought to be heterogeneously distributed across the membrane until rather recently, even though evidence as early as the 1970s suggested as such.\textsuperscript{4,6-10} Compositionally distinct cholesterol and sphingolipid-enriched domains, known as 'lipid rafts,' are now believed to play vital roles in many cell functions, including signaling, transport, and sorting.\textsuperscript{11-17}

Still unclear, however, are questions concerning the precise composition of lipid rafts within the cell membrane and the mechanism that drives lipid heterogeneity, whether it is lipid-protein and/or lipid-lipid (specifically lipid-cholesterol) interactions. This is in part due to the indirect methods with which these properties are assessed. For example, while detergent-resistance based extraction may point to
physiological relevant biases in lateral composition, and fluorescence microscopy methods may provide mechanistic information of protein and lipid transport across the membrane, these methods are commonly used without regard for the artifacts that can be generated in the native membrane by using these techniques. Consequently, how cell membrane organization influences biological processes is still mostly unknown, likely due to a lack of suitable imaging methods.

**Model Membranes**

Due to the complexity of natural cellular membranes, many different model systems have been created that retain the same lipid bilayer structure, yet simplify the membrane system. By employing model membranes, one is afforded the possibility of visualizing the organization and dynamics of individual components within the membrane, and can, consequently, investigate their roles within the membrane. There are many different models and approaches that have been developed and studied, as illustrated in Figure 1.1. Perhaps the two most commonly studied types of model membranes are giant unilamellar vesicles (GUVs) and supported lipid bilayers (SLBs), which are frequently used to characterize phase behavior of binary and ternary lipid mixtures. In particular, these models have been vital to investigations that probe the composition of heterogeneous lipid domains and in determining whether the mechanisms of lipid-lipid and/or lipid-cholesterol
interactions are sufficient to form lipid domains with analogous properties to cellular membrane lipids rafts.

Figure 1.1. A collection of model lipid membrane systems surrounding a schematic diagram of a cell that emphasizes the large number of different membrane surfaces. Clockwise, beginning from the upper left: (a) giant unilamellar vesicles (GUVs) and blebs; (b) networks of giant vesicles connected by lipid microtubules; (c) ruptured GUV's on solid supported bilayers; (d) membrane nanodiscs containing transmembrane proteins; (e) supported lipid bilayers (SLBs) analyzed by NanoSIMS and AFM; (f) ruptured cell membranes on solid supports; (g) bilayers containing ion channels tethered to a solid support; (h) vesicles tethered to a SLB by DNA; (i) visual representation of multi-scale simulations. Figure reproduced with permission from Ref 25.
GUVs are generally created from well-defined combinations of pure lipids and cholesterol, are typically 1-10 µm in size, and have been of great value to understanding the phase behavior of lipid mixtures. The basic experimental technique for attaining phase information from these model membranes is fluorescence microscopy, where phase separation can be imaged (as seen in Figure 1.1A) using fluorescent lipophilic dyes that selectively partition into the gel, liquid-ordered (L$_o$), or liquid-disorder (L$_d$) phase. In general, these models can only be analyzed by a narrow range of analytical techniques, where fluorescence microscopy, as stated being the workhorse method, has some deficiencies of its own. These include a limited lateral resolution, the need for high intensity light sources that can cause artificial phase behavior, and that compositional information must be inferred.

Conversely, SLBs offer a planar configuration, which make them amendable to a greater variety of analytical approaches that include surface-sensitive techniques originally developed for material sciences, geochemistry, and catalysis. SLBs can be formed a number of ways, but bilayers formed by slow-cooled vesicle fusion are novel in that they exhibit similar phase behavior to that of free standing GUVs. When bilayers are created by the vesicle fusion process, a few nanometers-thick layer of water is present separating the bilayer from the substrate, enabling the lipids to diffuse laterally within each leaflet analogous to a cell membrane. However, these bilayers are only stable in aqueous solutions, and will delaminate from the
support upon contact with air.\textsuperscript{33,34} Numerous studies have successfully demonstrated the viability of flash-freezing followed by freeze-drying the SLBs to remove the water layer, yielding the ability for in-air analyses.\textsuperscript{25,29,30,35} SLBs preserved in this fashion are referred to as \textit{supported lipid membranes}. Once preserved, a supported lipid membrane can be analyzed by a number of surface characterization techniques in succession, providing complementary information about the membranes properties.\textsuperscript{29,36,37} Considering the advantages, phase-separated supported lipid membranes make an excellent model for determining chemical composition of lipid domains and simultaneously provide insight into lipid phase behavior.

### Common Characterization Techniques

Phase-separated SLBs have been studied by an array of methods, including fluorescence microscopy, atomic force microscopy (AFM), near-field scanning optical microscopy, X-ray photoelectron spectrometry, quartz crystal microbalance, surface plasmon resonance, and secondary ion mass spectrometry (SIMS).\textsuperscript{38-43} All of these methods possess their own advantages, and each provides a unique insight into the properties of phase-separated lipid membranes. However, the discussion here will be limited to techniques most pertinent to this thesis, which are fluorescence microscopy, AFM, and SIMS.

Imaging phase separation within supported lipid membranes using fluorescence microscopy is commonly performed by adding a small amount of a
fluorescently labeled lipid to the lipid mixture, pre-bilayer formation.\textsuperscript{37,44} The labeled molecule will be selectively excluded from the more densely packed, ordered lipid phase (gel or L\text{\textsubscript{a}}), thus the less densely packed, disordered phase (L\text{\textsubscript{d}}) will appear bright under the fluorescence microscope. The fluorescence microscopy experiments reported in this thesis are diffraction limited, making nanometer-size features difficult to resolve. Regardless of this limitation, fluorescence microscopy is a valuable technique for rapidly evaluating sample quality, and can generate image mosaics of the entire sample that are used to relocate specific areas of the supported lipid membrane for characterization with additional techniques.

Phase-separation in supported lipid membranes can also be imaged using AFM, by which topographical changes resulting from different membrane thicknesses are detected. Membrane thickness is directly attributed to variations in acyl chain packing, where the more densely packed gel-phase domains are thicker than L\text{\textsubscript{a}}-phase domains, which are thicker than the loosely packed L\text{\textsubscript{d}}-phase domains. Thus, when measuring supported lipid membranes with the AFM, gel-phase domains will be the tallest and L\text{\textsubscript{d}}-domains will be the shortest.\textsuperscript{45-47} AFM offers excellent lateral and height resolution of <2 nm and <0.1 nm, respectively, for supported lipid membranes, and similar to fluorescence microscopy, can be performed on hydrated SLBs or freeze-dried supported lipid membranes.\textsuperscript{39} While fluorescence microscopy and AFM provide dynamic and microstructural information about the membrane, respectively, composition must be inferred.
Secondary Ion Mass Spectrometry

SIMS is a direct approach to composition analysis. Though largely underutilized for biological sample analysis, SIMS is positioned to be at the vanguard of membrane analysis because it has the potential to provide the location-specific compositional information on cell and model lipid membranes that is currently missing.\textsuperscript{29,38,48,49} In SIMS, an accelerated ion beam bombards the surface, sputtering superficial molecules and molecular fragments that are within the beam’s focal area. The masses of the sputtered ionized species, called secondary ions, provide information about the composition of the sample’s surface (Figure 1.2). Time-of-flight SIMS (TOF-SIMS) is the most popular SIMS technique for analyzing biological membranes. In this approach, the pulsed ion beam produces molecular secondary ions, enabling a full mass spectrum to be recorded at each beam position using a TOF mass spectrometer (Figure 1.3).\textsuperscript{50} When using TOF-SIMS, the detection of molecular ions and high-mass molecular fragment ions, specific to the parent molecule, is desirable. By rastering the ion beam across the sample, an image
revealing the distribution of these distinctive ions, and thus the components that produced them, is created. The high chemical specificity, and its application to unlabeled species, renders TOF-SIMS a powerful method for analyzing the lateral composition of biological membranes.\textsuperscript{48,51,52} Unfortunately, characteristic secondary ions that permit unambiguous component identification commonly do not produce sufficient yields to enable composition imaging at submicron lateral resolution.\textsuperscript{38} Typically, biological membrane analysis by TOF-SIMS has a lateral resolution of a micron, whereas lipid domains are expected to be submicron in size.\textsuperscript{53}

\textbf{Figure 1.3.} A general schematic of a time-of-flight SIMS (TOF-SIMS) instrument. TOF-SIMS is typically operated in static mode, where the primary ion source is pulsed generating molecular secondary ions that are detected based upon their mass-to-charge ($m/z$) with a TOF mass spectrometer. Figure taken from Ref 50 (open source).

Alternatively, dynamic SIMS uses high yielding atomic and diatomic secondary ions for component identification. High lateral resolution (50 nm) has been attained for dynamic SIMS performed on the NanoSIMS 50 (Cameca; Figure 1.4).\textsuperscript{38} In this
configuration, a cesium or oxygen ion beam extensively fragments the molecules within the beam’s focal area. Up to five or seven atomic and diatomic secondary ions with different mass-to-charge (m/z) ratios, depending on the instrument model, are detected in parallel by a magnetic sector mass spectrometer with high mass resolving power (i.e., $^{12}\text{C}^{15}\text{N}^-$, 26.9996 amu and $^{13}\text{C}^{14}\text{N}^-$, 27.0059 amu can be separated). The intensities of the preselected ions measured at each location are used to create maps of the sample’s elemental and isotopic distributions. In order to differentiate molecules with the same elemental composition, molecules of interest must be labeled with a distinct stable isotope so that the isotopically enriched atomic and diatomic secondary ions generated during analysis encode for component identity.

Recently, the distributions of isotopically labeled lipids within phase-separated supported lipid membrane have been successfully imaged with 100 nm-lateral resolution by the NanoSIMS. The lipid-specific $^{13}\text{C}^{1}\text{H}^-$ and $^{12}\text{C}^{15}\text{N}^-$ secondary ions revealed the distributions of $^{13}\text{C}$-labeled 1,2-distearoylphosphatidylcholine ($^{13}\text{C}$-DSPC, gel phase) and $^{15}\text{N}$-labeled 1,2-dilauroylphosphatidylcholine ($^{15}\text{N}$-DLPC, fluid phase), respectively (Figure 1.5A and B, respectively), in the membrane. Comparison of the NanoSIMS images to the AFM topographic data (Figure 1.5C and D) acquired at the same sample locations prior to the NanoSIMS analysis enabled determination of the lipid composition within specific structures that were detected by AFM. The lipid composition at small regions of the membrane could also be quantified by using
standard samples to calibrate the normalized lipid-specific secondary ion signal intensities.\textsuperscript{30,54} Consequently, the NanoSIMS is capable of providing compositional information on a length scale that is relevant to biological membrane organization, which complements the structural and dynamic information that can be acquired with AFM and fluorescence microscopy, respectively.

\textbf{Figure 1.4.} Schematic of the NanoSIMS 50 (Cameca) illustrating the major components for imaging. Here, the SIMS is operated in the dynamic mode and the primary ion beam is constantly bombarding the surface, generating atomic and diatomic ions of the parent surface molecule. The co-axial optics enable co-propagation of the primary and secondary ions, thereby reducing the beam’s focal area and enhancing secondary ion collection. Secondary ions are analyzed based upon their mass-to-charge (\textit{m}/\textit{z}) ratio in a magnetic sector mass spectrometer.
**Figure 1.5.** NanoSIMS analysis of a freeze-dried, phase-separated supported lipid membrane composed of $^{15}\text{N}$-1,2-dilauroylphosphatidylcholine ($^{15}\text{N}$-DLPC) and $^{13}\text{C}$-1,2-distearoylphosphatidylcholine ($^{13}\text{C}$-DSPC) (A-C) and the atomic force microscopy (AFM) topography image of the same membrane location (D). (A) The normalized $^{12}\text{C}^{15}\text{N}^{-}$ secondary ion signal intensity (green) reveals the distribution of $^{15}\text{N}$-DLPC in this area, and (B) the normalized $^{13}\text{C}^{1}\text{H}^{-}$ secondary ion signal (red) shows the distribution of $^{13}\text{C}$-DSPC in the membrane. (C) Overlay of the two lipid-specific ion signals.

**Principal Component Analysis**

While the NanoSIMS is capable of achieving compositional information about model membranes at unprecedented lateral dimensions, label-free approaches are desirable for sample preparation and SIMS analysis of native cell membranes. TOF-SIMS analysis of membranes, as discussed previously, does not require the use of labels, but the types of fragment ions used to discriminate between species generate insufficient ion yields to achieve submicron resolution.$^{55,56}$ Low molecular weight
fragments ions, conversely, do produce ample yields to achieve submicron resolution, but are common to many parent species, which prohibits their use for identification. However, the extensive data sets generated by TOF-SIMS analysis, resulting from obtaining an entire mass spectrum of a surface, is especially suitable for incorporating the use of multivariate analysis (MVA) methods.

Principal component analysis (PCA), a method of MVA, has been particularly useful in classifying and identifying many biomolecules based upon variances between TOF-SIMS spectra.\textsuperscript{57-60} PCA is capable of identifying parent molecules by using several mass fragments to distinguish the similarities and discrepancies in the TOF-SIMS data acquired from different samples.\textsuperscript{61,62} Briefly, in PCA the data complexity is reduced by calculating new variables, or principal components (PCs), which represent linear combinations of the original variables and capture the greatest variation in the data set.\textsuperscript{63} As a result, the abundant low mass ions, not utilized for univariate spectra interpretation, can be used for component identification based on the variance in relative ion yields.\textsuperscript{61} PCA can be applied to images as well, by unfolding the \( n \) number of ion images that are \( I \) by \( J \) pixels, and reshaping the data to form a single 2-D array matrix with \( n \) columns and \( I \) multiplied by \( J \) rows.\textsuperscript{55,64} PCA is then performed on the resulting matrix and scores are calculated for each pixel based upon how they correlate to a given PC. The data is reassembled into an image with \( I \) by \( J \) pixels in which the score value on each PC is encoded in a pseudo-color scale.
By applying PCA to TOF-SIMS images, an increase in image contrast and improvements in specificity for component identification can be achieved.\textsuperscript{61,62,65-67}

**Scope and Aim of Thesis**

This thesis describes the development of SIMS techniques for analyzing supported lipid membranes. The use of SIMS and multivariate analysis to discriminate cells according to variations in the chemical composition of the cell surface is also explored.

In Chapter 2, high-resolution SIMS performed with a NanoSIMS instrument is extended to three-component lipid membranes. By coupling complementary information gained by using AFM and the NanoSIMS, the effect of cholesterol on phase separation was studied. This project endeavored to determine if cholesterol would induce lipid intermixing between the gel- and fluid-phase lipid components, and if cholesterol-lipid interactions were sufficient to form L\textsubscript{c}-phase domains, which are thought to be analogous in phase to cellular lipid rafts.\textsuperscript{24,26}

While implementing the NanoSIMS is one part of this thesis, the majority of this thesis focuses on the using TOF-SIMS. In Chapters 3 and 4, TOF-SIMS coupled with PCA was employed to discriminate saturated lipid species containing the same phosphocholine headgroup that differed only in the length of the hydrocarbon tails. PCA was then used on TOF-SIMS images of phase-separated lipid membranes to reveal domains of differing lipid composition (Chapter 3). By using PCA to
discriminate between isotope-labeled lipids and the analogous unlabeled (natural abundance) lipid, the lipid-related TOF-SIMS fragments could be identified (Chapter 4). This a priori knowledge of the lipid-related fragments improved PCA discrimination between lipid species and verified that discrimination is a function of composition, as opposed to sample-specific contaminants. As a final project for developing this method for analysis of supported lipid membranes, another MVA technique called partial least squares regression (PLSR),\textsuperscript{68,69} was used to acquire quantitative information about the composition of homogenous supported membranes containing a lipid species and cholesterol (Chapter 5). This method could be used for quantifying lipids and cholesterol within distinct domains of phase-separated supported lipid membranes. Lastly, this thesis discusses work in progress to use TOF-SIMS and MVA to differentiate different cell types according to cellular membrane composition (Chapter 6). Here, different cellular types were discriminated and classified based upon the composition of their respective cellular membranes using PLS discriminate analysis (PLS-DA), and, in an unrelated study, PCA was used to determine cellular membrane differences between normal human endothelial cells and cells modified with a vascular endothelial growth factor.

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Chapter 2.
CORRELATED AFM AND NANOSIMS IMAGING TO PROBE
CHOLESTEROL-INDUCED CHANGES IN PHASE BEHAVIOR
AND NON-IDEAL MIXING IN TERNARY LIPID MEMBRANES

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Introduction

Lateral variations in component distribution within the plasma membrane are required to coordinate membrane-mediated cellular functions. The nonrandom distributions of certain proteins within the plasma membrane are well-established. Lipids and cholesterol are also believed to be spatially organized in biological membranes, but whether their organization is driven by lipid-lipid or lipid-protein interactions is not clear. In one model of plasma membrane organization, differential affinities between cholesterol and other membrane components are hypothesized to drive the formation of liquid-ordered, cholesterol- and sphingolipid-enriched nanoscale domains (diameters < 300 nm) that are referred to as lipid rafts. Thus, characterizing the effects of cholesterol-dependent interactions on component distribution within the plasma membrane is the goal of much research.

Insight into how lipid-cholesterol and lipid-lipid interactions might influence lipid organization within biological membranes has been acquired by studying model lipid membranes. In the absence of cholesterol, membranes composed of a low- and high-melting temperature lipid component homogeneously mix when heated above the melting transition temperature ($T_m$) of both lipid species, but separate into disordered fluid-phase and ordered gel-phase domains when cooled below the $T_m$ of the high-melting temperature lipid species. The addition of cholesterol to the membrane affects the lipid miscibility and domain microstructure observed at room temperature in a manner that depends on the degree of saturation in the low-melting
temperature lipid.\textsuperscript{16-19} At low cholesterol concentrations, membranes composed of a di-unsaturated low T\textsubscript{m} lipid, such as 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), and a high T\textsubscript{m} lipid species exhibit macroscopic fluid- and gel-phase domains.\textsuperscript{14,19} At higher cholesterol concentrations (mole fraction \(> \sim 0.16\)), the gel-phase is replaced by macroscopic liquid-ordered domains that coexist with the disordered fluid-phase.\textsuperscript{14,16,18,20-22} Above a threshold cholesterol concentration (mole fraction \(> \sim 0.4\)), phase separation ceases and homogeneous lipid mixing occurs.\textsuperscript{14,16,18,20-22} In contrast, macroscopic liquid-ordered domains are usually not detected in cholesterol-containing ternary membranes in which the lipid species with the low T\textsubscript{m} is saturated, such as 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC).\textsuperscript{14,16,18,19,23-26} The gel-phase domains that coexist with the disordered fluid-phase undergo a reduction in size without the formation of macroscopic, liquid-ordered domains as the cholesterol concentration increases.\textsuperscript{19,25,26} For example, Figure 2.1 shows the ternary phase diagram determined by Zhao et al. for giant unilamellar vesicles (GUVs) composed of DLPC/1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC)/cholesterol at room temperature.\textsuperscript{26} At a threshold cholesterol concentration (0.15 – 0.2 mole fraction), phase separation could not be visualized in GUVs using conventional fluorescence microscopy, but fluorescence resonance energy transfer (FRET) measurements indicate that the membrane is not homogeneously mixed.\textsuperscript{18,26} This FRET behavior is postulated to be due to the presence of nanoscopic liquid-ordered domains in the membrane, even though tiny
gel-phase domains and distinct lipid clusters transiently produced by non-ideal mixing may also be consistent with these results.$^{18, 21, 23, 26, 27}$

Figure 2.1. Ternary phase diagram for giant unilamellar vesicles composed of DLPC/DSPC/cholesterol at room temperature. Uniform fluorescence and phase separation was observed at compositions marked by “○” and “●”, respectively. Green crosses “X” mark the compositions of the vesicles used to form the supported lipid membranes that were characterized with AFM and NanoSIMS in this study (an equal molar ratio of DLPC/DSPC with 0, 3, 7, 15, and 19 mol% cholesterol). The molar concentration of cholesterol in each lipid mixture used to make the supported lipid membranes was determined using commercially available enzymatic assays. Reprinted from Ref. 26 with permission from Elsevier. Phase notation for liquid-ordered ($L_o$), gel ($L_g$) phase and fluid ($L_a$) phase from Gosku and Longo (Ref. 28) have been added.

Although saturated, low $T_m$ lipids are rarely found in cellular membranes, identification of how this change in lipid structure alters membrane phase behavior provides a better understanding of the physiological significance of fatty acid chain
unsaturation in biological membranes. Moreover, demonstration of the existence of nanoscopic liquid-ordered domains in model lipid membranes would indicate that lipid-lipid and lipid-cholesterol interactions are sufficient to produce liquid-ordered domains with dimensions similar to those that may exist in biological membranes. Despite the significance of such a finding, whether the nanoscale heterogeneity detected in membranes composed of DLPC, DSPC, and a threshold cholesterol concentration is due to the presence of liquid-ordered domains, gel-phase domains, or non-ideally mixed transient lipid assemblages has not been definitively established.

A key difference between phase-separated domains and non-ideally mixed transient lipid clusters is that the composition and microstructure would be constant between nanoscopic phase-separated domains of the same phase, as opposed to varying from one structure to another in non-ideally mixed lipid assemblages. Liquid-ordered and gel-phase domains can be discriminated by their microstructures, as the edges of liquid-ordered domains are smooth and rounded, whereas the perimeters of gel-phase domains are angular and jagged. The membrane thickness may also differ between gel-phase and liquid-ordered domains. Therefore, information on the composition and microstructure of individual domains is required to identify whether the lipid structures detected at the threshold cholesterol concentration are non-ideally mixed lipid assemblages or phase-separated domains in the gel- or liquid-ordered phase. The microstructure, and therefore phase, of
submicron-sized lipid assemblages within supported lipid bilayers can be characterized with atomic force microscopy (AFM) even when the domain sizes are below the lateral resolution of conventional fluorescence microscopy.\textsuperscript{17,19,23,25,31,32}

Assessing the lipid composition of individual lipid domains is more challenging because few analytical techniques yield spatially resolved and chemically specific information. Secondary ion mass spectrometry (SIMS) is one of the few approaches that enables imaging specific lipid species within supported lipid membranes with both chemical and spatial specificity.\textsuperscript{32-42} Time-of-flight SIMS (TOF-SIMS) is a molecular imaging approach that has the advantage of not requiring labels for lipid identification, but the lateral resolution and sensitivity is seldom sufficient to characterize submicron-sized lipid domains.\textsuperscript{43} Recently, a combination of high-resolution SIMS performed with a Cameca NanoSIMS 50 and lipid-specific isotope labeling has permitted the distributions of two isotopically labeled lipid species in phase-separated supported lipid membranes to be imaged with 100-nm-lateral resolution and quantified, rendering this a promising approach to analyze the lipid composition within nanoscopic lipid domains (diameters <300 nm).\textsuperscript{36} The Cameca NanoSIMS 50 is a magnetic sector mass spectrometer that can image differences in elemental and isotopic composition.\textsuperscript{44} To identify and image different lipids using a NanoSIMS, each lipid species must contain a distinct stable isotope so that the secondary ions generated during analysis can be linked to the parent lipid species.\textsuperscript{44}
In this chapter, we use AFM and NanoSIMS to characterize the effects of cholesterol addition on the microstructure and composition of lipid domains in ternary membranes composed of the saturated, low $T_m$ lipid $^{15}$N-1,2-dilauryl-sn-glycero-3-phosphocholine ($^{15}$N-DLPC), and the high $T_m$ lipid 1,2-distearoyl-D$_{70}$-sn-glycero-3-phosphocholine (D$_{70}$-DSPC). We investigated cholesterol concentrations between 0 and 19 mol%. Over this range of cholesterol concentrations, GUVs composed of DLPC/DSPC exhibit macroscopic phase-separated domains and nanoscale heterogeneous mixing that is postulated to signify the presence of tiny liquid-ordered domains.$^{18,26}$ Supported lipid membranes are the subject of these studies to permit analyses with NanoSIMS and AFM. Although discrepancies in the phase behavior of supported membranes and GUVs have been reported, we avoided the non-equilibrium effects potentially induced by the solid support by using slow-cooled vesicle fusion to prepare the supported membranes, as demonstrated by Longo and co-workers.$^{23,25}$ Analysis of the same regions of the membrane with both AFM and NanoSIMS enabled correlation of domain microstructure with lipid composition, and allowed us to assess whether the nanoscopic lipid assemblages that occur near the threshold cholesterol concentration are non-ideally mixed clusters, or phase-separated lipid domains in either a gel or liquid-ordered state. As shown later, the AFM and NanoSIMS data indicate that the nanoscopic lipid assemblages within the 19 mol% cholesterol membrane are gel-phase domains.
Results and Discussion

The cooling rate employed for bilayer formation determines whether the phase-separation approaches equilibrium. All of the samples we studied were cooled to room temperature at the same rate to ensure that any variations in domain morphology were not due to differences in their thermal history. For compatibility with NanoSIMS analysis, the room temperature $^{15}$N-DLPC/D$_{70}$-DSPC/cholesterol supported lipid membranes were flash-frozen and freeze-dried to remove the water without perturbing lipid organization. Previous studies demonstrated that flash-freezing and freeze-drying does not induce further phase-separation or other changes in membrane organization, and the preserved membrane structure reflects that present at room temperature prior to membrane preservation. The lipids in the membrane do not exhibit lateral mobility after freeze-drying, and the membrane organization does not change in the time between AFM imaging and NanoSIMS analysis (2 – 6 weeks). Note that the use of isotopically labeled lipids is not expected to alter membrane phase behavior because D$_{70}$-DSPC has nearly the same $T_m$ as natural abundance DSPC (50.5 °C and 55.0 °C, respectively), and the single nitrogen-15 isotope in $^{15}$N-DLPC should not affect its $T_m$.

Cholesterol-free phase-separated supported lipid membranes

To correlate domain structure with lipid composition, we imaged the same locations within the phase-separated supported lipid membrane using both AFM and
NanoSIMS. Similar to previous AFM studies of phase-separated supported lipid membranes, we observed circular gel-phase domains with a bimodal size distribution in the cholesterol-free membrane (Figure 2.2).\textsuperscript{32,37,46} Small gel-phase domains with diameters below 200 nm were primarily located near the perimeters of the large gel-phase structures that were several microns in diameter (Figure 2.2a-c). Nanoscopic fluid-phase subdomains were also trapped inside of the micron-sized gel-phase domains. We measured a height difference of 1.3 nm at the interface between the gel- and fluid-phase domains (Figure 2.2b, inset). This height difference is smaller than that previously reported between gel- and fluid-phase domains (1.8 nm) and is closer to the height difference between an asymmetric domain, with one gel-phase and one fluid-phase leaflet, and a symmetric fluid-phase domain.\textsuperscript{37,46} However, NanoSIMS analysis of these domains (Figure 2.2d-f) indicated they contain too little $^{15}$N-DLPC to be asymmetric $^{15}$N-DLPC/$^{70}$-DSPC domains (see later). The smaller height difference we measured between the gel- and fluid-phases is likely due to our use of a repulsive tip-sample interaction for AFM imaging.\textsuperscript{47} AFM imaging of similar samples performed with a repulsive tip-sample interaction yielded a height difference of 1.3 nm at the gel/fluid interface, but with a tip-sample interaction that fluctuated between repulsive and attractive, we measured a height difference of 1.6 nm at the same location (not shown), which is close to the 1.8 nm height difference as previously reported.\textsuperscript{37,46}
Figure 2.2. AFM (a and b) and high-resolution SIMS images (d and e) of a phase-separated supported lipid membrane containing an equal molar ratio of D$_{70}$-DSPC and $^{15}$N-DLPC without cholesterol. (a) AFM image shows gel- and fluid-phase membrane domains. (b) Higher resolution AFM image of the region outlined in (a). The line scan below the AFM image was acquired at the location indicated by the dashed line. (d) The NanoSIMS images showing an overlay of the normalized $^{12}$CD$^{-}$ (red) and $^{12}$C$^{15}$N$^{-}$ (green) ion signals that localize D$_{70}$-DSPC and $^{15}$N-DLPC, respectively, were acquired at the same site as the AFM image (a). The absence of lipid-specific ions at the regions that appear dark is due to the low intensity of the minor isotopes. (e) Detail of the region outlined in (a), which corresponds to the AFM image in (b). Additional AFM and NanoSIMS images (c and f) taken at a different membrane region. The NanoSIMS images were acquired with ~100-nm-lateral resolution and smoothed over a lateral resolution of ~150 nm.

The NanoSIMS analysis was performed at the same membrane locations that were imaged with AFM. The normalized $^{12}$CD$^{-}$ ions (red) and $^{12}$C$^{15}$N$^{-}$ ions (green)
were used to locate D$_{70}$-DSPC and $^{15}$N-DLPC, respectively. The absence of lipid-specific ions at some pixels is due to the low intensity of the minor isotopes, as the AFM image of the freeze-dried membrane does not show damage at these sites (Figure 2.2a-c). Similar to previous reports, the area occupied by D$_{70}$-DSPC within the membrane appeared to be slightly lower than that expected based on the mol% D$_{70}$-DSPC in the vesicles used for membrane preparation. This difference in the D$_{70}$-DSPC content within the supported membranes and the phase-separated lipid membranes is likely due to the selective adsorption of these different lipid species onto the substrate, or from a lipid exchange process.

![Figure 2.3](image)

**Figure 2.3.** Calibration curve correlating the normalized $^{12}$C$^{15}$N ion intensity to the mol% $^{15}$N-DLPC in the membrane, constructed by performing several NanoSIMS measurements on homogeneous lipid membranes of known mol% $^{15}$N-DLPC. Error bars represent one standard deviation around the mean.

Using the NanoSIMS calibration curves described later (Figure 2.3), we determined that the $^{15}$N-DLPC concentration was $\sim$97 mol% in the fluid-phase domains, and $\sim$6 mol% in the gel-phase domains. Because NBD-PC (see Materials and Methods section) is excluded from the gel-phase, the D$_{70}$-DSPC
concentration in the gel-phase regions of the cholesterol-free membrane is ~94 mol%. These results are consistent with previous NanoSIMS studies of DLPC/DSPC membranes and phase diagram predictions that the gel-phase contains ≤10 mol% DLPC and the fluid-phase contains ≤10 mol% DSPC. The one exception was at the edges of the large gel-phase domains, where the data occasionally suggest the fractions of D$_{70}$-DSPC and $^{15}$N-DLPC within the same pixel (yellow areas, Figure 2.2d-f) were more nearly equal. This might suggest that the amount of lipid mixing at the edges of the domains was greater than that predicted by phase-diagrams. However, the AFM images acquired at these sites show the edges of the gel-phase domains are not smooth, but instead consist of many small crevices and peninsulas. Thus, the detection of elevated amounts of D$_{70}$-DSPC and $^{15}$N-DLPC at the same pixel occurred because these structures were not resolved in these NanoSIMS images, which were acquired with a beam diameter of ~100 nm and were smoothed to the equivalent of ~150 nm lateral resolution.

Phase-separated supported lipid membranes with 3 - 15 mol% cholesterol

The addition of cholesterol to the membrane induced an elongation of the macroscopic gel-phase domains. At 3 mol% cholesterol, the macroscopic D$_{70}$-DSPC-enriched gel-phase domains were oblong and had jagged borders (Figure 2.4). A network of gel-phase domains was observed at 7 mol% cholesterol (Figure 2.5). Similar to the cholesterol-free membrane, fluid-phase subdomains were entrapped
within the macroscopic gel-phase structures, and gel-phase microdomains were dispersed throughout the fluid phase in the 3 and 7 mol% cholesterol membranes. A networked gel-phase structure that contained entrapped fluid-phase subdomains was also observed in the 15 mol% cholesterol membrane, but the edges of the gel-phase structures were smoother than those observed in the other membranes (Figure 2.6). The area occupied by the D$_{70}$-DSPC-rich domains in the membranes that contained 7 and 15 mol% cholesterol was higher than that expected based on the compositions of the vesicles used for membrane formation. This difference in surface coverage was consistent across the membrane (see additional images in Figure 2.6). We attribute these results to the phenomena occurring during bilayer formation, such as the selective adsorption of the lipids species onto the substrate or lipid exchange, as opposed to selective incorporation of cholesterol into the ordered domains, because cholesterol is expected to evenly distribute between the two lipid phases.\textsuperscript{14} Moreover, the increase in D$_{70}$-DSPC domain surface coverage seems larger than the fraction of cholesterol in the membrane. The height difference between the gel- and fluid-phase regions of the 3, 7, and 15 mol% cholesterol membranes did not change from that measured in the cholesterol-free membrane (~1.3 nm). Similarly, the $^{15}$N-DLPC concentration within the D$_{70}$-DSPC-enriched domains remained constant at ~6 mol%, indicating that the solubility of $^{15}$N-DLPC within the D$_{70}$-DSPC-rich domains did not change over this range of cholesterol concentrations. We did not attempt to quantify the mol% of $^{15}$N-DLPC in the fluid phase due to the high likelihood that the numerous
nanoscopic gel-phase domains detected within the fluid phase by AFM would inadvertently be included in the measurement.

![Image](image.png)

**Figure 2.4.** AFM (a-c) and corresponding high-resolution SIMS (d-f) images of phase-separated membranes composed of a 1:1 molar ratio of D_{70}-DSPC to ^{15}N-DLPC and 3 mol% cholesterol. The line scan below the AFM image in (a) was taken at the area indicated by the dashed line. Additional AFM and NanoSIMS images (b and c, and e and f, respectively) taken at a different membrane region. The NanoSIMS images were acquired with ~100-nm-lateral resolution and smoothed over a lateral resolution of ~150 nm.
Figure 2.5. AFM (a-c) and corresponding high-resolution SIMS (d-f) images of phase-separated membranes composed of a 1:1 molar ratio of D$_{70}$-DSPC to $^{15}$N-DLPC and 7 mol% cholesterol. The line scan below the AFM image in (a) was taken at the area indicated by the dashed line. Additional AFM and NanoSIMS images (b and c, and e and f, respectively) taken at a different membrane region. The NanoSIMS images were acquired with ~100-nm-lateral resolution and smoothed over a lateral resolution of ~150 nm.
Figure 2.6. AFM (a-c) and corresponding high-resolution SIMS (d-f) images of phase-separated membranes composed of a 1:1 molar ratio of D$_{70}$-DSPC to $^{15}$N-DLPC and 15 mol% cholesterol. The line scan below the AFM image in (a) was taken at the area indicated by the dashed line. Additional AFM and NanoSIMS images (b and c, and e and f, respectively) taken at a different membrane region. The NanoSIMS images were acquired with ~100-nm-lateral resolution and smoothed over a lateral resolution of ~150 nm.
Figure 2.7. AFM (a-c) and correlated high-resolution IMS (d-f) images of a phase-separated membrane composed of a 1:1 molar ratio of D<sub>70</sub>-DSPC to <sup>15</sup>N-DLPC and 19 mol% cholesterol. AFM images (a and b) of two different areas of the phase-separated membrane and corresponding IMS images (d and e, respectively). (c) Higher resolution AFM scan of the area outlined with a rectangle in (b). The line scan below (c) was acquired at the edge of a cluster of nanoscopic domains, indicated by the dashed line in (c). The line scan below (f) was acquired at the nanoscopic lipid domain indicated by the circle in (c). (f) Enlarged IMS image of the area indicated by the black rectangle in (b), which corresponds to the AFM image in (c). The NanoSIMS images were acquired with ~100-nm-lateral resolution and smoothed over a lateral resolution of ~150 nm.
Supported lipid membranes containing 19 mol% cholesterol and greater

Our AFM and NanoSIMS images of the 19 mol% cholesterol membrane clearly show heterogeneity in membrane composition and structure (Figure 2.7). Individual and micron-sized aggregates of nanoscopic ordered domains enriched with D$_{70}$-DSPC are visible in the membrane. The height difference measured with AFM between the gel- and fluid-phases in the 19 mol% cholesterol membrane did not vary with domain size (~1.2 nm), and was similar to those measured in membranes with lower cholesterol levels (~1.3 nm). The $^{15}$N-DLPC concentration within the submicron-size domains that were resolved in the NanoSIMS images was ~7 mol%, within one standard deviation of the value measured on the 0 – 15 mol% cholesterol membranes. Thus, a significant increase in lipid intermixing did not occur in these domains. However, elevated amounts of D$_{70}$-DSPC and $^{15}$N-DLPC were detected at the same pixel more frequently in the 19 mol% cholesterol membrane than in the other membranes (yellow areas, Figure 2.7d-f). Comparison to the AFM images acquired at these locations revealed the presence of nanoscopic lipid structures that were smaller than the resolution of the NanoSIMS images (Figure 2.7a-c). Therefore, the detection of significant amounts of D$_{70}$-DSPC and $^{15}$N-DLPC at the same pixel in the 19 mol% cholesterol membrane signifies the presence of a phase-separated domain that is smaller than the lateral resolution of the NanoSIMS image. The lack of an observable change in the height of the nanoscopic lipid structures or lipid intermixing denotes the presence of phase-separated domains, and not non-ideally
mixed lipid clusters, in the 19 mol% cholesterol membrane. Because AFM imaging also shows that both the micron-sized and nanoscopic lipid domains have irregular borders, we conclude that the domains enriched with D$_{70}$-DSPC remained in the gel-phase at 19 mol% cholesterol.

We also attempted to characterize supported lipid membranes that contained >19 mol% cholesterol (27 and 35 mol% cholesterol). These membranes exhibited numerous small height changes (<1 nm) and taller features indicative of debris by AFM, whereas patches of $^{15}$N-DLPC and very little D$_{70}$-DSPC were detected on the substrate using the NanoSIMS (not shown). These features are not characteristic of those we have observed in poorly preserved membranes, nor do they resemble the dehydration-induced defects characterized by others.$^{50}$ These results suggest that high-quality supported lipid membranes did not form under the same conditions as those used to create the membranes with lower mol% cholesterol.

_Lipid organization in $^{15}$N-DLPC/D$_{70}$-DSPC/cholesterol membranes_

Using a combination of AFM and high-resolution SIMS to acquire correlated information on the structure and lipid distribution within $^{15}$N-DLPC/D$_{70}$-DSPC/cholesterol membranes at room temperature, we found that the addition of up to 19 mol% cholesterol induced significant changes in domain morphology, but it produced no observable change in the miscibility of $^{15}$N-DLPC within the gel-phase. Unlike previous results in which the phase behavior of analogous supported
membranes differed from that of GUVs,\textsuperscript{23} our results are in agreement with those reported for DLPC/DSPC/cholesterol GUVs and mica-supported membranes.\textsuperscript{26,28} This good agreement indicates that the slow-cooled vesicle fusion process we employed for membrane formation likely allowed the membrane to approach equilibrium, and neither the silicon substrate nor the isotope labels appeared to alter membrane phase behavior.

According to the DLPC/DSPC/cholesterol phase diagram (Figure 2.1), the miscibility point for a 1:1 molar ratio of DLPC/DSPC is \(~19\) mol\% cholesterol, and non-ideal mixing postulated to be caused by nanoscopic, liquid-ordered domains has been previously detected with FRET at slightly higher cholesterol concentrations (between 20 and 25 mol\%).\textsuperscript{18,26} Yet gel-phase domains were present in our supported lipid membranes composed of DLPC/DSPC (1:1 molar ratio) and 19 mol\% cholesterol. A higher amount of D\textsubscript{70}-DSPC than \textsuperscript{15}N-DLPC in the membrane, which could have occurred due to small gravimetric and volumetric errors in measuring the lipids, would shift the miscibility point to a higher cholesterol concentration (\(~20\) mol\%) than we studied.\textsuperscript{26} However, we expect that nanoscopic gel-phase domains would also be present in our membranes at 20 – 21 mol\% cholesterol because we detected a small number of micron-sized, gel-phase domains at 19 mol\% cholesterol (Figure 2.7a and b, lower left), and the gel-phase domains in supported lipid membranes undergo a reduction in size due to domain pinning before complete miscibility is reached.\textsuperscript{19,28} This expectation is supported by a recent report by Gosku
and Longo. The speckling in Gosku and Longo’s fluorescence microscopy images of mica-supported DLPC/DSPC/cholesterol (40/40/20 mol:mol:mol) membranes suggests the presence of tiny phase-separated domains that could be detected by AFM, but are too small to be clearly visualized with fluorescence microscopy. We hypothesize that the small inconsistency in the location of the boundary for phase separation between our results and the published DLPC/DSPC/cholesterol phase diagram is due to the difference in techniques used to detect phase separation, as the AFM and NanoSIMS techniques we used are better suited to unambiguously detect submicron-sized domains than the conventional fluorescence microscopy.

The fusion of DLPC/DSPC (1:1 mol ratio) vesicles that contained 27 and 35 mol% cholesterol to the silicon substrates under conditions identical to those employed for the other cholesterol concentrations did not produce continuous, defect-free membranes. Increasing the cholesterol concentration within vesicles composed of phase-separating lipids is reported to hinder vesicle rupture, causing an increase in the critical number of vesicles that must adsorb to the silicon substrate in order to initiate vesicle fusion. Because others have used vesicle fusion to form DLPC/DSPC (1/1 mole ratio) membranes that contained 30 mol% cholesterol on mica substrates, we hypothesize that the vesicle concentration or incubation time we employed for vesicle fusion was insufficient for the formation of defect-free membranes that contained 27 or 35 mol% cholesterol.
Significance and implications

Our data indicate that interactions between saturated lipids, such as DLPC and DSPC, and cholesterol are insufficient to drive the formation of liquid-ordered domains that are similar to those expected to exist within biological membranes. Our results, and those reported by others, suggest that molecular interactions between the fluid-phase lipid and cholesterol affect the structure of the ordered lipid domains that form in the membranes.\textsuperscript{19,25} Although the DLPC/DSPC/cholesterol membranes we studied are not compositionally or biophysically representative of biological membranes, our findings do provide insight into the physiological significance of fatty acid chain saturation in biological membranes. Specifically, our results support the hypothesis that the presence of unsaturation in the fatty acid chains of the low-melting lipids found in biological membranes is required for the formation of a liquid-ordered phase. Furthermore, we propose that the absence of saturated, low $T_m$ lipids in cellular membranes is functionally significant, as their presence may hinder the formation of liquid-ordered membrane domains.

This work also establishes a direct approach to acquire correlated information on the microstructure and composition of individual lipid domains. This approach could be extended to quantifying the amounts of DSPC and cholesterol in the lipid domains detected in these membranes by also incorporating a different distinct stable isotope into the cholesterol, and creating separate calibration curves for DSPC and cholesterol using sets of standard lipid membrane samples. Such studies would
permit identifying whether the cholesterol concentration is equal in both lipid phases, as indicated by original experiments,\textsuperscript{14} or if the cholesterol concentration is higher in the ordered domains, as found for other lipid mixtures.\textsuperscript{18} Our approach could also be used to construct more detailed phase diagrams that include tie lines for DLPC/DSPC/cholesterol or other more physiologically relevant lipid mixtures. Such studies may clarify uncertainties in membrane phase behavior, and provide a better understanding of how cholesterol-dependent interactions contribute to cell membrane organization.

**Conclusions**

By analyzing the same locations in supported lipid membranes with AFM and high-resolution SIMS, we were able to acquire correlated information on the microstructure and composition of the individual lipid domains found in the membranes at room temperature. We showed that the nanoscopic, D\textsubscript{70}-DSPC domains present in the 19 mol\% cholesterol membrane were in the gel phase. The \textsuperscript{15}N-DLPC concentration in the D\textsubscript{70}-DSPC domains was relatively constant (6 – 7 mol\%) for cholesterol concentrations between 0 and 19 mol\%, indicating that cholesterol did not induce a significant increase in lipid intermixing in these domains. Our results support the finding of Zhao et al. that nanoscopic, phase-separated domains are present in the membrane at ∼19 mol\% cholesterol,\textsuperscript{26} but we found no evidence for the presence of liquid-ordered domains. We conclude that the
interactions between cholesterol and saturated lipids, such as DLPC and DSPC, are not sufficient to create liquid-ordered domains. Consequently, the absence of saturated, low-melting lipids in biological membranes may be required for the formation of the liquid-ordered domains that are expected to exist in cellular membranes.

Materials and Methods

Materials

The chrome-patterned, oxidized silicon substrates (10-nm-thick oxide layer) were prepared as previously described\textsuperscript{37} using silicon wafers that were a generous gift from Prof. Steven G. Boxer (Stanford University, Stanford, CA). The lipids 1-palmitoyl-2-\{12-\[(7-nitro-2-1,3-benzoziadazol-4-yl)amino]lauroyl\}-sn-glycero-3-phosphocholine (NBD-PC) and D\textsubscript{70}-DPSC were purchased from Avanti Polar Lipids, Inc (Alabaster, AL), and (\textsuperscript{15}N-DLPC) was synthesized as reported.\textsuperscript{37} To minimize possible oxidation, cholesterol was freshly synthesized from i-cholesteryl methyl ether (Sigma) as reported.\textsuperscript{52} The cholesterol and phospholipid concentrations in the vesicle solutions were measured with the Amplex\textsuperscript{®} Red Cholesterol Assay Kit and the Amplex\textsuperscript{®} Red Phospholipase D Assay Kit, respectively, from Molecular Probes (Eugene, OR). Millipore (18 mΩ) water was used in all experiments.
Preparation of supported lipid membranes

Small unilamellar vesicles were created from an equal molar ratio of $^{15}$N-DLPC ($T_m = -1 \degree C$) to $D_{70}$-DPSC ($T_m = 50.5 \degree C$), and approximately 0, 2.5, 5, 10, 15, 20, or 35 mol% cholesterol. The actual cholesterol concentrations in the final vesicle solutions measured with enzymatic assays (see section below) were 0, 3, 7, 15, 19, 27, and 35 mol%. A small amount (1 mol%) of the fluorescent lipid, NBD-PC, was added to the mixture to allow the evaluation of membrane integrity and the presence of phase separation using fluorescence microscopy. These components were dissolved in chloroform, dried under nitrogen, and placed under vacuum to remove residual chloroform. The lipid film was resuspended in 65 °C water to a final lipid concentration of 0.5 mg/mL. The lipid solution was heated in a 65 °C water bath for 15 min, vortexed, transferred to a plastic tube, and sonicated using a tip sonicator (Branson Tip Sonifier Model 250, Branson Ultrasonics, Danbury CT) for 30 s intervals until it became transparent. The vesicle solution was reheated to 65 °C in a water bath, and 2.5 mL of 65 °C vesicle solution was added to a 60-mm-diameter x 15-mm-tall polystyrene culture dish containing multiple chrome-patterned, oxidized silicon substrates submerged in 2.5 mL of 65 °C water. The culture dish was covered, incubated at room temperature for 40 min to allow for bilayer formation, and then was transferred into a 65 °C water bath. The substrates were gently shaken under water to dislodge vesicles adhered to the bilayer’s surface, and were transferred to a fresh 65 °C water bath. The water bath containing the substrates was placed in a
programmable oven (ECHOterm™ IN35 Programmable Chilling/Heating Incubator, Torrey Pines Scientific, San Diego, CA), maintained at 70 °C for 1 h, and then was slowly cooled to room temperature at a rate of 5 °C/h to induce phase separation. To prepare the lipid membranes for SIMS analysis, the samples were flash-frozen in liquid ethane and freeze-dried as previously described.\textsuperscript{35-37} To evaluate sample quality, fluorescence imaging was performed on a Leica DM6000 B upright fluorescence microscope equipped with a fluorescence filter cube (GFP, Leica) that matches the excitation and emission spectra for NBD-PC.

\textit{Measurement of mol\% cholesterol}

The moles of cholesterol and phosphatidylcholine (PC) in each vesicle solution were measured using the Amplex® Red Cholesterol Assay Kit and the Amplex® Red Phospholipase D Assay Kit, respectively. The assays were performed in 96-well plates purchased from Costar® (Corning, NY), and the fluorescence intensity was read using a Synergy HT Multi-Mode Microplate Reader Model SIAFRT (Biotek® Instruments, Inc., Winooski, VT). Cholesterol and PC standards were used to create the calibration curves. Eight replicates were performed on each vesicle and standard solution. The \( \mu \)mole of cholesterol and PC measured in each small unilamellar vesicle solution was used to calculate the mol\% of cholesterol. The mol\% cholesterol in the supported lipid membranes is assumed to be the same as the cholesterol content in the vesicle solution used for bilayer formation.
**AFM imaging of lipid membranes**

AFM analysis was performed in ambient air and temperature using an Asylum MFP-3D™ Stand Alone AFM. AFM images were acquired of the phase-separated membranes within specific grid boxes, which were relocated and analyzed with the NanoSIMS. Measurements were taken with standard tapping 300 kHz AFM probes (Tap300Al-G, Budget Sensors, Bulgaria) in AC (tapping) mode operated in the repulsive tip-sample interaction regime to preserve tip lifetime. Images were flattened to the first order. The line scans were averaged over three pixels perpendicular to the line-section path to minimize random variations in height due to noise. The line scans made on the 19 mol% cholesterol membrane were not averaged because the domains were often smaller than the 3-pixel averaging width. For each cholesterol concentration investigated, the height difference between the gel- and fluid-phase regions was measured at ten different domain interfaces.

**High-resolution SIMS analysis**

SIMS was performed on a Cameca NanoSIMS 50 instrument (Cameca, France) at Lawrence Livermore National Laboratory (Livermore, CA). Measurements were made with a ~0.8 pA, 16 keV Cs⁺ primary ion beam focused onto a ~100 nm-diameter spot with an analysis area of 25 µm x 25 µm. A beam diameter of 102 nm was determined using the reported knife-edge method. Measurements consisted of
six replicate scans of 512 x 512 pixels with a dwell time of 0.3 ms/pixel, and corresponded to a primary ion dose of $\sim 3.8 \times 10^{14}$ ions/cm$^2$. The analysis conditions used in this work were chosen to provide sufficient ion counts to characterize the samples while minimizing analysis time. Based on our analysis conditions, a total sputter time of 472 s, and the sputtering rate of 2.5 nm$\cdot$μm$^2$/pA$\cdot$s determined on other biological samples,$^{53}$ we estimate that the sputtering depth was $\sim 1.5$ nm for these measurements. Based on the thickness of the bilayer, the analysis time could have been $\sim 3$ times longer, though the secondary ion intensities obtained in each additional replicate scan would be lower than the previous scan. The pixel size in the NanoSIMS ion images was 49 nm x 49 nm, which is smaller than the beam diameter. The secondary ion signals $^{12}$C$^-$, $^{12}$CD$^-$, and $^{12}$C$^{15}$N$^-$, and the secondary electron signal were collected simultaneously using multi-collection mode. A mass resolving power of $\sim 7500$ was used to separate isobaric interferences from the isotopes of interest, e.g., $^{12}$C$^2$H from $^{13}$C$^1$H and $^{12}$C$^1$H$_2$ at a nominal mass of 14 amu, and $^{12}$C$^{15}$N from $^{13}$C$^{14}$N at a nominal mass of 27 amu. Data were analyzed using a custom software package (L’image, L.R. Nittler, Carnegie Institution of Washington) run on the PV-Wave platform (Visual Numerics, Inc., Houston, TX). The lipid-specific ions ($^{12}$CD$^-$ and $^{12}$C$^{15}$N$^-$) were smoothed over 3 x 3 pixels (147 nm x 147 nm). Then the lipid-specific ion signal was normalized by dividing the intensity for each ion by the $^{12}$C$^-$ intensity measured at the same pixel in order to minimize random, concentration-independent changes in the signal intensity. Regions of interest (ROIs) on the
calibration set of homogeneous lipid membranes were defined on areas of the substrate that were covered by the membrane, and excluded areas where the chrome grid or obvious defects were visible in the secondary electron image that was collected in parallel to the secondary ion images. ROIs on the phase-separated membranes that consisted of only gel-phase or fluid-phase regions were defined based on the correlated AFM images.

_Determination of lipid composition within lipid phases_

A calibration curve correlating the normalized $^{12}\text{C}\text{^{15}N}$ secondary ion intensity to the mol\% $^{15}\text{N}$-DLPC in the membrane was made as previously reported.$^{36, 37}$ Briefly, NanoSIMS analysis was performed on homogeneous lipid bilayers that systematically varied in mol \% $^{15}\text{N}$-DLPC to unlabeled DLPC. These samples were used instead of $^{15}$N-DLPC/cholesterol membranes in order to obtain a calibration curve that was accurate at both high and low $^{15}$N-DLPC concentrations. The same analytical conditions were used for the calibration and phase separated samples. We assessed the validity of these calibration samples by calculating the relative sensitivity factors (RSF)$^{39}$ for the $^{12}\text{C}\text{^{15}N}$ using $^{12}\text{C}$ as the reference ion. For membranes composed of $^{15}$N-DLPC/NBD-PC (99/1 mol\%:mol\%), $^{15}$N-DLPC/cholesterol/NBD-PC (84/15/1 mol\%/mol\%/mol\%), and $^{15}$N-DLPC/cholesterol/NBD-PC (69/30/1 mol\%/mol\%/mol\%), the RSF for $^{12}\text{C}\text{^{15}N}$ was 0.0089 ± 0.0003, 0.0093 ± 0.0017, and 0.0093 ± 0.0011, respectively. Note that
these values are low because the intensity of the $^{12}$C$^{-}$ reference ion is very high. Because the RSF for $^{12}$C$^{15}$N$^{-}$ was $\sim$ 4.5% higher in the presence of cholesterol, we expect that the mol% $^{15}$N-DLPC that we calculate in membranes containing cholesterol may be overestimated by up to 5 mol%.

To create the calibration curve, the normalized $^{12}$C$^{15}$N$^{-}$ intensity ($^{12}$C$^{15}$N$^{-}$/2C$^{-}$) was measured for several ROIs so that an area of at least 1000 $\mu$m$^2$ was analyzed on each homogeneous membrane sample of specified mol% $^{15}$N-DLPC. The mean values and standard deviation of the normalized $^{12}$C$^{15}$N$^{-}$ signal were calculated for each mol% $^{15}$N-DLPC. The normalized $^{12}$C$^{15}$N$^{-}$ intensity was plotted against the mol% $^{15}$N-DLPC in the membrane (Figure 2.3) where the error bars show one standard deviation, and the best-fit line for the calibration data was calculated by linear regression. For every cholesterol composition studied, the amount of $^{15}$N-DLPC in the gel-phase was determined using the calibration curve to convert the normalized $^{12}$C$^{15}$N$^{-}$ intensity measured within gel-phase regions where trapped fluid-phase subdomains were not detected by AFM into mol% $^{15}$N-DLPC. The normalized $^{12}$C$^{15}$N$^{-}$ intensity was measured at several ROIs on each sample, and the average mol% $^{15}$N-DLPC in the gel-phase and standard deviation were calculated. Because NBD-PC is excluded from the gel-phase, the average amount of D$_{70}$-DSPC in the gel-phase regions of the cholesterol-free membrane was determined by subtracting the mol% $^{15}$N-DLPC measured at membrane locations identified as gel-phase by AFM from 100 mol%.
References


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Chapter 3.
DISCRIMINATING AND IMAGING DIFFERENT PHOSPHATIDYLCHOLINE SPECIES WITHIN PHASE-SEPARATED MODEL MEMBRANES BY PRINCIPAL COMPONENT ANALYSIS OF TOF-SIMS IMAGES

Notes and Acknowledgements

This chapter was coauthored by Bita Vaezian, Christopher R. Anderton, and Mary L. Kraft, and originally appeared in Analytical Chemistry.¹ Bita Vaezian’s contributions include generating the research idea, making and analyzing the initial pure lipid film samples, making the phase-separated DPPC/DLPC membrane, and performing TOF-SIMS analysis on all of the phase-separated samples. Christopher R. Anderton’s contributions include making and analyzing the additional pure lipid film samples, making the phase-separated DSPC/DLPC membrane, and doing the post-sampling data processing that included downbinning and performing PCA on the TOF-SIMS images. This was reprinted in its entirety, to provide a complete narrative of this work, with permission from the American Chemical Society. The material is based upon work funded by a Career Award at the Scientific Interface from the Burroughs Wellcome Fund held by Mary L. Kraft. Portions of this work were carried out in the Frederick Seitz Materials Research Laboratory Central Facilities, Univ. of Illinois, which are partially supported by the U.S. Department of Energy under grants DE-FG02-07ER46453 and DE-FG02-07ER46471.
Introduction

Comprehensive characterization of lipid mixing behavior within model lipid membranes provides valuable information on how lipid-lipid interactions may contribute to cell membrane organization and function.\textsuperscript{2,3} The biophysical and structural properties of model lipid membranes are routinely probed by using fluorescence microscopy and atomic force microscopy.\textsuperscript{4-8} Spatially resolved compositional information that complements this biophysical and structural data is also essential for understanding biological membrane organization, but is presently more difficult to acquire. The lipid distribution within model membranes can be imaged in a chemically specific manner by using secondary ion mass spectrometry (SIMS).\textsuperscript{9-21} However, additional advances in SIMS methodologies are necessary to improve the specificity, sensitivity, and lateral resolution of lipid identification and imaging in label-free systems.

Time-of-flight SIMS (TOF-SIMS) is a molecular imaging technique that reveals the chemical composition at the surface of a sample with as high as submicron lateral resolution.\textsuperscript{11,22,23} For the analysis of lipid membranes using TOF-SIMS, lipid components are usually identified according to a limited number of characteristic lipid fragment ions in the mass spectra, such as those corresponding to lipid headgroups, fatty acids, and backbone segments.\textsuperscript{9,10,14-17,24,25} However, the use of these types of fragment ions to discriminate between structurally similar lipids, such as different phosphatidylycerine species, is especially problematic. Though low molecular weight
fragment ions (i.e., phosphocholine and hydrocarbon ions) generate sufficient ion yields to achieve submicron lateral resolution, these ions are common to many structurally similar lipid species, prohibiting their use for species identification. Molecular ions and large fragment ions may be unique to a single component, but they usually have very low yields that compromise the working lateral resolution of the resulting TOF-SIMS image. Structurally similar lipids can be endowed with characteristic fragment ions through the use of stable isotope labeling, but label-free approaches are desired to facilitate sample preparation and SIMS analysis of native cell membranes.

Principal component analysis (PCA) is a multivariate analysis technique that is used to identify linear combinations of multiple mass peaks that exhibit similarities and differences in the TOF-SIMS data acquired from different samples. Because PCA distinguishes the spectra according to variations in the relative intensities of many TOF-SIMS mass peaks, the abundant low mass ions that are common to structurally similar molecules, and thus, not utilized for univariate spectra interpretation, can be exploited for component identification. This use of a larger fraction of the TOF-SIMS mass peaks for component identification increases image contrast and improves the specificity of component identification. The TOF-SIMS spectra of structurally similar molecules, including monosaccharide stereoisomers, proteins, and alkane thiols, have been successfully discriminated using PCA. PCA has also been used to identify unlabeled lipids with
different headgroups and deuterated lipids in model membranes by their TOF-SIMS data.\textsuperscript{26,29} However, successfully discriminating the TOF-SIMS data from unlabeled structurally similar lipid species (i.e., lipids with the same headgroups and degrees of saturation in the fatty acid tails) using PCA has not been reported.

In this chapter, we investigate the ability to distinguish and image structurally similar lipids within supported lipid membranes by using PCA to interpret TOF-SIMS images. Because high mass ions have low intensities in the spectra acquired from lipid membranes with TOF-SIMS, we first evaluate the feasibility of discriminating four different unlabeled saturated phosphatidylcholine species that are frequently studied in model membranes, 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), by PCA of the low mass ions ($m/z \leq 200$) in the TOF-SIMS data. Discrimination of these lipid species by TOF-SIMS is especially challenging because each contains a phosphocholine headgroup, glycerol backbone, and two saturated fatty acid tails that differ only in length (12, 14, 16, and 18 carbons per fatty acid in DLPC, DMPC, DPPC, and DSPC, respectively). Next, we assess whether each lipid species within supported lipid membranes composed of DSPC/DLPC and DPPC/DLPC can be differentiated, visualized, and chemically identified by PCA of TOF-SIMS images. These lipid mixtures were selected because they are known to phase-separate into DLPC-rich fluid-phase domains, and DSPC- or DPPC-rich gel-phase domains at
In addition, the chemically specific SIMS imaging of the lipid organization within these membranes has only been accomplished by using stable isotope labeling.\textsuperscript{18,21} To evaluate whether the principal component (PC) scores images generated from the TOF-SIMS images of the phase-separated membranes show the sizes and shapes of the gel- and fluid-phase domains, we compare the PC scores images to AFM images of the phase-separated domains that were acquired at the same membrane locations prior to TOF-SIMS analysis. Finally, we investigate whether the lipid composition at these distinct locations can be identified by projecting their TOF-SIMS data onto PC models developed using spectra from lipid standards.

**Results and Discussion**

*PCA discrimination of TOF-SIMS spectra of saturated phosphatidylcholine species*

Representative TOF-SIMS positive ion mass spectra of four saturated phosphatidylcholine species are shown in Figure 3.1. The unique mass peaks that could be used to discriminate DLPC, DMPC, DPPC, and DSPC include the molecular ions (\(m/z\) 622, 678, 734, and 789, respectively), diacylglycerol fragment ions (\(m/z = 439, 495, 551,\) and 607, respectively), monoacylglycerol fragment ions (\(m/z = 257, 285, 313,\) and 341, respectively), and fatty acids (\(m/z = 183, 211, 239, 267,\) respectively). However, high molecular weight ions (\(m/z >200\)), including many of the aforementioned fragment ions, usually have low intensities in the TOF-SIMS spectra.
acquired from supported lipid membranes,\textsuperscript{14,16,24} though new primary ion sources may enhance the intensities of high mass fragment ions.\textsuperscript{37} Few characteristic mass peaks with $m/z \leq 200$ are present in the spectra of the lipid standards due to their similar structures.

\textbf{Figure 3.1.} The TOF-SIMS positive-ion spectrum and structures (inset) of four different saturated phosphatidylcholine species: (A) DLPC, (B) DMPC, (C) DPPC, and (D) DSPC. The spectra were obtained from pure dried lipid films of these lipid using an ion dose of $2.4 \times 10^{12}$ ions/cm$^2$. 

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Figure 3.2. Scores and loadings plots from PCA of the TOF-SIMS positive-ion spectra of DLPC, DMPC, DPPC, and DSPC lipid films. These four saturated phosphatidylcholine species differ only in the lengths of their fatty acid tails. (A) The scores plot on PC1 and PC2 differentiates the four phosphatidylcholine lipid species. (B) The loadings plot illustrates the positive ion mass peaks that contribute to the variation captured by each PC. PCA was performed on the ion peaks with \( m/z \) from 30 to 200 amu, excluding the potassium \((m/z = 39)\), calcium \((m/z = 40)\), hydrocarbon impurity \((m/z = 41)\), chrome \((m/z = 52)\) and PDMS contaminant \((m/z = 73 \text{ and } 147)\) peaks. These peaks were normalized to the total counts obtained over this mass range and mean-centered. The ellipses in the score plot that are outlined with a solid line represent the border for each group at the 95% confidence limit. The ellipse outlined with a dotted blue line represents the border for the entire PC model at the 95% confidence limit.

PCA was performed using the mass peaks with \( m/z \) from 30 to 200, excluding known contaminants, because these ions \((m/z \leq 200)\) are relatively abundant in the TOF-SIMS positive-ion spectra of lipid membranes.\(^{14,24}\) The resulting PC scores plot of the lipid spectra projected onto PC1 and PC2 (Figure 3.2A) demonstrates that these phosphatidylcholine species can be differentiated and classified. The ellipse around each group in the scores plot shows the 95% confidence limit for the boundary of the group.\(^{38}\) The ellipse outlined with a dotted blue line represents the border for the entire PC model at the 95% confidence limit. The majority of the variation between lipid species is captured by PC1, which separates the lipids according to the lengths of their fatty acid tails. The mass peaks responsible for this
lipid-specific separation have high loadings on PC1, and are shown in the loadings plot (Figure 3.2B). These values are partially tabulated in Table 3.1. Both hydrocarbon and headgroup fragments have large loadings on PC1, indicating the normalized intensities of these ions vary significantly between the phosphatidylcholine species. The hydrocarbon ions with \( m/z \) of 55 (\( \text{C}_4\text{H}_7^+ \)), 57 (\( \text{C}_4\text{H}_9^+ \)), 67 (\( \text{C}_5\text{H}_7^+ \)), 69 (\( \text{C}_5\text{H}_9^+ \)), 71 (\( \text{C}_5\text{H}_{11}^+ \)), 79 (\( \text{C}_6\text{H}_7^+ \)), 81 (\( \text{C}_6\text{H}_9^+ \)), and 83 (\( \text{C}_6\text{H}_{11}^+ \))\(^{29,39}\) have negative loadings on PC1 and therefore, relatively higher normalized intensities in the spectra from DSPC and DPPC. Headgroup fragments with \( m/z \) of 58 (\( \text{C}_3\text{H}_8\text{N}^+ \)), 59 (\( \text{C}_3\text{H}_9\text{N}^+ \)), 86 (\( \text{C}_5\text{H}_{12}\text{N}^+ \)), 102 (\( \text{C}_5\text{H}_{12}\text{NO}^+ \)), 104 (\( \text{C}_5\text{H}_{14}\text{NO}^+ \)), 166 (\( \text{C}_5\text{H}_{13}\text{NPO}_3^+ \)), and 184 (\( \text{C}_5\text{H}_{15}\text{NPO}_4^+ \))\(^{10,29,40}\) and the dodecanoic acid fragment at \( m/z = 183 \) load positively on PC1, and thus, have relatively higher normalized intensities in the spectra of DMPC and DLPC. Although all four lipid species have the same phosphocholine headgroup, the headgroup ions have a relatively higher normalized intensity in the spectra of DLPC and DMPC, likely because DLPC and DMPC have shorter fatty acid tails that contribute fewer hydrocarbon ions to their spectra. On PC2, the loadings for the mass peaks that correspond to lipid headgroups (\( m/z = 184, 166, 104, 86, \) and 58) are negative, and the small hydrocarbon ion at \( m/z = 43 \) has a large positive loading. PC2 may separate the samples according to disparities in their surface coverage, as opposed to lipid-specific chemical variations, because the intensities of the higher mass fragment ions and low mass hydrocarbon ions are influenced by surface coverage to
This ability to discriminate the lipid spectra according to the relative intensities of their low mass ions suggests that PCA of TOF-SIMS images might enable discriminating and imaging saturated phosphatidylcholine species within phase-separated lipid membranes.

Table 3.1. Information on select peaks in the TOF-SIMS positive ion spectra of DLPC, DMPC, DPPC, and DSPC.

<table>
<thead>
<tr>
<th>m/z</th>
<th>Assignment</th>
<th>Comments</th>
<th>Loadings on PC1</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>CH₃N⁺ or C₂H₆⁺</td>
<td>Headgroup or hydrocarbon fragment</td>
<td>0.044</td>
</tr>
<tr>
<td>43</td>
<td>C₃H₇⁺ or C₂H₂O⁺</td>
<td>Hydrocarbon chain or fatty acid fragment</td>
<td>0.181</td>
</tr>
<tr>
<td>55</td>
<td>C₄H₇⁺</td>
<td>Hydrocarbon chain fragment</td>
<td>-0.251</td>
</tr>
<tr>
<td>57</td>
<td>C₄H₈⁺</td>
<td>Hydrocarbon chain fragment</td>
<td>-0.293</td>
</tr>
<tr>
<td>58</td>
<td>C₃H₈N⁺</td>
<td>Headgroup fragment</td>
<td>0.452</td>
</tr>
<tr>
<td>59</td>
<td>C₃H₉N⁺</td>
<td>Headgroup fragment</td>
<td>0.127</td>
</tr>
<tr>
<td>67</td>
<td>C₅H₇⁺</td>
<td>Hydrocarbon chain fragment</td>
<td>-0.243</td>
</tr>
<tr>
<td>69</td>
<td>C₅H₉⁺</td>
<td>Hydrocarbon chain fragment</td>
<td>-0.366</td>
</tr>
<tr>
<td>71</td>
<td>C₅H₁₁⁺</td>
<td>Hydrocarbon chain fragment</td>
<td>-0.122</td>
</tr>
<tr>
<td>72</td>
<td>C₄H₁₀N⁺</td>
<td>Headgroup fragment</td>
<td>0.010</td>
</tr>
<tr>
<td>74</td>
<td>C₅H₁₂N⁺</td>
<td>Headgroup fragment</td>
<td>0.044</td>
</tr>
<tr>
<td>79</td>
<td>C₅H₇⁺</td>
<td>Hydrocarbon chain fragment</td>
<td>-0.112</td>
</tr>
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<td>81</td>
<td>C₅H₉⁺</td>
<td>Hydrocarbon chain fragment</td>
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<td>83</td>
<td>C₅H₁₁⁺</td>
<td>Hydrocarbon chain fragment</td>
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<td>86</td>
<td>C₅H₁₂N⁺</td>
<td>Headgroup fragment</td>
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</tr>
<tr>
<td>95</td>
<td>C₅H₁₁⁺</td>
<td>Hydrocarbon chain fragment</td>
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</tr>
<tr>
<td>97</td>
<td>C₇H₁₃⁺</td>
<td>Hydrocarbon chain fragment</td>
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<tr>
<td>102</td>
<td>C₅H₁₂NO⁺</td>
<td>Choline</td>
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<tr>
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<td>C₅H₁₄NO⁺</td>
<td>Choline</td>
<td>0.146</td>
</tr>
<tr>
<td>150</td>
<td>C₅H₁₃NPO₂⁺</td>
<td>Headgroup fragment</td>
<td>0.022</td>
</tr>
<tr>
<td>166</td>
<td>C₅H₁₃NPO₃⁺</td>
<td>Headgroup fragment</td>
<td>0.022</td>
</tr>
<tr>
<td>183</td>
<td>C₁₂H₂₃O⁺</td>
<td>Dodecanoic acid fragment</td>
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<tr>
<td>184</td>
<td>C₅H₁₅NPO₄⁺</td>
<td>Phosphocholine</td>
<td>0.006</td>
</tr>
</tbody>
</table>

PCA of TOF-SIMS images of phase-separated supported lipid membranes

Typically, TOF-SIMS analysis is performed using an ion dose below 10¹³ ions/cm² to prevent exceeding the static limit.²⁹,⁴¹ However, our initial studies
demonstrated that analysis within the static limit yielded insufficient counts to visualize the lipid domains with PCA (Figure 3.3). Instead, we obtained higher ion counts, and better contrast and sensitivity in the resulting PC scores, images by slightly exceeding the static limit. Therefore, we used an ion dose of $2.8 \times 10^{13}$ ions/cm$^2$ to image the phase-separated lipid membranes. To further improve the image quality, the TOF-SIMS images were downbinned to 128 pixels x 128 pixels. Figure 3.4 shows select TOF-SIMS ion images of a DSPC/DLPC membrane (1:2 molar ratio). For comparison, the AFM image acquired at the same membrane location prior to TOF-SIMS analysis is shown in Figure 3.6A. In the AFM image, the DSPC-rich gel-phase domains have diameters between 5 to 25 microns (light gray regions, Figure 3.6A), and protrude ~1.5 nm above the surrounding DLPC-rich fluid-phase membrane regions (dark gray areas, Figure 3.6A). This height difference is slightly smaller than that previously measured between gel- and fluid-phase domains in DSPC/DLPC membranes,\textsuperscript{18,21,42} which may be due to our use of a repulsive tip-sample interaction for AFM imaging.\textsuperscript{18,43,44} Submicron-size fluid-phase sub-domains were also detected by AFM within the gel-phase domains.

Comparison between the AFM and TOF-SIMS ion images of the same membrane location indicates the hydrocarbon fragments with $m/z$ of 43 (C$_3$H$_7^+$), 57 (C$_4$H$_9^+$), 67 (C$_5$H$_7^+$), 69 (C$_5$H$_9^+$), and 83 (C$_6$H$_{11}^+$) have higher counts (Figure 3.4) and normalized intensities (Figure 3.5) on the gel-phase DSPC-rich domains. In contrast, the ions with $m/z$ of 58 (C$_3$H$_8$N$^+$) and 183 (C$_{12}$H$_{23}$O$^+$) have higher counts (Figure 3.4)
Figure 3.3. (A) AFM image shows the morphologies of the DSPC-enriched gel-phase domains, and the surrounding DLPC-enriched fluid-phase regions in the DSPC/DLPC (1:2 molar ratio) phase-separated membrane. The PC1 (B) and PC2 (D) scores images were created by PCA of the TOF-SIMS spectra that was acquired using an ion dose of $4 \times 10^{12}$ ions/cm$^2$ at the same membrane location as that shown in (A). Phase separation is barely discernable in these PC scores images. For both PC1 and PC2, the gel-phase domains have positive scores (yellow), and the fluid-phase locations have negative scores (cyan). Loadings on PC1 (C) and PC2 (E) show the mass peaks with the largest variability between the gel- and fluid-phase membrane regions. Mass peaks with positive loadings have higher normalized intensities on the DSPC-rich gel-phase regions, and peaks with negative loadings have higher normalized intensities on the DLPC-rich fluid-phase domains.
Figure 3.4. Select TOF-SIMS positive ion images acquired on phase-separated supported lipid membranes composed of DSPC/DLPC (1:2 molar ratio) show the intensity of the specified mass peak. The images were obtained in unbunched mode for optimal lateral resolution. The TOF-SIMS images were acquired of an 85 µm × 85 µm area with 256 x 256 pixels, downbinned to 128 pixels x 128 pixels, and cropped to show a 65 µm × 65 µm area of the membrane.

Figure 3.5. Downbinned (128 pixels x 128 pixels) TOF-SIMS images showing the counts of the indicated mass peak normalized to the total ion counts at the same region of the phase-separated DSPC/DLPC (1:2 molar ratio) membrane shown in Figure 3.4.
and normalized intensities (Figure 3.5) on the fluid-phase DLPC-rich regions. These observations are fairly consistent with the PC1 loadings calculated for the TOF-SIMS data from the pure lipid films (Figure 3.2). Although the PC model of the pure lipid films indicates the relative normalized intensity of the phosphocholine ion \((m/z = 184)\) is higher on DLPC, the contrast in the \(m/z = 184\) ion image is too low to identify the phase-separated membrane domains. PCA of the TOF-SIMS images yielded PC scores images that graphically show the score value at every pixel in the TOF-SIMS image (Figure 3.6B). Comparison of the PC1 scores image to the AFM image acquired at the same location (Figure 3.6A) confirms that the PC1 scores image reveals phase separation in the membrane. The fluid-phase regions that are enriched with DLPC have positive scores (green, yellow, and red areas), and the gel-phase domains that are enriched with DSPC have negative scores (blue regions). The contrast values for the PC1 scores image (Figure 3.6B) and the individual TOF-SIMS ion images (Figure 3.4) were calculated as previously reported,\(^{30,45}\) and are tabulated in Table 3.2. The higher contrast values of the PC scores image confirms that the PC1 scores image reveals the phase-separated lipid domains with better contrast than that obtained in the individual TOF-SIMS ion images. Therefore, although PC1 accounts for only 2.4% of the pixel-to-pixel variation in the normalized TOF-SIMS peak intensities, this variation is significant. The improvement in the sensitivity and
Figure 3.6. (A) AFM image shows gel- and fluid-phase domains that are enriched with DSPC and DLPC, respectively, in the DSPC/DLPC (1:2 molar ratio) membrane. (B) The PC1 scores images generated by PCA of the downbinned TOF-SIMS image of the same membrane location. The gel-phase domains have negative scores (blue), and the fluid-phase locations have positive scores (green, yellow, and red). (C) The loadings on PC1 show the mass peaks with the largest variability between the gel- and fluid-phase regions of the membrane. The mass peaks with positive loadings have higher normalized intensities on the fluid-phase DLPC-rich regions, and the peaks with negative loading have elevated normalized intensities on the gel-phase DSPC-rich domains.

The specificity of lipid detection obtained with PCA is further demonstrated by the detection of the ~5-µm-wide gel-phase domain at the top right corner of the AFM and PC1 scores images (Figure 3.6A and B), as this domain was not visible in any of the individual TOF-SIMS ion images (Figure 3.4). However, the submicron-size fluid-phase subdomains entrapped within the large gel-phase domains that are shown in the AFM image (Figure 3.6A) were not resolved in the PC1 scores image. These
domains were also not detectable in the PC scores images produced by PCA of the unbinned (256 pixels x 256 pixels) TOF-SIMS data (Figure 3.7), which indicates these features were smaller than the lateral resolution of the TOF-SIMS analysis.

Table 3.2. Contrast calculated for the individual TOF-SIMS ion images (Figure 3.4) and PC scores image (Figure 3.6B) of the same region of a phase-separated DSPC/DLPC membrane.

<table>
<thead>
<tr>
<th>Image</th>
<th>Contrast</th>
</tr>
</thead>
<tbody>
<tr>
<td>m/z 43</td>
<td>1.13</td>
</tr>
<tr>
<td>m/z 57</td>
<td>1.10</td>
</tr>
<tr>
<td>m/z 58</td>
<td>0.45</td>
</tr>
<tr>
<td>m/z 67</td>
<td>1.00</td>
</tr>
<tr>
<td>m/z 69</td>
<td>1.23</td>
</tr>
<tr>
<td>m/z 83</td>
<td>0.90</td>
</tr>
<tr>
<td>m/z 183</td>
<td>0.40</td>
</tr>
<tr>
<td>m/z 184</td>
<td>0.18</td>
</tr>
<tr>
<td>PC1</td>
<td>1.97</td>
</tr>
</tbody>
</table>

Figure 3.7. (A) PC1 scores image of the unbinned (256 pixels x 256 pixels) TOF-SIMS images acquired from the same location of a phase-separated DSPC/DLPC membrane as shown in Figure 3.6 (contrast = 1.31). (B) Loadings on PC1 show the mass peaks with the largest variability on PC1.
The plot of the loadings on PC1 (Figure 3.6) shows which mass peaks have large positive loadings and, therefore, higher normalized intensities on the fluid-phase DLPC-rich areas, and the peaks with large negative loadings and thus, higher normalized intensities on the gel-phase DSPC-rich domains. Many of the mass peaks that correspond to DSPC and DLPC according to the PC model developed on the pure lipid films are correlated with the DSPC-rich gel-phase and DLPC-rich fluid-phase domains, respectively, based on the PC1 loadings for the PC model of the phase-separated membrane. This agreement suggests PC1 separates the spectra according to differences in lipid composition, and not variations in lipid packing or topography. Furthermore, sample topography is not a likely source of the spectral variation captured by PC1 because the loadings for the phase-separated membrane have both positive and negative values, and topographic effects in TOF-SIMS images are often isolated on a single PC that primarily loads in a single direction.27

We investigated the generality of PCA of TOF-SIMS spectra to discriminate and image different saturated phosphatidylcholine species by applying this approach to phase-separated DPPC/DLPC (1:2 molar ratio) membranes. Unlike the individual TOF-SIMS ion images of the DSPC/DLPC membrane (Figure 3.4), the domains are barely discernable in the TOF-SIMS images of the counts and normalized intensities of specific ions acquired from the DPPC/DLPC membrane (Figures 3.8 and 3.9). For comparison, the AFM image acquired at the same location prior to TOF-SIMS analysis (Figure 3.10A) shows gel-phase domains that are several microns in
diameter and protrude ~1 nm above the surrounding fluid phase. This height difference is slightly smaller than that previously reported (1.4 nm), which, as stated above, is likely due to our use of a repulsive tip-sample interaction for AFM imaging. Submicron-size gel-phase domains are also visible within the fluid phase.

![Figure 3.8](image)

**Figure 3.8.** Select TOF-SIMS positive-ion images acquired on phase-separated supported lipid membranes composed of DPPC/DLPC (1:2 molar ratio) show the intensity of the specified mass peak. The images were obtained in unbunched mode for optimal lateral resolution. The TOF-SIMS images were acquired of a 65 µm × 65 µm area with 256 pixels x 256 pixels, downbinned to 128 pixels x 128 pixels, and cropped to show a 60 µm × 60 µm region of interest. Phase-separated domains are barely visible in these images.

The PC1 scores image (Figure 3.10B) clearly shows distinct regions that correlate with the gel- and fluid-phase domains detected with AFM (Figure 3.10A). Calculation of the contrast values, which are listed in Table 3.3, confirms that the contrast between the compositionally distinct gel- and fluid-phase domains is higher in the PC1 scores image than the individual TOF-SIMS ion images that are shown in
Figure 3.8. Again, the submicron-size gel-phase domains that are visible in the AFM image (Figure 3.10A) were not resolved in either the downbinned and unbinned PC1 scores images (Figure 3.10B and 3.11, respectively), indicating these domains are likely smaller than the lateral resolution of the TOF-SIMS analysis. The plot of the PC1 loadings shows the mass peaks that have negative loadings, and therefore, higher normalized intensities on the gel-phase DPPC-rich domains, and the peaks with positive loadings and thus, higher normalized intensities on the fluid-phase DLPC-rich domains (Figure 3.10C). Again the presence of both positive and negative loadings on PC1 indicates topography is not likely responsible for the spectral variation captured by PC1.

Figure 3.9. Downbinned (128 pixels x 128 pixels) TOF-SIMS images show the counts of the indicated mass peak normalized to the total ion counts at the same region of the phase-separated DPPC/DLPC (1:2 molar ratio) membrane shown in Figure 3.8.
Figure 3.10. (A) AFM image shows the morphologies of the DPPC-enriched gel-phase domains, and the surrounding DLPC-enriched fluid-phase regions in the DPPC/DLPC (1:2 molar ratio) phase-separated membrane. (B) PC1 scores image created by PCA of the downbinned TOF-SIMS data of the same membrane location as (A). The gel-phase domains have negative scores (blue), and the fluid-phase locations have positive scores (green, yellow and red). (C) Loadings on PC1 show the mass peaks with the largest variability between the gel- and fluid-phase membrane regions. Mass peaks with negative loadings have higher normalized intensities on the DPPC-rich gel-phase regions, and positives loadings have higher normalized intensities on the DLPC-rich fluid-phase domains.

Table 3.3. Contrast calculated for the individual TOF-SIMS ion images (Figure 3.8) and PC scores image (Figure 3.10B) of the same region of a phase-separated DPPC/DLPC membrane.

<table>
<thead>
<tr>
<th>Image</th>
<th>Contrast</th>
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</thead>
<tbody>
<tr>
<td>m/z 43</td>
<td>0.66</td>
</tr>
<tr>
<td>m/z 57</td>
<td>0.55</td>
</tr>
<tr>
<td>m/z 58</td>
<td>0.28</td>
</tr>
<tr>
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<tr>
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<td>m/z 83</td>
<td>0.43</td>
</tr>
<tr>
<td>m/z 183</td>
<td>0.26</td>
</tr>
<tr>
<td>m/z 184</td>
<td>0.04</td>
</tr>
<tr>
<td>PC1</td>
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</tr>
</tbody>
</table>
Figure 3.11. PCA of the unbinned (256 pixels x 256 pixels) TOF-SIMS images acquired from the same location of a phase-separated DPPC/DLPC membrane as shown in Figure 3.10A. (A) Phase separation is not visible in the PC1 scores image. The contrast calculated between the DLPC-rich fluid-phase domains and the DPPC-rich gel-phase domains in the PC1 scores image is 0.24. (B) Loadings on PC1 show the mass peak with the largest variability on PC1 is the small hydrocarbon fragment at $m/z$ 43. Most of the other mass peaks load negatively on PC1. (C) PC2 scores image of the same membrane location reveals phase separation in the membrane (contrast = 0.68). (D) Loadings on PC2 show the mass peaks responsible for this contrast.

Determination of lipid composition within distinct membrane domains by projection onto the PC model of lipid standards

To identify the lipid composition within the gel- and fluid-phase domains (Figure 3.6B and 3.10B), we projected the TOF-SIMS data that we extracted from regions corresponding to gel- or fluid-phase domains in the DSPC/DLPC and
DPPC/DLPC membranes onto the PC model developed using the DLPC, DMPC, DPPC, and DSPC spectra, which was shown in Figure 3.2. The resulting PC scores plot is shown in Figure 3.12. The PC1 scores for the TOF-SIMS spectra acquired from the DSPC/DLPC membrane designate that the gel-phase domains correspond to DSPC, in agreement with phase diagrams and previous reports. The fluid-phase regions had scores on PC1 that were most similar to DMPC, followed by DLPC. Because these are model membranes with known composition, we conclude that the fluid-phase domains were enriched with DLPC, and not DMPC. We suggest the lower scores on PC1 may be due to the presence of tiny gel-phase domains in the fluid-phase. The scores on PC1 for the spectra acquired on the DPPC/DLPC membrane indicate that the gel-phase domains are composed of DPPC, as expected from the phase diagrams for this lipid mixture. The fluid-phase regions of the DPPC/DLPC membrane had PC1 scores that were consistent both DLPC and DMPC. Again, because the sample did not contain DMPC, we conclude that the fluid-phase membrane regions were enriched with DLPC. We speculate that tiny gel-phase domains were present within the fluid-phase regions that had lower scores on PC1. The agreement between the compositions we determined by projecting onto PC models developed using lipid standards and previous reports suggests the chemical variations identified in the PC scores images were not due to the presence of the small amount (1 mol%) of fluorophore-labeled lipid in the fluid phase or difference in lipid packing between the two phases. We note that the spectra from the
lipid membranes had large residuals on the PC model of the lipid standards, indicating the lipid membranes were not well described by this PC model. This imperfect fit may be due to differences in the surface coverage, lipid orientation, or ion dose used to analyze the lipid standards and membranes, and suggests these conditions should be keep constant to optimize the fit. Nonetheless, these results demonstrate that the lipid composition within distinct membrane domains can be identified through the use of PC models developed using spectra from lipid standards.

Figure 3.12. Projection of the TOF-SIMS data extracted from gel- and fluid-phase regions of the DSPC/DLPC and DPPC/DLPC membranes onto the PC scores plot developed using the positive-ion spectra of the four phosphatidylcholine species: DLPC, DMPC, DPPC, and DSPC. The PC1 scores of the TOF-SIMS data extracted from the phase-separated membranes identifies the lipid composition at these sites.
Conclusions

The distributions of saturated phosphatidylcholine species within phase-separated lipid membranes can be differentiated and visualized by slightly exceeding the static limit of TOF-SIMS analysis, and using PCA to interpret the TOF-SIMS images. Projection of the TOF-SIMS data from phase-separated domains onto PC models of pure lipid samples enables the identification of the lipid composition within subregions of the membrane. This method can be used to image and identify the lipid composition at small areas within label-free model membranes composed of less well-characterized lipid mixtures. The development of PC models constructed using the spectra from additional lipid species and cholesterol may also allow the discrimination and chemical imaging of structurally similar lipids within more complex membranes. Further improvements in TOF-SIMS imaging and multivariate analysis approaches may also permit the detection of submicron-sized membrane domains.

Materials and Methods

Preparation of thin films of pure phosphatidylcholine lipids

DLPC, DMPC, DPPC, and DSPC were purchased in chloroform from Avanti Polar Lipids (Alabaster, AL) and used without further purification. A thin film of each lipid standard was created by spotting a small droplet of the lipid dissolved in chloroform onto a 5 mm x 5 mm silicon substrate that was patterned with a chrome grid to facilitate sample positioning. For each lipid standard, three different samples
were prepared on different days. The sample was subjected to vacuum for a minimum of 3 h to evaporate the solvent.

*Preparation of phase-separated supported lipid membranes*

The lipid mixtures used to create phase-separated supported lipid membranes consisted of a 1:2 molar ratio of DSPC/DLPC or DPPC/DLPC, plus 1 mol% of the fluorescent lipid, 1-palmitoyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]auroyl}-sn-glycero 3-phosphocholine (NBD-PC, Avanti Polar Lipids, Alabaster, AL) for visualization with fluorescence microscopy. Small unilamellar vesicles (SUVs) composed of DSPC/DLPC/NBD-PC were prepared by tip sonification (Branson Tip Sonifier Model 250D, Branson Ultrasonics, Danbury, CT). SUVs composed of DPPC/DLPC/NBD-PC were made with an extruder because this method produced larger phase-separated domains. Supported lipid membranes were formed by slow-cooled vesicle fusion onto the silicon substrates described above. The DSPC/DLPC and DPPC/DLPC bilayers were annealed at 70 °C and 55 °C, respectively, for 1 h, and then were slowly cooled at a rate of 2.6°C/hr and 1.5°C/hr, respectively, to induce phase-separation.

To permit analyzing the samples with TOF-SIMS, which is performed under ultra-high vacuum (UHV), the samples were flash-frozen in liquid ethane, and then the ice was sublimed from the membrane using an oil-free scroll pump (Triscroll 300, Varian, Inc. Palo Alto, CA). Fluorescence microscopy (Leica DM6000 B, Q-Imaging
EXi Blue Fluorescence Microscope) was used to evaluate membrane quality, and to generate optical maps of the samples that enabled imaging the same sample locations with both AFM and TOF-SIMS.

**AFM analysis of phase-separated lipid membranes**

The freeze-dried supported lipid membranes were imaged with an Asylum MFP-3D™ Stand Alone AFM (Asylum Research, Santa Barbara, CA) in ambient air and temperature. Measurements were taken in AC mode (tapping) in the repulsive tip-sample interaction regime with standard 300 kHz AFM probes (Tap300Al-G, Budget Sensors, Bulgaria). AFM images were flattened to the second-order, and line scans between the gel- and surrounding fluid-phase domains were made to verify that symmetric phase-separated domains were present.

**TOF-SIMS analysis**

TOF-SIMS analysis was performed on a PHI Trift-III TOF-SIMS (Physical Electronics Incorporated, Chanhassen, MN) using a gold liquid ion gun operated at 22 kV. A $^{197}\text{Au}^+$ primary ion beam with 3.7 nA and 1 nA was used to analyze the lipid films and phase-separated lipid membranes, respectively. TOF-SIMS data from the lipid films were acquired from $100 \mu m \times 100 \mu m$ sample regions using bunched mode. TOF-SIMS spectra were acquired at twelve different regions on each pure lipid film.
TOF-SIMS analysis of the phase-separated supported lipid membranes was performed in unbunched mode for optimal lateral resolution. TOF-SIMS images with 256 pixels x 256 pixels were acquired from 85 µm x 85 µm or 65 µm x 65 µm areas on the membrane, and were cropped to show subregions of the membrane. TOF-SIMS spectra were obtained with a mass range of 0 to 800 amu and a mass resolution of M/ΔM = 850 at the C₃H₇⁺ peak (m/z 43). The analysis time varied according to the size of the acquisition area in order to keep a constant primary ion dose of 2.4 × 10¹² ions/cm² for the pure lipid films, and 2.8 × 10¹³ ions/cm² for the phase-separated supported lipid membranes. To improve the image quality, downbinned TOF-SIMS images were created by summing the intensities of the mass peaks over 2 pixels x 2 pixels, producing individual TOF-SIMS ion images with 128 pixels x 128 pixels.

PCA of TOF-SIMS spectra of pure lipid films and phase-separated lipid membranes

PCA was performed using the PLS Toolbox and the MIA Toolbox (v.5.2.2 and v.1.0.7, respectively, Eigenvector Research, Manson, WA), which were run in MATLAB (v.7.8.0, MathWorks Inc., Natick, MA). Unit mass binning was applied to each spectrum acquired from a pure lipid film. The mass peaks from m/z 30 to 200 were used for analysis, but the peaks with m/z of 39 (potassium), 40 (calcium), 41 (hydrocarbon contaminant), 52 (chrome grid), 73 (PDMS contaminant), and 147 (PDMS contaminant) were removed from the spectra. The TOF-SIMS data was
arranged in a matrix such that the different samples formed the rows and the individual mass peaks formed the columns. Each mass spectrum of a pure lipid film was normalized to the total intensity of the selected peaks and mean-centered. A PC model was constructed using the mass spectra from the four different lipid species. Score plots were generated that illustrate the projection of the TOF-SIMS data from the phosphatidylcholine species onto the new PCs. Loadings plots were created that exhibit the linear combination of mass peaks that contributed to each PC. Mass peaks with large positive or negative loadings on a PC tend to have relatively higher intensities in the normalized spectra of the samples with positive or negative scores, respectively, on the same PC.28

For PCA of the TOF-SIMS images acquired on the phase-separated supported lipid membranes, the mass peaks with counts of at least 0.1% of the highest observed count rate were imported directly into the PLS toolbox. The intensities of the mass peaks were summed over 2 pixels x 2 pixels, producing downbinned images with 128 x 128 pixels. If the chrome grid that is used for sample positioning was visible in the TOF-SIMS image, PCA was performed only on the region of the image where the membrane was located. As in the analysis of the lipid standards, the mass peaks listed above (m/z 0 to 29, 39, 40, 41, 52, 73, and 147) were removed from the spectra. The resulting spectra were normalized to the total intensity of the selected peaks and autoscaled. Finally a PC model was created that converted the TOF-SIMS data from n ion images of 128 pixels x 128 pixels, where n is the number
of mass peaks, into a two dimensional array with n columns and 16,384 rows that contained the TOF-SIMS data collected at every pixel. A score value was calculated for each pixel, and the data was reassembled into a 128 pixel × 128 pixel image in which the score value at each pixel was encoded by a pseudo-color scale. We refer to this graphic representation of the scores values as a PC scores image. A loadings plot for each PC that shows the linear combination of mass peaks that contribute to the PC was generated.

*Calculation of image contrast*

To compare the quality of the individual TOF-SIMS images and PC scores images, contrast between the gel- and fluid-phase membrane domains within the images was calculated according to the following equation, as previously reported. $^{30,45}$

$$c_{gel, fluid} = \frac{|I_{gel} - I_{fluid}|}{\sigma_{gel, fluid}}$$

Where $I_{gel}$ is the average intensity in the gel-phase domains, $I_{fluid}$ is the average intensity in the fluid-phase domains, and $\sigma_{gel, fluid}$ is the standard deviation of the intensity within both regions. Intensity refers to counts in the individual TOF-SIMS ion images, and to the PC score values in the PC scores images.

*Identification of lipid composition in the gel- and fluid-phase domains within phase-separated membranes using PCA*
TOF-SIMS data were extracted from 12 regions of interest (ROIs) on the gel- and fluid-phase membrane regions, which were identified by comparison to the AFM image of the DSPC/DLPC and DPPC/DLPC membranes (24 ROIs per membrane, 48 ROIs in total). Unit mass binning was applied to each spectrum acquired from a ROI on the membrane. For each of the resulting 48 TOF-SIMS spectra, the normalized peaks from m/z 30 to 200 that did not correspond to the impurity peaks listed above were loaded as the validation block in the PC model developed using the pure lipid films. The mass spectra from the lipid membranes were arranged in a matrix as described above, normalized to the total intensity of the selected peaks each spectrum, mean-centered, and then projected onto the PC model developed using the pure lipid films. Although the spectra from the lipid standards were acquired with higher mass resolution than the TOF-SIMS images of the phase-separated lipid membranes, the mass resolution in the data set was ultimately limited by the unit mass binning applied to all the spectra prior to data processing.

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(2) Feigenson, G. W. BBA - Biomembranes 2009, 1788, 47-52.


Chapter 4. 
ENHANCING TOF-SIMS IMAGING OF LIPID MEMBRANES USING LIPID-RELATED PEAKS SETS AND PRINCIPAL COMPONENT ANALYSIS

Notes and Acknowledgements

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Introduction

Lateral heterogeneity in the component distribution within the cellular plasma membrane is widely believed to be vital for a variety of cell functions. The distributions of specific proteins can be visualized by using functionalized affinity labels or genetically encoded fluorescent protein constructs. Unfortunately, fluorophores can alter the molecular interactions and the distributions of the lipids that they label, so far fewer approaches are available for elucidating the organization of specific lipid species. To unambiguously identify the lipid distribution within cellular
or model membranes, spatially well-resolved chemical information is required. The distributions of different lipid classes within model membranes can be chemically imaged with as high as submicrometer lateral resolution by using time-of-flight secondary ion mass spectrometry (TOF-SIMS).\textsuperscript{1-3} However, discriminating between structurally similar, unlabeled lipid species within a TOF-SIMS image is very challenging. High mass fragment ions (i.e., molecular ions and large fragment ions) are often characteristic of a single lipid species, but typically have low yields that results in insufficient signal-to-noise ratios for chemical imaging with submicron lateral resolution.\textsuperscript{4,5} Lipids also produce low mass fragment ions (m/z < 200) that have higher yields and better signal-to-noise ratios, but these ions are often common to multiple lipid species, prohibiting unambiguous identification.

Multivariate analysis techniques, such as principal component analysis (PCA), are statistical methods that can be used to distinguish various molecules according to combinations of numerous mass peaks that may be common to multiple samples.\textsuperscript{6-12} Multivariate analysis of TOF-SIMS data has enabled discriminating and imaging structurally similar organic molecules, including monosaccharide isomers,\textsuperscript{12} proteins,\textsuperscript{13-15} alkane thiols,\textsuperscript{7} and lipids.\textsuperscript{16-19} We recently demonstrated that unlabeled, saturated phosphatidylcholine lipids could be successfully discriminated by PCA of their TOF-SIMS spectra.\textsuperscript{19} PCA of the TOF-SIMS images allowed visualizing the distributions of saturated phosphatidylcholine species within phase-separated lipid
membranes with quantifiably higher contrast than that present in any of the individual TOF-SIMS ion images.\textsuperscript{19}

The ability of PCA to discriminate the TOF-SIMS data of structurally similar molecules is influenced by the number of mass peaks in the spectra that are not generated by the molecules of interest. If sample-to-sample variation in the spectra is dominated by mass peaks related to contaminants or the sample substrate, then the spectral variation associated with the molecules of interest can be difficult to detect using PCA.\textsuperscript{7} In addition, whether classification by PCA of TOF-SIMS data is truly based on chemical differences between the samples, as opposed to sample-related contamination, is a concern. However, these problems can be circumvented by performing PCA on a restricted data set that contains only the mass peaks that are related to the molecules of interest.\textsuperscript{7,15} For example, PCA of proteins is often performed using a restricted data set that consists of mass peaks associated with amino acids.\textsuperscript{13,14,16,20-22} Consequently, the establishment of a restricted set of mass peaks that are associated with lipids could improve the identification of various lipid species in the TOF-SIMS images acquired from model membranes, and facilitate identifying the origins of mass peaks acquired from native cell membranes.

In this chapter, we identify the TOF-SIMS peaks that are associated with phosphatidylcholine lipids by using PCA to detect the peaks that differentiate an isotope-substituted lipid from the analogous unlabeled, natural abundance molecule. Because the lipid isotopologues are chemically identical and differ only in isotope
composition, the characteristic mass shift between ions containing different isotopes can be used to confirm the isotopologue-associated peaks are lipid fragments, and not ions produced by sample-specific contaminants. To identify the peaks associated with the lipid headgroup, we used PCA to characterize the spectral differences between natural abundance 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC) and $^{15}$N-DLPC. The mass peaks related to the lipid tails were discriminated according to the spectral differences between natural abundance 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and a DSPC isotopologue with perdeuterated fatty acid tails (D$_{70}$-DSPC). We use the Fisher’s ratio, which is a quantitative measure of the discrimination between groups on each multivariate axis,$^{15,23}$ to assess how use of the restricted set of mass peaks influences the classification of standard lipid samples by PCA. We also evaluated how use of these lipid-related peak sets influences visualization of lipid distribution within phase-separated lipid membranes.

![Figure 4.1. Positive-ion spectra and structures (inset) of DLPC isotopologues: (A) DLPC and (B) $^{15}$N-DLPC. The spectra were obtained using an ion dose of 9.7 x 10$^{11}$ ions/cm$^2$.](image)
Results and Discussion

Identification of Mass Peaks Related to Lipid Headgroups

The mass spectra acquired from unlabeled, natural abundance DLPC and $^{15}$N-DLPC are shown in Figure 4.1. To detect the headgroup-related differences in the spectra from the DLPC isotopologues, PCA was performed on the 12 TOF-SIMS data sets acquired from each lipid (Figure 4.2). Each TOF-SIMS data set consisted of the mass peaks in the 1-300 m/z range, with the exception of known inorganic ions ($\text{Li}^+$, $\text{Na}^+$, $\text{Si}^+$, and $\text{K}^+$). This mass range was selected because secondary ions with $m/z > 300$ usually have intensities that are too low to be utilized for TOF-SIMS imaging of lipid membranes with submicron-scale lateral resolution.\textsuperscript{5,17} Figure 4.2A shows the scores on PC1 and PC2 from PCA of 12 TOF-SIMS data sets acquired from $^{15}$N-DLPC and natural abundance DLPC. PC1 captures the majority of variance in the data (90.9%), and separates the samples according to their isotope composition. PC2 captures a much smaller percentage of the variance in the data (5.5%) that is not related to the lipid isotope composition (see below).

The mass peaks that contribute highly to differentiating the lipid spectra by isotope composition have high loadings on PC1, and are shown in the PC1 loadings plot in Figure 4.2B. Note that the fatty acid tails in $^{15}$N-DLPC and DLPC are chemically and isotopically identical, so the fatty acid fragment ions from the DLPC isotopologues should be identical, and, therefore, have negligible loadings on PC1.
Figure 4.2. Scores and loadings plots from PCA of the positive-ion spectra acquired from natural abundance DLPC and $^{15}$N-DLPC. These two lipid species differ only in the nitrogen isotope in the headgroup. PCA was performed on the peaks with $m/z$ from 1 to 300 amu, excluding the lithium ($m/z = 7$), sodium ($m/z = 23$), and potassium ($m/z = 39$) ions. The peaks were normalized to the total counts in the data set and mean-centered. (A) The scores on PC1 differentiate the lipids by their nitrogen isotope. The variance captured on PC2 is not isotope-specific. The ellipses outlined with solid and dashed lines represent the border for each group, and the whole model, respectively, at the 95% confidence limit. (B) The loadings plot shows the mass peaks that are responsible for the majority of the variance in the spectra of DLPC and $^{15}$N-DLPC. (C) Loadings plot shows the mass peaks that are responsible for the spectral variance captured on PC2.
Table 4.1. Headgroup-related mass peaks and assignments identified by PCA of the positive ion spectra of DLPC isotopologues that were acquired with TOF-SIMS.

<table>
<thead>
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<th>m/z</th>
<th>Proposed assignments</th>
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<td>DLPC[^{15}\text{N}]\text{-DLPC}</td>
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<tr>
<td>184/185</td>
<td>C$<em>6$H$</em>{13}$NPO$_4$[^{15}\text{N}^+$/C$<em>2$H$</em>{18}$[^{15}\text{N}^+$/</td>
</tr>
<tr>
<td>190/191</td>
<td>C$<em>7$H$</em>{13}$NPO$_4$[^{15}\text{N}^+$/C$<em>2$H$</em>{18}$[^{15}\text{N}^+$/</td>
</tr>
<tr>
<td>194/195</td>
<td>C$<em>7$H$</em>{13}$NPO$_4$[^{15}\text{N}^+$/C$<em>2$H$</em>{18}$[^{15}\text{N}^+$/</td>
</tr>
<tr>
<td>196/197</td>
<td>C$<em>7$H$</em>{13}$NPO$_4$[^{15}\text{N}^+$/C$<em>2$H$</em>{18}$[^{15}\text{N}^+$/</td>
</tr>
<tr>
<td>198/199</td>
<td>C$<em>7$H$</em>{13}$NPO$_4$[^{15}\text{N}^+$/C$<em>2$H$</em>{18}$[^{15}\text{N}^+$/</td>
</tr>
<tr>
<td>206/207</td>
<td>C$<em>7$H$</em>{13}$NPO$_4$Na[^{15}\text{N}^+$/C$<em>2$H$</em>{18}$[^{15}\text{N}^+$/</td>
</tr>
<tr>
<td>210/211</td>
<td>C$<em>7$H$</em>{17}$NPO$_4$[^{15}\text{N}^+$/C$<em>2$H$</em>{18}$[^{15}\text{N}^+$/</td>
</tr>
<tr>
<td>212/213</td>
<td>C$<em>7$H$</em>{17}$NPO$_4$[^{15}\text{N}^+$/C$<em>2$H$</em>{18}$[^{15}\text{N}^+$/</td>
</tr>
<tr>
<td>224/225</td>
<td>C$<em>7$H$</em>{18}$NPO$_4$[^{15}\text{N}^+$/C$<em>2$H$</em>{18}$[^{15}\text{N}^+$/</td>
</tr>
<tr>
<td>226/227</td>
<td>C$<em>7$H$</em>{18}$NPO$_4$[^{15}\text{N}^+$/C$<em>2$H$</em>{18}$[^{15}\text{N}^+$/</td>
</tr>
<tr>
<td>238/239</td>
<td>C$<em>8$H$</em>{18}$NPO$_4$[^{15}\text{N}^+$/C$<em>2$H$</em>{18}$[^{15}\text{N}^+$/</td>
</tr>
<tr>
<td>240/241</td>
<td>C$<em>8$H$</em>{18}$NPO$_4$[^{15}\text{N}^+$/C$<em>2$H$</em>{18}$[^{15}\text{N}^+$/</td>
</tr>
<tr>
<td>246/247</td>
<td>C$<em>8$H$</em>{19}$NPO$_4$Na[^{15}\text{N}^+$/C$<em>2$H$</em>{18}$[^{15}\text{N}^+$/</td>
</tr>
<tr>
<td>252/253</td>
<td>C$<em>9$H$</em>{19}$NPO$_4$[^{15}\text{N}^+$/C$<em>2$H$</em>{18}$[^{15}\text{N}^+$/</td>
</tr>
<tr>
<td>254/255</td>
<td>C$<em>9$H$</em>{19}$NPO$_6$[^{15}\text{N}^+$/C$<em>2$H$</em>{18}$[^{15}\text{N}^+$/</td>
</tr>
<tr>
<td>256/257</td>
<td>C$<em>9$H$</em>{19}$NPO$_6$[^{15}\text{N}^+$/C$<em>2$H$</em>{18}$[^{15}\text{N}^+$/</td>
</tr>
<tr>
<td>282/283</td>
<td>C$<em>9$H$</em>{19}$NPO$_7$[^{15}\text{N}^+$/C$<em>2$H$</em>{18}$[^{15}\text{N}^+$/</td>
</tr>
</tbody>
</table>
The mass peaks with positive loadings have higher relative intensities in the spectra of $^{15}\text{N}$-DLPC, and should include $^{15}\text{N}$-labeled lipid headgroup fragments. In contrast, the mass peaks with negative loadings have higher relative intensities in the spectra of natural abundance DLPC, and should include unlabeled lipid headgroup fragments. Sample-specific contaminants may also contribute to the variation between the spectra, and have high loadings on PC1. Therefore, we identified the peaks that correspond to lipid headgroup fragments according to the presence of a 1 nominal mass that had higher intensity on the other DLPC isotopologues. For instance, the C$_3$H$_{10}$N$^+$ fragment at $m/z$ 60, respectively, was masked by the C$_3$H$_9^{15}$N$^+$ ion with the same molecular weight. A few mass peaks with high loadings on PC1 were associated with contaminants due to the absence of an oppositely loading peak with the characteristic 1-amu shift. These contaminant-related peaks include small hydrocarbons ($m/z$ 15, 27, 29, and 41) and the silicon substrate ($m/z$ 28).

The loadings on PC2 are shown in Figure 4.2C. The peaks with high loadings on PC2 include ions related to the silicon substrate ($m/z$ 28), hydrocarbon fragments ($m/z$ 27, 29, 41, and 43), and polydimethylsiloxane (PDMS) contaminants ($m/z$ 73, 147, 207, 221, and 281). A few ions that are characteristic of lipid headgroup fragments ($m/z$ 104, 166, 167, 184, and 185) are also present. Therefore, PC2 likely captures variance in the sample spectra that is related to the amount of lipid surface coverage and contamination on the substrate.
Identification of Mass Peaks Related to Lipid Fatty Acid Tail Groups

Representative mass spectra for DSPC and D$\text{}_{70}$-DSPC are shown in Figure 4.3. These DSPC isotopologues have the longest fatty acid tails of any lipid frequently studied in model membranes. Therefore, their spectra should contain both the mass peaks from larger hydrocarbon ions that are related to lipids with long fatty acid tails, and the smaller hydrocarbon fragment ions that are generated by all lipid species. In addition, these hydrocarbon fragments will also reflect those produced by lipids with unsaturated fatty acid tails because fragmentation of the saturated alkyl moieties yields both alkyl and alkenyl carbocations. To detect the fatty acid-related mass peaks in the spectra, PCA was performed on the 12 positive-ion spectra acquired from each isotopologue with TOF-SIMS. Figure 4.4A shows the scores for each sample on PC1 and PC2. The vast majority of the variance in the data (99.9%) is captured by PC1, which separates the samples according to their isotope composition. In contrast, PC2 captured only a very small amount of the variation in the data (0.05%), and will not be discussed further. The mass peaks responsible for discriminating DSPC and D$\text{}_{70}$-DSPC, and contributing to the variation on PC2 are shown in the PC1 and PC2 loadings plots in Figure 4.4B and C. The mass peaks with positive loadings on PC1 are characteristic of DSPC and are expected to include the natural abundance fatty acid fragments. In contrast, the mass peaks with negative loadings on PC1 have higher normalized intensities in the spectra of D$\text{}_{70}$-DSPC, and are expected to correspond to perdeuterated fatty acid fragments. The isotopologue-
associated peaks that corresponded to fatty acid tails were identified by the presence of oppositely loading mass peaks with the characteristic mass shift between fragment ions that differ in hydrogen isotope composition. In addition, examination of the individual mass spectra also indicated that the DSPC-related $C_2H_6^+$ fragment at $m/z$ 42 was masked on PC1 by overlap with the $D_{70}$-DSPC-related $C_2D_4O^+$ fragment with the same nominal mass. Table 4.2 lists the fatty acid-related peaks we identified. Note that PCA of DOPC and $D_{70}$-DSPC spectra confirmed that these peaks are also related to the DOPC fatty acid fragments (Figure 4.5). This indicated that this peak set is applicable to both saturated and unsaturated lipids, and can be used to assess the spectra of a range of model and cellular membranes.

![Figure 4.3](image)

**Figure 4.3.** Positive-ion spectra and structures (inset) of DSPC isotopologues: (A) DSPC and (B) $D_{70}$-DSPC. The spectra were obtained using an ion dose of $9.7 \times 10^{11}$ ions/cm$^2$. 
Figure 4.4. Scores and loadings plots from PCA of the spectra of natural abundance DSPC and D$_{70}$-DSPC. The protons within the fatty acid tails are completely substituted with deuterium in D$_{70}$-DSPC. PCA was performed on the peaks in the 1-300 m/z range, excluding the lithium (m/z = 7), sodium (m/z = 23), and potassium (m/z = 39) ions. The peaks were normalized to the total counts in the data set and mean-centered. (A) The scores on PC1 differentiate the lipids by isotope composition. PC2 captures a very small amount of spectral variance (<<1%), which is not isotope-specific. The ellipses outlined with solid and dashed lines represent the border for each lipid, and the whole model, respectively, at the 95% confidence limit. (B) The loadings plot shows the mass peaks that discriminate the spectra of DSPC and D$_{70}$-DSPC. (C) Loadings plot shows the mass peaks that are responsible for the spectral variance captured on PC2.
Table 4.2. Fatty acid tail-related mass peaks and assignments identified by PCA of the positive ion TOF-SIMS data acquired from DSPC and D$_{70}$-DSPC.

<table>
<thead>
<tr>
<th>m/z</th>
<th>Proposed assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSPC/D$_{70}$DSPC</td>
<td>DSPC/D$_{70}$DSPC</td>
</tr>
<tr>
<td>15/18</td>
<td>CH$_3^+$/CD$_3^+$</td>
</tr>
<tr>
<td>27/30</td>
<td>C$_2$H$_5^+$/C$_2$D$_5^+$</td>
</tr>
<tr>
<td>29/34</td>
<td>C$_2$H$_7^+$/C$_2$D$_7^+$</td>
</tr>
<tr>
<td>41/46</td>
<td>C$_3$H$_7^+$/C$_3$D$_7^+$</td>
</tr>
<tr>
<td>41/42</td>
<td>C$_3$HO/C$_3$DO</td>
</tr>
<tr>
<td>42/46</td>
<td>C$_3$H$_5^+$/C$_3$D$_5^+$</td>
</tr>
<tr>
<td>43/50</td>
<td>C$_3$H$_7^+$/C$_3$D$_7^+$</td>
</tr>
<tr>
<td>53/58</td>
<td>C$_4$H$_9^+$/C$_4$D$_9^+$</td>
</tr>
<tr>
<td>54/60</td>
<td>C$_4$H$_7^+$/C$_4$D$_7^+$</td>
</tr>
<tr>
<td>55/62</td>
<td>C$_4$H$_5^+$/C$_4$D$_5^+$</td>
</tr>
<tr>
<td>57/66</td>
<td>C$_4$H$_9^+$/C$_4$D$_9^+$</td>
</tr>
<tr>
<td>67/74</td>
<td>C$_5$H$_9^+$/C$_5$D$_9^+$</td>
</tr>
<tr>
<td>69/78</td>
<td>C$_5$H$_5^+$/C$_5$D$_5^+$</td>
</tr>
<tr>
<td>71/82</td>
<td>C$_5$H$_11^+$/C$_5$D$_11^+$</td>
</tr>
<tr>
<td>81/90</td>
<td>C$_6$H$_9^+$/C$_6$D$_9^+$</td>
</tr>
<tr>
<td>83/94</td>
<td>C$_6$H$_11^+$/C$_6$D$_11^+$</td>
</tr>
<tr>
<td>85/98</td>
<td>C$_6$H$_13^+$/C$_6$D$_13^+$</td>
</tr>
<tr>
<td>91/98</td>
<td>C$_7$H$_9^+$/C$_7$D$_9^+$</td>
</tr>
<tr>
<td>93/102</td>
<td>C$_7$H$_11^+$/C$_7$D$_11^+$</td>
</tr>
<tr>
<td>95/106</td>
<td>C$_7$H$_13^+$/C$_7$D$_13^+$</td>
</tr>
<tr>
<td>97/110</td>
<td>C$_7$H$_13^+$/C$_7$D$_13^+$</td>
</tr>
<tr>
<td>111/126</td>
<td>C$_8$H$_15^+$/C$_8$D$_15^+$</td>
</tr>
</tbody>
</table>

Figure 4.5. Scores and loadings plots from PCA of the positive-ion spectra acquired from DOPC and D$_{70}$-DSPC (structures inset). DOPC differs from DSPC only in fatty-acid chain saturation. PCA was performed on the peaks with m/z from 1 to 300 amu, excluding the lithium (m/z = 7), sodium (m/z = 23), and potassium (m/z = 39) ions. The peaks were normalized to the total counts in the data set and mean-centered. (A) The scores on PC1 differentiate the lipids by isotope composition. PC2 captures a very small amount of spectral variance (<<1%), which is not isotope-specific and is not shown here. (B) The loadings plot shows the mass peaks that discriminate the spectra of DOPC and D$_{70}$-DSPC, which has very similar loadings to that of PCA of DSPC and D$_{70}$-DSPC, illustrated in Figure 4.4B.
Classification of Lipid Standards

We evaluated how the use of the lipid-related mass peak set influences the discrimination between four unlabeled phosphatidylcholine lipid species: DLPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), and DSPC. The TOF-SIMS spectra of these phosphatidylcholine species are difficult to discriminate because each lipid contains two saturated fatty acid tails, a phosphocholine headgroup, and a glycerol backbone. PCA was performed using four different TOF-SIMS peak sets with \( m/z \) in the 1-300 range that were acquired from DLPC, DMPC, DPPC, and DSPC (Table 4.3). A complete peak set that consisted of all mass peaks with \( m/z \) 1-300 except for \( m/z \) 7, 23, 28, and 39 from Li\(^+\), Na\(^+\), Si\(^+\) and K\(^+\), respectively, was selected as the first peak set, as PCA is typically performed on a complete peak set in which only salt cations (Li\(^+\), Na\(^+\), and K\(^+\)) and substrate ions are excluded. A contaminant-free peak set that consisted of all peaks with \( m/z \) 30-300 except for \( m/z \) 39 (K\(^+\)), 40 (Ca\(^+\)), 41 (hydrocarbon), 52 (Cr\(^+\)), 73 (PDMS), 147 (PDMS), 207 (PDMS), 221 (PDMS), and 281 (PDMS) was used for the second peak set because very low mass peaks (\( m/z <30 \)) that have poor molecular specificity\(^1\),\(^9\),\(^12\),\(^19\),\(^20\),\(^26\) and peaks produced by known contaminants are often removed from TOF-SIMS data sets prior to PCA\(^9\),\(^12\),\(^19\),\(^20\),\(^26\).

The third data set was a lipid-related peak set that consisted only of the ion peaks that are listed in Tables 4.1 and 4.2. The fourth peak set was a contaminant-free lipid-related peak set that consisted of the lipid-related peaks that are listed in Tables
4.1 and 4.2, but peaks with \( m/z < 30 \) and those related to known contaminants (\( m/z \) 39, 40, 41, 52, 73, 147, 207, 221, and 281) were excluded.

**Table 4.3.** Description of the four peak sets that were constructed using the same TOF-SIMS data that was collected from DLPC, DMPC, DPPC, and DSPC. Each peak set consisted of the mass peaks with \( m/z \) 1-300, but different masses were excluded from each peak set. The Fisher's ratio (FR) calculated on PC1 is a quantitative measure of the lipid discrimination.

<table>
<thead>
<tr>
<th>Peak set (( m/z ) 1 - 300)</th>
<th>Excluded peaks</th>
<th>FR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>( m/z ) 7, 23, 28 and 39</td>
<td>6.6</td>
</tr>
<tr>
<td>Contaminant-free</td>
<td>( m/z &lt; 30, 39, 40, 41, 52, 73, 147, 207, 221, 281 )</td>
<td>59</td>
</tr>
<tr>
<td>Lipid-related</td>
<td>Peaks not listed in Tables 4.1 and 4.2</td>
<td>14</td>
</tr>
<tr>
<td>Lipid-related and contaminant-free</td>
<td>Peaks not listed in Tables 4.1 and 4.2, and ( m/z &lt; 30, 41, 147 )</td>
<td>55</td>
</tr>
</tbody>
</table>

The scores and loadings plots produced by PCA of the four peak sets are shown in Figure 4.6. For all peak sets, the PC1 scores separate the lipids according to the length of their fatty acid tails, whereas the PC2 scores were not related to the lipid composition. We quantitatively assessed the lipid-specific discrimination of the different phosphatidylcholines by calculating the PC1 Fisher's ratio (Table 4.3). The lipid standards were not well-separated in the PC1 and PC2 scores plot (Figure 4.6A) by PCA utilizing the complete peak set, and the calculated PC1 Fisher’s ratio (6.6) confirmed that the lipid-specific separation was low. The loadings plot for the complete peak set (Figure 4.6B) shows the masses that contribute significantly to the spectral variation captured by PC1 include \( m/z \) 27, 29, 41 and 43. Thus, the poor separation may have occurred because both lipid-related hydrocarbons and contaminants contributed to these peaks. Visual inspection of the PC1 and PC2
scores plot (Figure 4.6C) obtained by PCA of the contaminant-free peak set indicated excellent separation between the lipid standards. We calculated a Fisher's ratio of 59 on PC1, verifying that significantly higher lipid-specific separation was achieved. The PC1 and PC2 scores plot produced with the lipid-related peak set (Figure 4.6E) indicated that restriction of the data set to the lipid-related peaks produced better discrimination of the phosphatidylcholine species than that obtained with the complete data set, but poorer discrimination than that achieved using the contaminant-free peak set. This was confirmed by the PC1 Fisher's ratio we calculated for the lipid-related peak set, which was 14. The loadings plot for the lipid-related peak set (Figure 4.6F) shows hydrocarbon ions related to both lipid fragments and contaminants (m/z 27, 29, and 41) had high loadings on PC1, which likely compromised lipid separation. Lipid separation on PC1 and PC2 (Figure 4.6G) could be improved by removing the very low mass peaks (m/z<30) and those related to contaminants from the lipid-related peak set. A PC1 Fisher's ratio of 55 was calculated for the contaminant-free lipid-related peak set, indicating the lipid-specific discrimination was similar to that obtained using the contaminant-free peak set. The loadings plot from PCA of the contaminant-free lipid-related peak set (Figure 4.6H) also appeared to be similar to the loadings plot from PCA of the contaminant-free peak set (Figure 4.6D).
Figure 4.6. Scores and loadings plots on PC1 and PC2 from PCA of different sets of mass peaks in the spectra acquired from DLPC, DMPC, DPPC, and DSPC lipid films. Scores (A) and loadings plots (B) produced by PCA of the data sets consisting of all mass peaks with m/z 1-300 except for the known inorganic ions (Li, Na, Si, and K). Scores (C) and loadings plots (D) from PCA of TOF-SIMS data sets that consisted of the peaks with m/z 30-300 with the exclusion of known contaminants ions (m/z 39, 40, 41, 52, 73, 147, 207, 221, and 281). Scores (E) and loadings plots (F) from PCA of restricted TOF-SIMS data sets that contained only the mass peaks related to the lipid headgroups (Table 4.1) and fatty acid tails (Table 4.2). Scores (G) and loadings (H) plots from PCA of the restricted TOF-SIMS data sets containing only the lipid-related peaks with the exclusion of peaks with m/z > 30 and those of known contaminant ions (m/z 39, 40, 41, 52, 73, 147, 207, 221, and 281).
Scores Images of Phase-Separated Supported Lipid Membranes

We evaluated how the use of the restricted mass peak set influenced visualization of the lipid distribution within phase-separated lipid membranes by PCA of the TOF-SIMS images. Figure 4.7 shows the AFM image that was acquired from a phase-separated DSPC/DLPC supported lipid membrane prior to TOF-SIMS analysis. Here, we see phase separation between the DSPC enriched gel-phase domains, which are the taller features, and the surrounding DLPC enriched fluid-phase region, which are the shorter areas.\textsuperscript{19,27,28} PCA was performed on the TOF-SIMS analysis of this same membrane region using the peak sets noted in Table 4.3, and Figure 4.8 shows the resulting PC scores images and loadings plots.

\textbf{Figure 4.7.} AFM image of a phase-separated DSPC/DLPC membrane. The image shows gel- and fluid-phase domains that are enriched with DSPC and DLPC, respectively, in the DSPC/DLPC (1:2 molar ratio) membrane.

In the complete data set PCA analysis (Figure 4.8A and B), the phase-separation between the two chemically distinct membrane regions is revealed. The contrast between these two regions was calculated as described in Chapter 3,\textsuperscript{19} and was determined to be 1.74. The very low mass peaks ($m/z<30$) that have poor
Figure 4.8. PCA of a TOF-SIMS image taken at the same location of the phase-separated DSPC/DLPC membrane shown in Figure 6. Scores images resulting from PCA of the (A) complete peak set, (C) contaminant-free peak set, (E) lipid-related peak set, and (G) lipid-related and contaminant-free peak set (refer to Table 4.3). The loadings plots for the (B) complete peak set, (D) contaminant-free peak set, (F) lipid-related peak set, and (H) lipid-related and contaminant-free peak set show the extent that each peak contributed to the spectral variation captured by PC1.
molecular specificity,\textsuperscript{19,20,26} were removed in the contaminant-free analysis (Figure 4C and D), and the contrast between the DSPC and DLPC enriched membrane regions was determined to be 1.53, which is less than the complete peak set analysis. In the third analysis only the lipid-related peaks were included (Figure 4.8E and F), and the contrast between the gel- and fluid-phase domain was calculated to be 1.57, which is better than the contaminant-free analysis, but poorer than the complete data set analysis. Finally, the contaminant-free lipid-related peaks were used for PCA (Figure 4.8G and H), and the contrast value for this image (1.34) was the poorest of all four analyses. From this analysis it was established that the complete data set had quantifiably the greatest contrast. However, by sacrificing less than 10\% of the contrast, and using only the lipid-related peaks, one can be more confident PCA separation is based on the DSPC and DLPC content, as opposed to non-specific fragments that could be contaminants, such as $m/z=73$ (PDMS), which significantly contributes to the contrast in the complete data set (Figure 4.8B).

\textbf{Figure 4.9.} AFM image of a phase-separated DPPC/DLPC membrane. The image shows gel- and fluid-phase domains that are enriched with DPPC and DLPC, respectively, in the DPPC/DLPC (1:2 molar ratio) membrane.
Figure 4.10. PCA of a TOF-SIMS image taken at the same location of the phase-separated DPPC/DLPC membrane shown in Figure 4.9. Scores images resulting from PCA of the (A) complete peak set, (C) contaminant-free peak set, (E) lipid-related peak set, and (G) lipid-related and contaminant-free peak set (refer to Table 4.3). The loadings plots for the (B) complete peak set, (D) contaminant-free peak set, (F) lipid-related peak set, and (H) lipid-related and contaminant-free peak set show the extent that each peak contributed to the spectral variation captured by the corresponding PC.
We assess the effectiveness of utilizing these peak sets on another supported lipid membrane that contained DPPC/DLPC. Figure 4.9 shows the AFM image that was acquired from a DPPC/DLPC membrane prior to TOF-SIMS analysis. Figure 4.10 shows the PC scores images and loadings plots of the same membrane region as shown in Figure 4.9 using the peak sets noted in Table 4.3. Analogous to the PCA results of DSPC/DLPC, the best contrast between the DPPC enriched gel-phase domains and the DLPC enriched fluid-phase domains was in the complete data set PCA analysis (contrast= 1.44), followed by the lipid related (1.31) and the contaminant-free peak sets (1.30), and the worst contrast was in the contaminant-free lipid-related peak set (0.97), in which the domains were revealed only in the PC2 scores image.

Conclusions

In this study, we were able to determine lipid-related ion peaks by comparing the spectra of isotopologues with PCA, thus creating the first reported comprehensive positive-ion fragment list of these phosphatidylcholine lipid species. Moreover, we verified that these hydrocarbon fragments also reflected those produced by more physiologically relevant lipids with unsaturated fatty acid tails, as previously reported, by comparing D_{70}-DSPC with unsaturated lipid species. By using limited peak sets for building PCA models from dried lipid films of these phosphatidylcholine lipid species, it was determined that using a contaminant-free or a contaminant-free
lipid-related peak set created the best model. However, in order to enhance the discrimination of these lipid species, we suggest that using only contaminant-free lipid-related peak set is ideal for analysis. Finally, we evaluated the effectiveness of using limited peak sets for PCA of TOF-SIMS images, where it was determined that using the complete peak set gave the greatest quantifiable contrast between the gel- and fluid-phase regions of both DSPC/DLPC and DPPC/DLPC phase-separated supported lipid membranes. However, by relinquishing < 10% of the image contrast in both analyses through the use of the lipid related peak set, one can be more certain that PCA is revealing the phase-separated membrane regions based on lipid content as opposed to contaminants. These results can be used in further analysis of related phase-separated lipid membranes containing unsaturated lipid species. Furthermore, by having a comprehensive lipid-related fragment list, one can begin to identify the origins of peaks detected in the TOF-SIMS analyses of native cell membranes.

Materials and Methods

Preparation of Thin Film Lipid Samples

DLPC, DSPC, DOPC and D$_{70}$-DSPC were purchased in chloroform from Avanti Polar Lipids (Alabaster, AL) and used without further purification. $^{15}$N-DLPC was synthesized as previously reported. Thin films of each lipid standard were created by spotting a 5 µl droplet of 5 mg/ml lipid solution dissolved in chloroform
onto a 5 mm x 5 mm silicon substrate, which was patterned with a chrome grid to facilitate sample positioning. The samples were then placed under vacuum overnight to evaporate the solvent.

**Preparation of Phase-Separated Supported Lipid Membranes**

Supported lipid membranes composed of a 1:2 molar ratio of DSPC/DLPC and DPPC/DLPC, with 1 mol% of the fluorescent lipid, 1-palmitoyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]lauroyl}-sn-glycero 3-phosphocholine (NBD-PC, Avanti Polar Lipids, Alabaster, AL) for visualization with fluorescence microscopy, were formed, flash-frozen, and freeze-dried as previously described. Fluorescence microscopy (Leica DM6000 B, Q-Imaging EXi Blue Fluorescence Microscope) was used to evaluate membrane quality. Samples mosaics, enabling imaging of the same sample locations with both AFM and TOF-SIMS, were also made by fluorescence microscopy.

**AFM Imaging of Phase-Separated Lipid Membranes**

An Asylum MFP-3D™ Stand Alone AFM (Asylum Research, Santa Barbara, CA) was used to image the freeze-dried supported lipid membranes in ambient air and temperature as previously described. AFM images were flattened to the zeroth-order to remove any erratic tip “hops”, and then second-order plane-fitted in both the x- and y-plane.
TOF-SIMS Analysis of Lipid Films and Phase-Separated Lipid Membranes

TOF-SIMS analysis was performed on a PHI Trift-III TOF-SIMS (Physical Electronics Incorporated, Chanhassen, MN) using a $^{197}$Au$^+$ liquid ion gun operated at 22 kV. A primary ion beam with 8 nA and 1 nA was used to analyze the lipid films and phase-separated lipid membranes, respectively. TOF-SIMS spectra of the lipid films were acquired from 100 µm × 100 µm sample regions using bunched mode, and were acquired at a minimum of twelve different regions on each pure lipid film. TOF-SIMS analysis of the phase-separated supported lipid membranes was performed in unbunched mode for optimal lateral resolution and to minimize analysis time, as previously described in Chapter 4.19

PCA of TOF-SIMS Spectra

PCA was performed using the PLS Toolbox and the MIA Toolbox (v.5.8.1 and v.2.0.1, respectively, Eigenvector Research, Manson, WA), which were run in MATLAB (v.7.8.0, MathWorks Inc., Natick, MA). Pure film spectra were unit mass binned prior to importing into the PLS toolbox. The data were arranged in a matrix in which the different samples formed the rows and the individual mass peaks formed the columns. Each peak was normalized to the total intensity of the selected peaks and mean-centered. PCA produced PC score plots that show the projection of the samples onto the new PCs and loadings plots that show the peaks that contributed to each PC. Mass peaks with large positive or negative loadings on a PC have relatively
higher intensities in the normalized spectra of samples with positive or negative scores, respectively.\textsuperscript{30} The Fisher ratios were calculated as previously described in Chapter 4.\textsuperscript{15}

The TOF-SIMS images of the phase-separated supported lipid membranes were imported into MATLAB using unit mass binning, and were downbinned to 128 pixels x 128 pixels, as previously described.\textsuperscript{19} The resulting spectra were normalized to the total intensity of the selected peaks and autoscaled. A PC model was created, and a score value was calculated for each pixel, which was encoded by a pseudocolor scale in the resulting scores image. The loadings of each peak on the new PCs were also calculated. Image contrast between the gel- and fluid-phase membrane domains within each PC scores image was calculated as previously reported.\textsuperscript{8,19,31}

References

(3) Williams, P. J. Biol. 2006, 5, 18.


Chapter 5.
QUANTITATIVE ANALYSIS OF HOMOGENOUS BINARY MIXTURE SUPPORTED LIPID MEMBRANES BY TOF-SIMS AND PARTIAL LEAST SQUARES REGRESSION MODELING

Notes and Acknowledgements

This chapter describes work in progress. The work was carried out with the assistance of Robert L. Wilson and Mary L. Kraft. The material is based upon work funded by a Career Award at the Scientific Interface from the Burroughs Wellcome Fund held by Mary L. Kraft. Portions of this work were carried out in the Frederick Seitz Materials Research Laboratory Central Facilities, Univ. of Illinois, which are partially supported by the U.S. Department of Energy under grants DE-FG02-07ER46453 and DE-FG02-07ER46471.

Introduction

Cholesterol is an essential component of the eukaryotic cellular membrane. The interaction of cholesterol with sphingolipids and saturated phosphatidylcholine lipids is responsible for the formation of liquid-ordered (L_o) domains, which are assumed to have the same structural and physical properties as lipid rafts found within native cell membranes.\textsuperscript{1-3} The roles of lipid rafts within cell membranes are believed to be vital for many functions, including signaling, transport, and sorting.\textsuperscript{3-9} As a result, the use of model membranes to elucidate how the abundance of
cholesterol within distinct regions of the membrane influences the lipid membrane environment has been an area of considerable research.\textsuperscript{10-17} Previously, quantitative analysis has demonstrated that stoichiometric complexes between cholesterol and certain lipid species, namely sphingolipids or saturated phosphatidylcholine species, administer the creation of L\textsubscript{o} lipid domains.\textsuperscript{10-12,18} However, determining the lipid composition within the co-existing phases that are present in these membranes remains a challenge.\textsuperscript{18,19} Frequently, the compositions of different lipid phases present within model membranes can be identified only if phase diagrams have been established for the lipid mixture being studied.\textsuperscript{16-18,20,21} Nuclear magnetic resonance (NMR) has been used to determine the composition of different lipid phases and to construct phase diagrams of various lipid mixtures, but NMR provides bulk measurements and cannot be used to assess the composition within specific regions of the membrane.\textsuperscript{19,22} Investigations with electron paramagnetic resonance (EPR) spectroscopy have been useful in determining component miscibility between phases, but these analyses require the use of isotopically labeled or biradical lipid species, which increases the cost and complexity of sample preparation.\textsuperscript{18,23-26} Synchrotron X-ray diffraction studies have also proven valuable for studying model systems,\textsuperscript{27,28} and recently were used to determine cholesterol distribution within three co-existing lipid phases.\textsuperscript{13} However, the availability of this technique is very limited due to the need for a synchrotron light source, and these analyses are too cost prohibitive to be a workhorse method for
systematically studying the behavior of cholesterol within any of the numerous possibilities of ternary lipid mixtures.

Time-of-flight secondary ion mass spectroscopy (TOF-SIMS), on the other hand, is a more ubiquitous, label-free technique that is capable of imaging the spatial distribution of molecular species within model membranes. Quantitative measurements performed with TOF-SIMS or other SIMS techniques have been achieved on membrane structures, but the limitation of these studies are that they are either comparative (i.e. determine relative concentrations between samples), or require the use of stable isotope-labeled lipids to determine absolute species concentrations. However, the interpretation of TOF-SIMS data using multivariate analysis (MVA) is a promising approach for quantifying the concentration of specific species within lipid membranes.

Partial least squares regression (PLSR) is a MVA technique that has recently been used with TOF-SIMS for quantifying the composition of binary protein films. PLSR is a multivariate calibration method that attempts to find factors that both capture the variance and attain correlation between the TOF-SIMS peaks and the predicted variable. A detailed discussion of PLSR can be found elsewhere. Briefly, PLSR is a process that maximizes the covariance between the independent predictor variables, X, and the dependent predicted variables, Y. In the application of PLSR to TOF-SIMS data from lipid membranes, the independent predictor variables would be the ion peaks in the spectra, and the dependent
predicted variables would be the cholesterol or lipid concentration. Analogous to principal component analysis (PCA), PLSR reduces the data complexity by calculating a series of new variables, called latent variables (LVs), which are linear combinations of the original variables. The LVs are used to calculate the maximum variation between X and Y within the data set, while simultaneously correlating the predictor (X) variables to the predicted (Y) variables. Regression coefficients are determined from the LVs by multivariate linear regression, which describe how the variables contribute to the correlation between X and Y. Because PLSR creates a predictive model that can be used as a de facto calibration curve to quantitate the content of an unknown sample, it is desirable to use as few LVs as possible to generate a model with the lowest possible complexity. The SIMPLS algorithm is commonly used in commercially available software to calculate the PLSR parameters, and will be used in this study.

In this chapter, a TOF-SIMS PLSR model was created to determine the concentration of cholesterol within binary homogenous supported lipid membranes that contain cholesterol and 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC). A PLSR model was developed using DLPC/cholesterol samples of known composition that maximized the covariance between the TOF-SIMS ion peaks and the cholesterol concentration independently measured with enzymatic assays. The accuracy of the PLSR model was assessed using the root mean squared error of prediction.
This work establishes a foundation for using PLSR models developed with calibration samples to determine the cholesterol content within lipid membranes.

Results and Discussion

Development of PLSR model for quantifying cholesterol concentrations in DLPC supported lipid membranes

Binary lipid mixtures containing DLPC and approximately 0, 5, 10, or 15 mol% cholesterol were used to create homogenous supported lipid membranes. This range of cholesterol concentrations was selected because phase-separation often does not occur at higher cholesterol concentrations,\(^{35,47,48}\) and enabling quantification of the mol% cholesterol at distinct locations in phase-separated membranes is the longer-term goal of this research. Prior to membrane formation, the mol% cholesterol in each lipid vesicle solution used for membrane formation was measured using commercial enzymatic assays (see Materials and Methods section). The homogeneous supported lipid membranes used to create a calibration model were determined to contain 0, 5, 12, and 25 mol% cholesterol, respectively. Because a bilayer cannot be formed from pure cholesterol, a thin film of pure cholesterol was used as the 100 mol% cholesterol sample.

TOF-SIMS measurements were acquired from each sample. Analysis was performed only on the regions of the homogenous supported lipid membranes that were free of artifacts, and were uniformly fluorescent. PLSR was performed on the
resulting TOF-SIMS data using two peak sets. The first was the contaminant-free peak set described in Table 4.3 in Chapter 4, but the ion peak $m/z = 147$ was included because it is a significant cholesterol fragment. The second consisted of the lipid-related and contaminant-free peaks listed in Table 4.3, plus several peaks that were previously identified as cholesterol-related. Note that this second peak set may omit additional, unidentified, cholesterol-related peaks that are present in the spectra of Piehowski et al. For each model, the number of LVs was chosen by using continuous block cross validation to create a PLSR model, and then including the LVs that improved the root mean square error of cross validation (RMSECV) by $\geq 2\%$. Agreement between the TOF SIMS/PLSR and enzymatic assays of the cholesterol concentration within the lipid membrane was quantitatively assessed using the RMSEP. Figure 5.1 shows the predicted cholesterol concentrations in the calibration samples from PLSR performed on all the normalized and mean centered ion peaks with $m/z$ from 30 to 200 amu, excluding the potassium ($m/z = 39$), calcium ($m/z = 40$), hydrocarbon ($m/z = 41$), chrome ($m/z = 52$), and PDMS ($m/z = 73$) peaks. The four LVs retained in this PLSR model captured 97.9% of the variance in cholesterol concentration (98.3% of the spectral variance). The predicted cholesterol concentrations from this model are tabulated in Table 5.1. The PLSR model of the complete data set and the enzymatic measurements agreed within 3.6 mol% (RMSEP), indicating a satisfactory fit. While the model fit well as a whole, individual sample predictions did not fit as well. The 0 mol% cholesterol sample was predicted
to contain 3.3 mol% cholesterol, and the standard deviation of this sample is the largest relative to the predicted cholesterol content. The membranes containing cholesterol did, however, fit better, and were well within the standard deviation of their respective predicted values.

![Figure 5.1](image)

**Figure 5.1.** PLSR model from the TOF-SIMS analysis of DLPC/cholesterol supported lipid membranes. PLSR was performed on all the normalized ion peaks with m/z from 30 to 200 amu, excluding the potassium (m/z = 39), calcium (m/z = 40), hydrocarbon (m/z = 41), chrome (m/z = 52), and PDMS (m/z = 73) contaminant peaks. The included peaks were normalized to the total counts in the data set and mean-centered. Four latent variables (LVs) were used for this model (A), and 97.9% of the variance was retained in the cholesterol concentration. The loadings plot (B) for first and second latent variables (LV1 and LV2, respectively) combined captured 94.6% of the variance within the spectra.

**Table 5.1.** Average predicted cholesterol concentration of each homogenous supported lipid membrane containing DLPC/cholesterol for the contaminant-free PLSR model (Figure 5.1).

<table>
<thead>
<tr>
<th>Enzymatically-measured mol% cholesterol</th>
<th>Average predicted from Figure 5.1 (4 LVs)</th>
<th>σ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.3</td>
<td>3.9</td>
</tr>
<tr>
<td>5</td>
<td>4.9</td>
<td>1.9</td>
</tr>
<tr>
<td>12</td>
<td>11.7</td>
<td>1.6</td>
</tr>
<tr>
<td>25</td>
<td>21.8</td>
<td>3.9</td>
</tr>
<tr>
<td>100</td>
<td>101.0</td>
<td>0.6</td>
</tr>
</tbody>
</table>
The loadings plot in Figure 5.1B shows the mass peaks that maximize the covariance with cholesterol concentration. Here, LV1 captures the majority of the spectral variance (81.4%), and high yielding lipid headgroup- and tail group-related peaks \((m/z = 42, 43, 55, 57, 86, \text{ etc.})\) have substantial negative correlations to LV1, whereas cholesterol-related fragments \((m/z = 95, 109, \text{ and } 147)\) have less substantial positive correlations with LV1. LV2, on the other hand, captures the variance between carbonyl-containing fragments \((\text{C}_2\text{H}_2\text{O}^+, m/z = 42; \text{C}_2\text{H}_5\text{O}^+, m/z = 45; \text{C}_3\text{H}_8\text{O}^+, m/z = 58)\), which negatively correlate with LV2, and hydrocarbons not containing oxygen \((\text{C}_3\text{H}_7^+, m/z = 43; \text{C}_4\text{H}_7^+, m/z = 55, \text{C}_5\text{H}_{12}^N^+, m/z = 86, \text{ etc.})\), which positively correlates with LV2.

**Figure 5.2.** PLSR model from the TOF-SIMS analysis of DLPC/cholesterol supported lipid membranes. PLSR was performed on only the lipid-related peaks from \(m/z 30\) to \(200\) listed in Tables 4.1 and 4.2, and the previously reported cholesterol fragments \((m/z = 95, 109, 147, \text{ and } 161)\). The included peaks were normalized to the total counts in the data set and mean-centered. Three LVs were used for this model (A), and 96.1% of the variance was retained in the cholesterol concentration. The loadings plot (B) for LV1 and LV2 combined captured 93.4% of the variance within the spectra.
Table 5.2. Average predicted cholesterol concentration of each homogenous supported lipid membrane containing DLPC/cholesterol for the lipid- and cholesterol-related PLSR model (Figure 5.2).

<table>
<thead>
<tr>
<th>Enzymatically-measured mol% cholesterol</th>
<th>Predicted from Figure 5.2 (4 LVs)</th>
<th>σ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.2</td>
<td>4.1</td>
</tr>
<tr>
<td>5</td>
<td>3.7</td>
<td>3.3</td>
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<tr>
<td>12</td>
<td>11.8</td>
<td>4.4</td>
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<td>25</td>
<td>21.3</td>
<td>4.3</td>
</tr>
<tr>
<td>100</td>
<td>100.0</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Figure 5.2 shows the predicted cholesterol concentrations in the calibration samples from PLSR of only the lipid-related peaks from m/z 30 to 200 listed in Tables 4.1 and 4.2, and the previously reported cholesterol fragments (m/z= 95, 109, 147, and 161). Only three LVs were retained in this model, which is one less than that utilized in the PLSR model of the contaminant-free data set. Perhaps fewer LVs are needed for this model due to the reduced peak set. This PLSR model captured slightly less of the variance in cholesterol concentration (96.1%), as well as in the spectral variance (96.2%), than the contaminant-free model. The predicted cholesterol concentrations from this model are tabulated in Table 5.2. These predicted values were slightly less accurate than in the contaminant-free model. The membrane containing no cholesterol was predicted to have a greater cholesterol concentration than the 5 mol% cholesterol membrane. The other samples containing higher cholesterol concentrations did fit somewhat better than those containing less cholesterol, but the standard deviations of the predicted values were all greater than those of the contaminant-free model. Moreover, the PLSR model of this lipid- and cholesterol-related peak set and the enzymatic measurements agreed within 4.9
mol% (RMSEP), indicating that this model had a less satisfactory fit than the contaminant-free model.

Figure 5.2B shows the loadings plot for the lipid- and cholesterol-related PLSR model. Here, LV1 captures 82.9% of the spectral variance. Analogous to the contaminant-free model, high yielding lipid headgroup- and tail group-related peaks negatively correlate to LV1, and cholesterol-related fragments positively correlate with LV1. In addition, LV2 captures the variance between carbonyl-containing fragments, which in this model positively correlate with LV2, and other hydrocarbons not containing oxygen, which negatively correlate with LV2.

Conclusions

Quantifying the mol% cholesterol within supported lipid membranes using TOF-SIMS/PLSR is greatly influenced by the selection of mass peaks included in the data set. Better agreement between the mol% cholesterol measured with enzymatic assays and TOF-SIMS/PLSR was obtained from the contaminant-free peak set. However, use of only the identified lipid- and cholesterol-related peaks reduces the number of LVs needed to fit the data, consequently decreasing the complexity of the model. Because only four cholesterol-related peaks were included in the restricted data set, the performance of the PLSR model constructed using the lipid- and cholesterol-related peak set is expected to improve if a more complete cholesterol-related peak set were available.
These results demonstrate that the cholesterol concentration within lipid membranes can be quantified by creating PLSR calibration models using homogeneous lipid membranes that systematically vary in mol% cholesterol. By creating a separate PLSR model for cholesterol-lipid mixtures, in which the lipid component forms either an ordered or disordered phase, the cholesterol concentration in individual lipid domains within phase-separated lipid membranes might be determined. Additional studies on PLSR calibration models for lipid membranes may permit extending this calibration methodology to quantifying the mol% of specific lipid species within small regions of model membranes. Such capabilities would enable the construction of phase diagrams with tie lines that reveal the distribution of cholesterol within each lipid phase, and could provide a better understanding of how cholesterol-lipid interactions influence membrane organization.

**Materials and Methods**

**Materials**

The chrome-patterned, oxidized silicon substrates (9-nm-thick oxide layer) were prepared as previously described. The lipids 1-palmitoyl-2-{12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]lauroyl}-sn-glycero-3-phosphocholine (NBD-PC), DLPC, and cholesterol were purchased from Avanti Polar Lipids, Inc (Alabaster, AL). The cholesterol and phospholipid concentrations in the vesicle solutions were measured with the Amplex® Red Cholesterol Assay Kit and the Amplex® Red Phospholipase D
Assay Kit, respectively, from Molecular Probes (Eugene, OR). Millipore (18 mΩ) water was used in all experiments.

*Preparation of a pure cholesterol thin film*

A thin cholesterol film was created by spotting a small droplet of cholesterol dissolved in chloroform onto a 5 mm x 5 mm silicon substrate that was patterned with a chrome grid to facilitate sample positioning.37 The sample was subjected to vacuum for a minimum of 3 h to evaporate the solvent.

*Preparation of homogenous supported lipid membranes*

Homogenous supported lipid membranes were made as discussed elsewhere.35,37 Briefly, small unilamellar vesicles were created with a varying cholesterol molar concentration and DLPC. The actual cholesterol concentrations in the final vesicle solutions were measured with enzymatic assays (see section below).35 A small amount (1 mol%) of the fluorescent lipid, NBD-PC, was added to the mixture to allow for the evaluation of membrane formation and integrity using fluorescence microscopy. These components were dissolved in chloroform, dried under nitrogen, and placed under vacuum to remove residual chloroform. The lipid film was resuspended in room temperature water to a final lipid concentration of 0.5 mg/mL. The lipid solution was then vortexed until the entire lipid film was in solution. The solution was transferred to a plastic tube, and sonicated using a tip sonicator
(Branson Tip Sonifier Model 250, Branson Ultrasonics, Danbury CT) for 30 s intervals until the solution became transparent. The small unilamellar vesicle solution was filtered, and 2.5 mL of the filtered vesicle solution was added to a polystyrene culture dish containing multiple chrome-patterned, oxidized silicon substrates submerged in 2.5 mL water. The culture dish was covered, incubated at room temperature for 30 min to allow for bilayer formation, and then was transferred into a room temperature water bath to remove vesicles adhered onto the bilayer. To prepare the lipid membranes for the ultrahigh vacuum environment of the SIMS, samples were flash-frozen in liquid ethane and freeze-dried as previously described.\textsuperscript{36,37,50} To evaluate sample quality, fluorescence imaging was performed on a Leica DM6000 B upright fluorescence microscope equipped with a fluorescence filter cube (GFP, Leica) that matches the excitation and emission spectra for NBD-PC.

\textit{Measurement of mol$\%$ cholesterol}

The moles of cholesterol and phosphatidylcholine (PC) in each vesicle solution were measured using the Amplex® Red Cholesterol Assay Kit and the Amplex® Red Phospholipase D Assay Kit, respectively. The assays were performed in 96-well plates purchased from Costar® (Corning, NY), and the fluorescence intensity was read using a Synergy HT Multi-Mode Microplate Reader Model SIAFRT (Biotek® Instruments, Inc., Winooski, VT). Cholesterol and PC standards were used to create the calibration curves. Eight replicates were performed on each vesicle and standard
solution. The µmole of cholesterol and PC measured in each small unilamellar vesicle solution was used to calculate the mol% of cholesterol. The mol% cholesterol in the supported lipid membranes is assumed to be the same as the cholesterol content in the vesicle solution used for bilayer formation.

**TOF-SIMS analysis**

The freeze-dried homogenous supported lipid membranes were analyzed using a PHI Trift-III TOF-SIMS (Physical Electronics Incorporated, Chanhassen, MN). A $^{197}$Au$^+$ primary ion beam operated at 22 kV with an 8 nA current was used to analyze all membrane samples. TOF-SIMS images were acquired over 50 µm x 50 µm (256 pixels x 256 pixels) sample regions using unbunched mode, for optimal lateral resolution and to reduce acquisition time. Careful consideration was taken to ensure the chrome grid was never within the analysis region. Spectra were obtained with a mass range of 0.1 to 800 amu. TOF-SIMS spectra were acquired over enough regions to incorporate at least ten membrane locations of each cholesterol concentration used for modeling with PLSR analysis. Analysis times varied to keep a constant primary ion dose of 3.3 x $10^{13}$ ions/cm$^2$ for all cell samples.

**PLSR modeling of TOF-SIMS spectra**

PLSR was performed using the PLS Toolbox (v.6.0.1, Eigenvector Research, Manson, WA), which was run in MATLAB (v.7.8.0, MathWorks Inc., Natick, MA). Unit
mass binning was applied to each spectrum acquired from each homogenous supported lipid membrane sample. Each mass spectrum of the lipid/cholesterol samples was normalized to the total intensity of the selected peaks and mean-centered. For each model, the number of LVs was chosen by using continuous block cross validation to create a PLSR model, and then including the LVs that improved the root mean square error of cross validation (RMSECV) by ≥2% (Figure 5.3).

Figure 5.3. RMSECVs plots for the contaminant-free peak set (A) and the lipid- and cholesterol-related peak set (B) PLSR models.
Calculation of RMSEP

The RMSEP was calculated as previously described,\(^\text{46}\) using the following equation 1:

\[
RMSEP = \sqrt{\frac{1}{\eta} \sum_{i=1}^{\eta} (y_i - \hat{y}_i)^2}
\]

where \(y_i\) and \(\hat{y}_i\) are the reference and predicted value for the \(i^{\text{th}}\) test sample, respectively, of the \(n\) test samples.

References


(25) Megli, F. M.; Russo, L. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **2008**, *1778*, 143-152.


Chapter 6.
EVALUATING CELLULAR MEMBRANE COMPOSITION WITH TOF-SIMS AND MULTIVARIATE ANALYSIS

Notes and Acknowledgements

This chapter describes work in progress. The work was carried out collaboratively with the assistance of sample and cell preparation from Jessica F. Frisz, Emily Gonnerman, and John Schmidt, and with financial and intellectual assistance from Brendan A. Harley, Hyun Joon Kong, and Mary L. Kraft. The material is based upon work funded by a Career Award at the Scientific Interface from the Burroughs Wellcome Fund held by Mary L. Kraft. Portions of this work were carried out in the Frederick Seitz Materials Research Laboratory Central Facilities, Univ. of Illinois, which are partially supported by the U.S. Department of Energy under grants DE-FG02-07ER46453 and DE-FG02-07ER46471.

Introduction

The proteins, lipids, and glycans on the surface of the cell are often characteristic of cell type, (i.e., kidney cells and neurons), degree of maturation (i.e., stem cells versus differentiated cells), and disease state.\textsuperscript{1-7} Consequently, tools to evaluate the surface composition of individual cells may enable classifying cell type according to disease or development, and identifying the changes that occur during these processes. Such tools may be especially beneficial to the area of tissue
engineering. For example, the ability to detect cell type-specific differences in cell surface composition could provide insight into the cell-fate decisions made by stem cells, and how the extracellular environment influences this developmental process.\textsuperscript{8-13} This information could facilitate the design of the bioscaffolds used in tissue regeneration.\textsuperscript{14-16} In addition, a method to characterize the binding of certain vascular endothelial growth factors (VEGFs) to human endothelial cells (ECs) and the resulting changes in the cell surface composition\textsuperscript{17-19} can lead to delineation of the regenerative pathways for creating blood vessels, and potentially enable external promotion of blood vessel formation.\textsuperscript{18}

Conventional methods to analyze cell surface composition such as flow cytometry and immunoassays require the use of fluorescently labeled antibodies.\textsuperscript{20-24} The use of affinity labels to identify cell type, however, requires \textit{a priori} knowledge of which cell surface components are characteristic of cell type.\textsuperscript{25,26} Consequently, a label-free approach with the ability to simultaneously identify multiple components within the cell membrane is highly desirable.

Over the last decade, time-of-flight secondary ion mass spectrometry (TOF-SIMS) has been applied to analyzing the distinct amino acids, short peptide sequences, and lipids present within cellular membranes without the need for labels.\textsuperscript{6,27-32} TOF-SIMS is particularly well-suited for analyzing the membrane composition of single cells because secondary ion ejection, and consequently, detection, occurs predominately in the outer two monolayers at the sample surface,
minimizing any signal contributions of intracellular molecules. However, the primary ion beam often induces extensive fragmentation of the surface molecules, necessitating identification of many biological molecules according to secondary ions with $m/z < 200$. This fragmentation complicates identifying the membrane proteins, glycans, and/or lipids that are characteristic of a specific cell type, as their low mass ($m/z < 200$) molecular fragments often correspond to building blocks (i.e., amino acids, monosaccharides, and lipid headgroups) that are common to multiple biomolecules.

Multivariate analysis techniques (MVA) are well-suited for identifying sample-specific correlations in common mass peaks. Principal component analysis (PCA), an unsupervised pattern recognition technique, is perhaps the most popular of such post-sampling MVA methods. The capability of PCA to discriminate between samples of differing biomolecular composition is based upon the identification of characteristic linear combinations of mass peaks that are common to multiple samples. PCA has been used to differentiate the TOF-SIMS spectra from a variety of different biomolecules. In addition, PCA has been used to discriminate between different spores, cancer cell lines, and tissues according to variations in their chemical composition. However, supervised MVA techniques, which require a set of known samples that are employed for model development, may be better suited for classifying unknown specimens. Partial least squares discriminate analysis (PLS-DA) is one such supervised MVA method, which attempts to find factors that
maximize the covariance between the X variables (e.g. ion peaks in a TOF-SIMS spectrum) and the Y variables (e.g. cell types), and can be used to identity unknown samples.\textsuperscript{44} PLS-DA reduces the data complexity, analogous to PCA, by calculating a series of new variables called latent variables (LVs) that are linear combinations of the original variables. The LVs are used to calculate the maximum variation between the X and Y variables and simultaneously achieve correlation within the data set.\textsuperscript{43,44}

This chapter will describe the use of MVA methods for TOF-SIMS analysis of preserved cell samples to discriminate the cells according to the composition of their surface membrane. The first experiments described here were performed in collaboration with the Harley research group. PLS-DA was used to distinguish and classify two different cell types, HL-1 and MC3T3 cells. HL-1 cells are similar to cardiomyocytes,\textsuperscript{45} and serve as a model cellular system for proof-of-concept experiments with the long-term goal of determining how cardiac stem cells differentiate as a function of the ECM microstructure. The MC3T3 cells, which are mouse clonal osteogenic cells, were used in this proof-of-concept experiment because they are significantly different in morphology and cell membrane protein composition from HL-1 cells, and are expected to be differentiable by PLS-DA of the TOF-SIMS spectra. The second set of experiments discussed here were performed in conjunction with the Kong research group with the goal of determining whether label-free VEGF 165 bound to the surface of human ECs can be detected.
Results and Discussion

Differentiating cell types

TOF-SIMS was used to analyze HL-1 and MC3T3 cell samples cultured on silicon substrates in standard growth medium. The mass spectra of six individual cells per cell type were extracted from regions within the TOF-SIMS image. The mass spectra were unit-massed binned from \( m/z \) 30 to 200 amu and normalized to the total ion counts of the selected peaks within each spectrum, and mean centered. A PLS-DA model was created from the known classes of cells (either HL-1 or MC3T3), and used to predict the type of two test cells. In each model, the \( Y \) variable is set to either 1 or 0 to indicate whether or not the samples belong to a class (the class being the cell type for that model). Because unknown samples will not always be predicted to exactly 1 or 0, a threshold must be set, most commonly 0.5, to decide within confidence whether or not a sample belongs to a class.\(^{44}\)

The first PLS model was constructed using the normalized, mean centered ion peaks with \( m/z \) from 30 to 200, excluding the contaminants \( m/z \) 39 (K\(^+\)), 40 (Ca\(^+\)), 41 (hydrocarbon), and 73 (PDMS),\(^{46}\) and can be seen in Figure 6.1. Note that although peak \( m/z \) 147 was previously identified as the PDMS contaminant, it was retained in this data set because this peak is also cholesterol-related.\(^{47}\) The resulting model contained three LVs (based on RMSECV, see chapter 5) that captured 90.1\% of the spectral variance. In the HL-1 model (Figure 6.1A), the HL-1 test cell was correctly predicted to be a HL-1 cell (predictor = 0.71) whereas the MC3T3 test cell was not
predicted to be a HL-1 cell (predictor = 0.09). The MC3T3 model (Figure 6.1B) predicted the HL-1 test cell was not a MC3T3 cell (predictor = 0.29), and the MC3T3 test cell was correctly identified as a MC3T3 cell (predictor = 0.91).

![Figure 6.1](image.png)

**Figure 6.1.** PLS-DA classification of HL-1 and MC3T3 cell types from TOF-SIMS spectra of individual cells. PLS-DA was performed on all the normalized ion peaks with $m/z$ from 30 to 200 amu, excluding the potassium ($m/z = 39$), calcium ($m/z = 40$), hydrocarbon ($m/z = 41$), chrome ($m/z = 52$), and PDMS ($m/z = 73$) contaminant peaks. The included peaks were normalized to the total counts in the data set and mean-centered. Three latent variables (LVs) were used for this model, capturing 84.6% of the spectral variance. The test cells were projected onto the class predicting models for the HL-1 and MC3T3 cells, (A) and (B), respectively. The loadings plot for the first two LVs can be seen in (C).
Table 6.1. Positive-ion peaks of selected amino acids (taken from Ref 27 with permission).

<table>
<thead>
<tr>
<th>Mass</th>
<th>Species</th>
<th>Amino Acid</th>
<th>abbr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>CH₃N</td>
<td>glycine</td>
<td>Gly</td>
</tr>
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The loadings plot of the first two LVs used for PLS-DA (Figure 6.1C) suggests that classification is based on the relative abundance of lipids and amino acids between cell types. The mass peaks with high loadings on LV1 include $m/z = 42, 43, 55, 58, 86, 81, 95, \text{ and } 105$. Each of these mass peaks is related to fragments produced by both amino acids and lipids, and therefore may be indicative of variations in either the protein or lipid composition on the surface of the cell. For LV2, peaks with $m/z = 58, 86, 104, 166, \text{ and } 184$ have negative loadings, whereas peaks with $m/z = 43, 55, 57, 77, 81, \text{ and } 91$ have positive loadings. Although some of these peaks are found in the spectra of amino acids (Table 6.1), all of the peaks with high negative loadings on LV2 are related to lipid headgroups (Table 4.1), and all peaks with high positive loadings on LV2 are related to lipid tail fragments (Table 4.2). Therefore, discrimination on LV2 is more likely due to differences in the lengths of the fatty acid tails, and not from differences in the amino acids present on the surface of the cell.

However, the loadings on the LVs do not provide a clear indicator of the differences in membrane composition between these cell types, and how these differences are weighted in classification of each model. If one desired such information, then a MVA method like PCA would be more capable of establishing the dissimilarities between samples. Nevertheless, the PLS-DA approach could be used as a tool for bioengineering purposes, such as classifying how cardiac stem cells
differentiate and the influence of the extracellular environment on these cell-fate process.\textsuperscript{8-13} Moreover, the potential to do this \textit{in situ} could provide information about how the microstructure of the surrounding ECM affects such cell-fate decisions.

\textit{Identifying membrane modifications}

Next, TOF-SIMS/PCA was used in the attempt to detect the binding of VEGF 165 to the surface of human ECs and the resulting changes in the cell surface composition. TOF-SIMS analysis was performed on human ECs that were incubated for 10 min in the presence or VEGF 165. For comparison, human ECs that were not treated with the VEGF 165 were also analyzed. PCA was performed on the normalized, mean centered mass ion peaks with \textit{m/z} 30 to 200, excluding known contaminant ion peaks (Figure 6.2). Some separation between the VEGF-treated ECs and control ECs was achieved on PC1, but the 95\% confidence ellipses of both classes intersect in the score plot (Figure 6.2A). PC2 was ineffective at separating the VEGF-treated cells from the untreated cells. This relatively poor separation implies that either the 10 min VEGF treatment induced very little change in the chemical composition of the cell membrane, or that additional ion peaks resulting from the VEGF bound to the surface created insignificant spectral variance. In addition, the PC1 scores for the cells treated with VEGF had a relatively narrow distribution, whereas the PC1 scores for the control cells had a much larger range,
suggesting the issue may be due to experimental procedures, which is discussed in
the section below.

Even with the short duration of VEGF treatment, changes in cell membrane
composition will occur,\textsuperscript{48,49} therefore measureable differences may be attributed to
these changes, as well as the VEGF binding. Examination of the loadings plot for
PC1 (Figure 6.2B), which reveals the peaks responsible for the spectral variation
existing between the two cell sets, provides further insight into the compositional
differences between samples. Lipid headgroup-related ion peaks ($m/z = 58, 59, 68, 86, 104, 184$, etc., see Table 4.1) have positive PC1 loadings, and are therefore more
relatively abundant on the VEGF-treated ECs. The peaks that load negatively on
PC1, and are consequently more prevailing in the control ECs, are likely due to
amino acids ($\text{Tyr, } m/z = 55; \text{Lys, } m/z = 57; \text{Thr, } m/z = 69; \text{Asn/Arg/Leu/Pro, } m/z = 70; \text{Val, } m/z = 83; \text{Gly, } m/z = 85; \text{Arg, } m/z = 110$, see Table 6.1), though a few of these
peaks have also been associated with lipid tail groups (i.e., $m/z = 55, 57, 69, 83, 85$).
These loadings suggest that the control ECs have a higher protein to lipid ratio
on their cell surfaces, which is not intuitive, because the VEGF-treated cells should
presumably have a greater abundance of proteins on the cell surface due to the
presence of VEGF, which is another protein, unless the VEGF receptor and attached
VEGF is internalized. To further investigate whether this discrimination was based on
differences in the protein and lipid composition at the surface of the cell, or from the
binding of VEGF to the cell surface, PCA was used to identify the peaks related to
VEGF. The peaks that differentiated the TOF-SIMS spectra of a dried film of VEGF 165 on a fibronectin-modified substrate from a control fibronectin-modified substrate (Figure 6.3) were identified with PCA. However, PC1 and PC2 were not able to differentiate, within confidence the VEGF/fibronectin film from the pure fibronectin film (Figure 6.3A). This poor separation suggests that either VEGF and fibronectin are too similar in amino acid composition to differentiate their spectra, or there is not enough VEGF in the VEGF/fibronectin film to produce a detectable spectral difference. Nonetheless, the loadings plot (Figure 6.3B) shows that ion peaks related to hydrocarbon chains groups (C$_3$H$_7^+$, m/z= 43; C$_4$H$_7^+$, m/z= 55; C$_5$H$_7^+$, m/z= 67; C$_5$H$_9^+$, 

**Figure 6.2.** Scores and loadings plots from PCA of the TOF-SIMS positive-ion spectra of individual human endothelial cells (ECs) and vascular endothelial growth factor (VEGF) modified ECs. (A) The scores plot on PC1 and PC2 differentiates the cells, and (B) the loadings plot illustrates the positive ion mass peaks that contribute to the variation captured by each within PC1 and PC2. PCA was performed on all the normalized ion peaks with m/z from 30 to 200 amu, excluding the potassium (m/z = 39), calcium (m/z = 40), hydrocarbon (m/z = 41), and PDMS (m/z = 73) contaminant peaks. The included peaks were normalized to the total counts in the data set and mean-centered.
Figure 6.3. Scores and loadings plots from PCA of the TOF-SIMS positive-ion spectra of VEGF dried film and the fibronectin-coated substrate. (A) The scores plots on PC1 and PC2 differentiate the VEGF from the control substrate, and (B) the loadings plots illustrate the positive ion mass peaks that contribute to the variation captured within PC1 and PC2. PCA was performed on all the normalized ion peaks with $m/z$ from 30 to 200 amu, excluding the potassium ($m/z = 39$), calcium ($m/z = 40$), hydrocarbon ($m/z = 41$), and PDMS ($m/z = 73$) contaminant peaks. The included peaks were normalized to the total counts in the data set and mean-centered.

$m/z = 69; C_5H_{11}^+, m/z = 71; C_6H_9^+, m/z = 83; \text{and } C_6H_{11}^+, m/z = 85$) most positively correlate with the PC1, and thus the VEGF/fibronectin film, whereas hydrocarbon ion peaks relating to amino acids (Asn/Arg/Leu/Pro, $m/z = 70$; Gly/Val, $m/z = 72$; Glu/Lys, $m/z = 84$; Lue/Ili, $m/z = 84$; Phe, $m/z = 120$, see Table 6.1) most negatively correlate with PC1, and thus the pure fibronectin film. Comparison of the loadings plots in Figures 6.2B and 6.3B indicates there are no significant fragments that are shared between the loadings that correlate to the VEGF/fibronectin samples and the VEGF-treated cells. Therefore, separation between the control cells and VEGF-treated cells (Figure 6.2) may not be related to the presence of the VEGF, but instead to unidentified changes in cell membrane composition. Further optimization of this analysis would be required to definitively establish the basis of the PCA separation,
which could be addressed by troubleshooting the issues discussed in the next section. If achievable, then this could be a valuable tool for determining the influence of the VEGF on the proliferation of ECs, and would be useful in bioengineering the external promotion of blood vessel formation.

**Challenges of TOF-SIMS/MVA for cell analysis**

Extraction of mass spectra from the largest possible area of the cell’s surface within the TOF-SIMS image is desirable in order to ensure sufficient ion counts for the purpose of statistical analysis. Previously, cells have been identified within TOF-SIMS images by localized increases in ion peaks related to potassium ($m/z=39$) and phosphocholine ($m/z=184$) and a decrease in the sodium ion peak ($m/z=23$). However, identifying the cell body was a challenge in both of the studies described in this chapter because there was no appreciable contrast between cell and substrate in these ion images. Consequently, the spectra extracted from the regions of interest may be too small to have sufficient ion counts, or may include small areas of the substrate, resulting in the variations seen in the data presented above.

An example of a TOF-SIMS image of HL-1 cells seeded and preserved on a polylysine-coated substrate can be seen in Figure 6.4. The cells are somewhat discernable within the total ion image and the total counts of the phosphocholine-related peaks, $m/z=86$ and 184 (Figure 6.4A-C, respectively). However, this contrast is due to sample topography, and once the phosphocholine related peaks are
normalized to the total ion count no contrast is detectable within the individual ion images. As a result, it is difficult to verify by this method that the contrast from topography is due to the cell bodies. PCA was performed on these TOF-SIMS images in an attempt to separate the cells from the substrate (Figure 6.5). However, PCA can discriminate only between the normal areas and the areas of image shadowing, which arise when non-uniform current density of the primary ion beam is created from surface topography.\textsuperscript{53} Even by taking steps such as pixel downbinning or removing these areas from the image, and performing PCA on the remaining portions, discrimination between the cells and the substrates was not achievable (images not shown here).

Figure 6.4. TOF-SIMS ion images of HL-1 cells seeded and preserved on a polylysine coated Si substrate. The total ion image (A) and the total ion counts for $m/z=86$ and $184$ (B and C, respectively), which are both lipid headgroup related fragments. The normalized ion counts for $m/z=86$ and $184$ (E and F, respectively). The white light image (D) of the approximate region of the TOF-SIMS analysis.
Figure 6.5. PCA of the TOF-SIMS positive-ion image in Figure 6.5. (A) PCA was performed on all the normalized ion peaks with \( m/z \) from 30 to 200 amu, excluding the PDMS contaminant ion peaks \( (m/z=73 \text{ and } 147) \) and the non-specific contaminant peak, \( m/z=41 \). (B) PCA was performed on only the normalized lipid related ion peaks from 30 to 200 amu (see Tables 4.1 and 4.2), excluding the ion peaks of known contaminants \( (m/z=40, 41, \text{ and } 73) \). The included peaks were normalized to the total counts in the data set and autoscaled prior to PCA.

Figure 6.6. TOF-SIMS ion images of HL-1 cells cultured and preserved on a bare Si substrate. The total ion image (A) and the total ion counts for \( m/z=86 \) and 184 (B and C, respectively), which are both lipid headgroup related fragments. The normalized ion counts for \( m/z=86 \) and 184 (E and F, respectively). The white light image (D) of the approximate region of the TOF-SIMS analysis.
We hypothesized that the uniform phosphocholine ion counts observed on substrate was caused by the adherence of free lipids in the medium or material left on the substrate after cell migration to the ECM coating (polylysine in the HL-1 cells and fibronectin in the ECs experiments). To test this hypothesis, cells were cultured on bare silicon substrates that were not coated with polylysine prior to cell seeding.\textsuperscript{50} However, contrast was only visible in the total ion image and the total counts of the phosphocholine related peaks (Figure 6.6A-C, respectively), and not in the normalized individual ion images (Figure 6.6E and F). Investigations using a different cell preservation protocol,\textsuperscript{50} or methods of further extracting the topographical artifacts in the TOF-SIMS images\textsuperscript{54} may resolve this issue and improve the MVA analysis of these cell samples. However, due to the successful classification of the cell types, the issues with the ECs experiments may be due samples and not the analysis.

Conclusions

In this chapter, we successfully classified two cell types based on differences in cell membrane composition, HL-1 and MC3T3 cells, by capturing the variance within the TOF-SIMS spectra and correlating it to known cell samples using PLS-DA. This method has significant implications for determining how the ECM microstructure can influence cell-fate decisions of cardiac stem cells. For example, this can be used
as an *in situ* technique for determining how these discussions are affected by collagen pore sizes and microstructure.

We also were somewhat successful in differentiating untreated ECs from those treated with VEGF 165 for 10 min using PCA. However, the results were somewhat unexpected in that there was a relatively greater abundance of protein related fragments in the control ECs than in the VEGF-treated cells. Moreover, the determination of VEGF related fragments, by comparison to a control sample, was inconclusive, and it did not attain ion peaks loadings similar to that being differentiated in the PCA of VEGF-treated cells and control cells. Further analyses are required to definitely establish the differences between untreated and treated cells, the origin of these differences, and whether the binding of the VEGF can be detected.

**Materials and Methods**

*Preparation of HL-1 and MC3T3 cells*

Silicon chips were sonicated for 15 min in acetone and then autoclaved. After passaging, the cell pellet was resuspended in a predetermined amount of complete media to obtain a solution with the desired concentration of cells. Cells were seeded at constant volume (20 \( \mu \)L), with a seeding density of 5,000 cells/10\( \mu \)L onto polylysine coated/uncoated silicon wafers. The cells were allowed to attach for two hours after seeding, after which complete media was added to the culture dish (2 mL
media/well of 6 well plate). Cells were cultured for 24 hrs at 37 °C and 5% CO₂. HL-1 cells were cultured in Claycomb media supplemented with 10% FBS, 100 µg/mL penicillin/streptomycin, 2 mM L-glutamine, and 0.1 mM norepinephrine. MC3T3-E1 cells were cultured in Alpha-MEM supplemented with 10% FBS, 100 µg/mL penicillin/streptomycin, and 2 mM L-glutamine. Cell preservation was performed at room temperature. Silicon chips with adherent cells were removed from cell culture and rinsed with PBS (with Ca²⁺ and Mg²⁺) and Hendry’s Phosphate Buffer (HPB). Cells were subsequently fixed for 30 min in 4% glutaraldehyde diluted in HPB, then rinsed once for 5 min in HPB, and twice for 5 min in triple distilled water. The cells were further fixed in 0.4% osmium tetroxide solution for 15 min. The samples were rinsed three times for 5-10 min in water and air-dried.

*Preparation of VGEF-treated and untreated human ECs*

Silicon chips were sonicated for 15 min in acetone and then autoclaved. The sterile silicon chips were coated with recombinant human fibronectin at a concentration of 2 µg/cm² for 1 hr at 37°C. The chips were washed three times with sterile phosphate buffered saline solution. Human umbilical vein endothelial cells were seeded on the surface at a concentration of 500 cells/mL. The cells were allowed to adhere for 24 hrs. Subsequently, the cells were exposed to 10 ng/ml of VEGF for 10 min. The cells were fixed in a 10% formalin solution for 1 hr. The fixed
cells were washed with DI water three times over a 24 hr period and then allowed to dry overnight at room temperature.

**TOF-SIMS analysis**

The preserved cell samples were analyzed using a PHI Trift-III TOF-SIMS (Physical Electronics Incorporated, Chanhassen, MN). A $^{197}$Au$^+$ primary ion beam operated at 22 kV with an 8 nA current was used to analyze all cell samples. TOF-SIMS images of 256 pixels x 256 pixels were acquired over 100 µm × 100 µm sample regions using unbunched mode, for optimal lateral resolution and to reduce acquisition time. Spectra were obtained with a mass range of 0.1 to 1000 amu. TOF-SIMS spectra were acquired from enough regions to incorporate at least six cells of each type into all MVA analysis. Analysis times varied to keep a constant primary ion dose of $3.0 \times 10^{13}$ ions/cm$^2$ for all cell samples.

**MVA of TOF-SIMS spectra of preserved cellular samples**

The data files generated from the WinCadence software that operates the TOF-SIMS instrument were imported directly into MATLAB (v.7.8.0, MathWorks Inc., Natick, MA), and were unit mass binned. Using the MIA Toolbox (v.2.0.1, Eigenvector Research, Manson, WA) regions of interests with known cell bodies within the TOF-SIMS image were identified, and the mass spectra of each cell was individually extracted. Once completed, the mass spectra were compiled into an Excel
spreadsheet, where each spectrum was normalized to the total ion count from $m/z=$ 30-200. This data was then imported into the PLS toolbox (v.6.0.1 Eigenvector Research, Manson, WA), where the ion peaks were removed as indicated in the Results and Discussion section. The TOF-SIMS data was arranged in a matrix such that the different samples formed the rows and the individual mass peaks formed the columns. Each mass spectrum of an individual cell was normalized to the total intensity of the selected peaks and mean-centered, and either PLS-DA or PCA was performed on the resulting matrix.

References


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Chapter 7.
CONCLUSIONS AND FUTURE PROSPECTS

Secondary ion mass spectrometry (SIMS) has been demonstrated as a valuable technique for analyzing model and native cellular membranes. By coupling high resolution SIMS, on a Cameca NanoSIMS 50, with atomic force microscopy, unprecedented information about the phase behavior and microstructure of ternary mixture supported lipid membranes containing cholesterol was obtained. This investigation determined that gel-phase domains remained present within the phase-separated membrane, containing two saturated phosphocholine lipid species, until a critical cholesterol concentration was reached, and the presence of liquid-ordered domains was not detected as previously postulated. Further studies of other ternary lipid mixtures using this protocol could offer significant insight into the biophysical properties of these model membranes.

The development and optimization of multivariate analysis (MVA) techniques with time-of-flight SIMS (TOF-SIMS) provided a label-free method for membrane analysis. By using principal component analysis (PCA), the TOF-SIMS positive-ion spectra of four structurally similar phosphocholine lipid species were discriminated by the variance in the high-yielding, low mass lipid fragments that were common to all four species. PCA was also performed on TOF-SIMS images of phase-separated supported lipid membranes containing these structurally similar phosphocholine lipids, providing better lateral characterization of these membranes than did the
individual ion images. The mass spectra of the domain regions were extracted from the membrane images, and projected on to a PCA model of pure lipid films for component identification. To further optimize this analysis, PCA was used to identify the major ion peaks associated with these lipids by comparing isotopically labeled and unlabeled lipids. Once the lipid-related ion peaks were identified and compiled, they were used to systematically optimize the PCA discrimination of these phosphocholine lipids. The ability to acquire quantitative information about the composition of homogenous supported membranes, containing one of these lipid species and cholesterol, was achieved by using partial least squares regression (PLSR) modeling. These models in essence serve as a calibration curve that can be used for determining the cholesterol or specific lipid content of unknown supported lipid membranes. This has significant implications as a label-free tool for quantifying how cholesterol is distributed within phase-separated supported lipid membranes.

The chemical composition of native cellular membranes was evaluated by coupling TOF-SIMS analysis with post-sampling MVA methods. Different cellular types were discriminated and classified based upon the composition of their respective cellular membranes using PLS discriminate analysis (PLS-DA). These results serve as a proof-of-concept, and this method has significant implications for such studies as determining how the extracellular matrix microstructure can influence cell-fate decisions of cardiac stem cells. PCA was used to determine cellular membrane differences between normal human endothelial cells and cells modified
with a vascular endothelial growth factor. However, additional investigations are required for determining the ion peaks indicative of the binding of the growth factor to the membrane receptors. Further development of this method potentially could be used to understand and control the regenerative cellular pathways for creating blood vessels. Overall, SIMS is a great tool for direct analysis of local composition within model and native cell membranes, and can provide valuable insight into these systems.
APPENDIX.
IMAGING MODEL MEMBRANES WITH NON-TRADITIONAL ANALYTICAL TECHNIQUES

Notes and Acknowledgements

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Secondary Electron Microscopy Imaging

The ability to determine phase-separation within supported lipid membranes rapidly, label-free, and with lateral resolution applicable to sensing domain sizes on the order of cellular lipid rafts (10s of nm) is highly desirable. Fluorescence microscopy can image membranes in real-time, but conventionally is diffraction limited (~λ/2).\textsuperscript{1-4} More exotic techniques, such as fluorescence resonance energy transfer, have the ability to achieve more desirable lateral resolutions,\textsuperscript{4,5} yet these methods still require the use of labeled molecules, as do all fluorescence methods. Atomic force microscopy (AFM), on the other hand, does not require labels and offers exceptional lateral of <2 nm for supported lipid membranes.\textsuperscript{6} However, due to the serial nature of this method, real-time information is limited.
Conversely, the ability to measure sample thickness of biologically relevant samples has been demonstrated by measuring contrast differences in secondary electron microscopy (SEM) images. In these measurements, variations in the secondary electron yield are dependent on layer thickness, electronic structure, and the surface work function. Abrupt changes in the secondary electron yields, produced by lipid domains of differing thicknesses, has been detected within phase-separated membranes containing a 1:1 molar ratio of 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC) and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) during SIMS analysis (unpublished observations).

Figure A.1. (A) AFM topography image of a DSPC:DLPC (1:1 molar ratio) phase-separated supported lipid membrane. The topography image illustrates the height difference between the taller gel-phase domains (lighter regions) and the lower fluid-phase domains (darker regions). (B) Secondary electron microscope (SEM) image of the same membrane location as the AFM topography image in (A). The darker membrane regions in the SEM image have a lower secondary electron yield than the brighter membrane locations, which match with the AFM image where the gel-phase domains are thicker than the fluid-phase domain regions. AFM analysis was performed in ambient air and temperature using an Asylum MFP-3D™ Stand Alone AFM. Measurements were taken with standard tapping 300 kHz AFM probes (Tap300Al-G, Budget Sensors, Bulgaria) in AC (tapping) mode, and the topography image was flattened to the first order. SEM analysis was performed using a Hitachi S4700 SEM with an acceleration voltage of 1 kV.
With this in mind, phase-separated membranes containing DLPC:DSPC (1:1 molar ratio) that were made by vesicle fusion followed by slow-cooling, prepared as described elsewhere,\textsuperscript{11,12} were analyzed using SEM. Preliminary results (Figure A.1) confirms that phase separation within DLPC:DSPC membranes produces a detectable contrast variation in the SEM images. The contrast is in good agreement with the AFM image of the same membrane region (Figure A.1A), in which the darker regions in the SEM image (indicative of lower secondary electron yield) match with the thicker, densely packed gel-phase domains, and the brighter membrane regions correlate with the less densely packed fluid-phase area. The detection of phase separation in supported lipid membranes by SEM has not been reported, and it might serve as a fast, label-free alternative to evaluating phase separation on the nanometer scale.

**Atomic Force Microscopy Phase-Contrast Imaging**

Typically, AFM is used to measure topographical height differences resulting from differences in lipid packing between membrane phases.\textsuperscript{6,11,14} These measurements are commonly made in contact mode, in which the cantilever tip scans the sample in close contact with the surface of the membrane, and the deflection of the cantilever, as a function of changing height, is measured. However, by imaging membranes in AC mode (commonly known as tapping mode), wherein the cantilever is oscillated at a set frequency or amplitude and scans the surface by
making intermitted contact, changes in cantilever oscillation can be measured (Fig. A.2).\textsuperscript{13,15,16} This mode of operation is referred to here as AFM phase-contrast (AFM-PC) imaging, and is capable of providing lateral information about sample composition, adhesion, and friction.\textsuperscript{15,16}

![Figure A.2. Scheme of the AFM phase-contrast (AFM-PC) imaging operation, where the cantilever oscillation depends on the topography and the composition of the sample. The phase-shift signal in AC-AFM mode only changes with variation in the dissipated energy on the sample surface. The image shows two different local regions on the flat substrate (brown): the blue region (B), which is made of a different material and protrudes from the substrate, and the yellow region (Y), which is flat and only changes in materials properties with respect to the substrate. The blue region appears in the height ($h$) signal (topography), whereas the yellow region does not appear in the height trace. However, both regions are clearly distinguished from the substrate by recording and plotting the phase signal ($\phi$). Figure adapted from Ref 13.]

Here, AFM-PC was used to image phase-separated supported lipid membranes that contained 1:1 DLPC:DSPC and either 0 or 5 mol\% cholesterol (fabrication as described earlier in the appendix). Phase-contrast differences were
observed between the fluid- and gel-phase domains as seen in Fig. A.3. More interestingly, in the membrane that contained 5 mol% cholesterol, a phase-contrast difference is observed at small membrane patches (100-300 nm) within the fluid-domains (presumably enriched with DLPC) as seen in Fig. A.4. These patches of phase-contrast difference in the fluid phase are most abundant around the domain interfaces. Membrane patches that exhibit an AFM-PC shift have been reproducibly detected in many different supported membranes of similar compositions. We speculate that these areas either vary in cholesterol content or have different lipid packing. Unfortunately, complementary information gained from SIMS analyses about the lateral composition of these membranes has not been obtainable.

![Figure A.3. AC-AFM image of a DSPC:DLPC (1:1 molar ratio) phase-separated supported lipid membrane. (A) Topography image that illustrates the height difference between the taller gel-phase domains (lighter regions) and the lower fluid-phase domains (darker regions). (B) Phase-contrast image of the same membrane location as the topography image in (A). Without knowing the state of the cantilevers interactions (which is determined by force-curve analyses), it is not possible to state whether positive phase-shift (yellow-tinted regions) are attractive or repulsive tip-surface interactions, and vice-versa for the negative phase-shifts (purple-tinted regions). AFM analysis was performed in ambient air and temperature using an Asylum MFP-3D™ Stand Alone AFM. Measurements were taken with standard tapping 300 kHz AFM probes (Tap300Al-G, Budget Sensors, Bulgaria) in AC (tapping) mode, and both the topography and phase-contrast images were flattened to the first order.](image-url)
Figure A.4. AC-AFM image of a DSPC:DLPC (1:1 molar ratio) phase-separated supported lipid membrane that contains 5 mol% cholesterol. (A) Topography image that illustrates the height difference between the taller gel-phase domains (lighter regions) and the lower fluid-phase domains (darker regions). (B) Phase-contrast image of the same membrane location as the topography image in (A). As compared to the membrane that does not contain cholesterol (Figure A.3), there are many patches of discernable phase-contrast within the fluid-phase domain region. AFM analysis was performed as described in Figure A.3.

References


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VITA

Christopher R. Anderton was born in Colorado Springs, CO on June 29, 1983. He attended Thomas B. Doherty High School in Colorado Springs and graduated in 2001. Christopher received a Bachelor of Science degree in Chemistry at the University of Colorado at Colorado Springs in 2005, graduating Cum Laude with Highest Distinction. During his undergraduate studies he participated in many research projects. From his second semester of freshman year until he graduated, he worked under the direction of Professor David J. Weiss. He also participated in two summer National Science Foundation Research Experiences for Undergraduates at Syracuse University, under Professor Donald C. Dittmer, and at the University of California, Riverside, under Professor Francisco Zaera. Upon completing his undergraduate degree, he enrolled in the doctoral program of the Department of Chemistry at the University of Illinois at Urbana-Champaign in the fall of 2005. Christopher first joined the group of Professor Paul Bohn that fall, but upon the departure of Professor Bohn in the spring of 2006 he switched into the research group of Professor Ralph Nuzzo, where he worked on the development and characterization of novel nanohole array plasmonic sensors. In the spring of 2008, Christopher chose to shift the focus of his research to developing analytical techniques for determining lipid dynamics within model membranes under the direction of Professor Mary L. Kraft. During both his undergraduate and graduate studies he participated in numerous extracurricular activities and was part of many
student groups, where he often assumed leadership roles. After completing his doctoral studies, Christopher will assume his new position under the direction of Dr. Anne L. Plant at the National Institute of Standards and Technology (NIST) in Gaithersburg, MD where he received a NIST National Research Council Postdoctoral Research Associateship.
Veni, vidi, vici