Influence of Molecular Environment on the Analysis of Phospholipids by Time-of-Flight Secondary Ion Mass Spectrometry

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Understanding the influence of molecular environment on phospholipids is important in time-of-flight secondary ion mass spectrometry (TOF-SIMS) studies of complex systems such as cellular membranes. Varying the molecular environment of model membrane Langmuir−Blodgett (LB) films is shown to affect the TOF-SIMS signal of the phospholipids in the films. The molecular environment of a LB film of dipalmitoylphosphatidylcholine (DPPC) is changed by varying the film density, varying the sample substrate, and the addition of cholesterol. An increase in film density results in a decrease in the headgroup fragment ion signal at a mass-to-charge ratio of 184 (phosphocholine). Varying the sample substrate increases the secondary ion yield of phosphocholine as does the addition of proton-donating molecules such as cholesterol to the DPPC LB film. Switching from a model system of DPPC and cholesterol to one of dipalmitoylphosphatidylethanolamine (DPPE) and cholesterol demonstrates the ability of cholesterol to also mask the phospholipid headgroup ion signal. TOF-SIMS studies of simplistic phospholipid LB model membrane systems demonstrate the potential use of these systems in TOF-SIMS analysis of cells.

Introduction

Time-of-flight secondary ion mass spectrometry (TOF-SIMS) has become an increasingly useful tool for interrogating biological systems.[1−3] However, interpretation of TOF-SIMS spectra of biological samples is still complicated. Cellular samples are usually freeze-fractured which results in many water cluster peaks,[2] and when a cell is fractured, different parts of the cell can be exposed at the surface depending on the fracture.[3] Furthermore, cells, such as red blood cells, contain many lipid components including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine, cholesterol, spingomyelin, and glycolipids.[4] A specific phospholipid class is identified by the type of headgroup of the class. For example, dipalmitoylphosphatidylcholine (DPPC) comes from the phosphatidylcholine class of lipids. Static SIMS analysis has shown that the most abundant fragment ion that arises from the phosphatidylcholine class of lipids is the phosphocholine headgroup (at a mass-to-charge ratio (m/z) of 184 ($(C_5H_{15}PO_4N)^+$)) regardless of the fatty acids attached to it.[5−7] Due to the existence of many different fatty acids, a large variety of molecular weights exist for phospholipids in cells. Therefore, identification of phospholipid molecular ions in cells by TOF-SIMS is difficult. As a result, TOF-SIMS identification of phosphatidylcholine-containing lipids in cellular samples has been accomplished by the presence of phosphocholine.[2,3,5,7−10] Given that headgroup fragment ions identify the type of phospholipids found in cells, it is important to understand what factors affect the TOF-SIMS detection of these ions.

The ionization mechanism of phosphocholine has been investigated through the use of many mass spectrometric techniques including fast atom bombardment mass spectrometry (FAB-MS),[11] TOF-SIMS of dueterated forms of DPPC,[12] postsource decay matrix-assisted laser desorption/ionization (MALDI) TOF-MS of PC species ($[PC + H]^+$ and $[PC + Na]^+$),[13] and by electrospray ionization/tandem quadropole mass spectrometry of the $[M + H]^+$ and $[M + Li]^+$ species.[14] According to the mechanism determined by FAB-MS, phosphocholine (m/z 184) forms via an intramolecular rearrangement from the molecular ion.[11] Roddy et al. have shown that the area surrounding the molecule is also very important in the formation of phosphocholine in TOF-SIMS studies of different deuterated isotopes of DPPC, DPPC in D_2O, and frozen-hydrated DPPC samples. These studies suggested an additional extramolecular protonation required for ionization resulting in a frozen water matrix enhancement.[12]

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Langmuir−Blodgett (LB) films of phospholipids model cellular systems nicely because the molecules in these films are ordered and interactions among the molecules are similar to that found in cells. Thus, studies of LB films have become increasingly important for understanding cellular membrane dynamics[15−18]—including the study of phospholipid−cholesterol domains.[19] The addition of other cellular molecules, such as cholesterol, to LB films of phospholipids improves the correlation between these model systems and cells. In a TOF-SIMS study of LB films of DPPC, Bourdos et al. concluded that the amount of signal of DPPC fragment ions observed in the spectra depend on the molecular area of the LB film.[15] However, the signal from phosphocholine is not specifically reported.

In this report, we investigate the influence of the molecular environment on TOF-SIMS analysis of phospholipid LB model membrane systems. The change in the molecular environment of a DPPC LB film is accomplished by varying the film density, varying the sample substrate, and the addition of cholesterol. The results show that an increase in DPPC LB film density decreases the amount of phosphocholine while the addition of proton-donating molecules such as cholesterol to the film increase the yield of this ion. However, in a LB film of DPPE (dipalmitoylphosphatidylethanolamine), the addition of cholesterol is shown to mask the phospholipid headgroup fragment ion region of the spectrum. It is of profound interest to determine what affects the TOF-SIMS detection of the PC and PE headgroup ions, since they are used for the identification of DPPC and DPPE phospholipids in cells, respectively. When studying cells, it is necessary to know if certain phospholipid headgroup ion peaks can be hidden or enhanced by other cellular constituents such as cholesterol. A TOF-SIMS study of fast-frozen, freeze-fractured red blood cells, for example, showed the absence of phospholipid headgroup ion peaks in spectra of multilayer densities of cells,[20] possibly due to masking by the molecular environment. TOF-SIMS studies of simplistic model membrane LB films are vital for interpretation of mass spectra of cellular systems. These model systems can be expanded upon to gradually include more lipids for a stronger comparison to cellular systems.

Experimental Section

Materials. DPPC (Avanti Polar Lipids, Inc., Alabaster, AL), DPPE (Avanti), cholesterol (Sigma, St. Louis, MO), *n*-hexadecanethiol (Sigma-Aldrich Co., St. Louis, MO), 16-mercaptohexadecanoic acid,[21] 2-propanol, hexane, methanol, and chloroform were used without further purification. Water used in the production of all LB films was purified by a Milli-Q Synthesis system (Millipore, Burlington, MA) with a final resistivity of 18.2 MΩ·cm and a total organic content of <5 ppb.

Substrate Preparation. Single crystal (100) silicon wafers (Silicon Quest International, Santa Clara, CA) were used as LB substrates and as gold deposition substrates for self-assembled monolayers (SAMs). All silicon substrates were piranha etched (3:1 H_2SO_4/H_2O_2) before further treatment to ensure a hydrophilic surface. (Extreme caution must be exercised when using piranha etch. An explosion-proof hood should be used.) For the formation of SAMs, silicon substrates were deposited with 100 Å chromium followed by 2000 Å Au as described by Fisher et al.[22] Solutions (1 mM) of *n*-hexadecanethiol and 16-mercaptohexadecanoic acid were used for self-assembly onto gold. All processes of self-assembly and LB film preparation were confirmed by measuring the thickness of the overlying layer with a single-wavelength (632.8 nm, 3 mm spot size, 70° incidence angle) null ellipsometer (Rudolph AutoEL-II, Fairfield, NJ). Three spots within areas of interest were chosen for reproducibility.

Langmuir−Blodgett Film Preparation. LB films were prepared using a Kibron μTrough S-LB (Helsinki, Finland). In each experiment the subphase consisted of 60 mL of Millipore-purified water at 24 °C. For monolayers involving DPPC, a 2 mg/mL solution of DPPC in 3:2 hexane/2-propanol was used. For monolayers involving DPPE, a 1 mg/mL solution in 9:1 chloroform/methanol was used. Cholesterol was dissolved in 3:2 hexane/2-propanol and applied to the subphase separately from the corresponding phospholipid in a 2:1 mole ratio of phospholipid to cholesterol. Monolayers were allowed to equilibrate for at least 20 min before compression to ensure complete solvent evaporation. Trough barriers were computer controlled to allow for uniform compression of the lipid layer as well as constant feedback when depositing monolayers. Isotherms were taken at a rate of 14 Å^2/(molecule/min), and the surface pressure was measured with a Wilhemy wire interfaced to a personal computer (Dell Inspiron, Round Rock, TX). For film density studies, films were deposited at the appropriate molecular area onto a SiO_2/Si substrate at constant pressure at a rate of 3 mm/min in the LE region (3.5 mN/m, 84 Å^2/molecule) and in the LC region (35 mN/m, 46 Å^2/molecule). Films pulled in the LC/LE region (6 mN/m, 60 Å^2/molecule) were deposited at constant area due to the high compressibility in this region. For substrate studies involving methyl-terminated SAMs, DPPC films were deposited (44 mN/m, 46 Å^2/molecule) horizontally to prepare a monolayer film using a technique reported by Meuse et al.[23] DPPC films were deposited onto acid-terminated SAMs and SiO_2/Si substrates at 35 mN/m (46 Å^2/molecule). DPPC/cholesterol films were deposited onto acid-terminated SAMs at approximately the same molecular area (40 Å^2/molecule) as the other DPPC films although this corresponds to a pressure of 8 mN/m. The DPPE film was deposited onto an acid-terminated SAM at 35 mN/mm (46 Å^2/molecule). The film involving DPPE and cholesterol was deposited at a surface pressure of 30 mN/m (40 Å^2/molecule).

Instrumentation. Mass spectrometry was performed with a TOF-SIMS (Kore Technology, U.K.) equipped with a 15 keV Ga^+ liquid metal ion gun (Ionoptika, Southampton, U.K.). The mass spectrometer has been described in detail by Braun et al.[24] Spectra were acquired at room temperature with an ion dose no greater than 10^{12} ions/cm^2. For SIMS imaging studies, a mass spectrum was acquired by counting single ions at each pixel (128 × 128 pixels) to generate a total ion image of the area being analyzed. Charge compensation was not required. Postionization data were acquired with a Ti−sapphire laser system (Clark-MXR, Inc., Dexter, MI).[25] The amplified beam is compressed to 150 fs with 3.5 mJ per pulse energy. The beam is coupled to the analysis chamber by a 25 cm focal length CaF_2 lens. Moving the lens mounted on an *x*, *y*, *z* manipulator outside the chamber controls the laser spot size and position over the sample. The resulting ionized neutrals were collected for TOF analysis with the same extraction conditions as for the SIMS studies. In contrast to the SIMS mode, however, the charge produced by the ion detector was directly digitized, thus allowing more than one ion of the same mass to be detected in a single pulse.

Results and Discussion

Effect of LB Film Density on Phosphocholine Peak Area. The molecular structure of DPPC is shown in Figure

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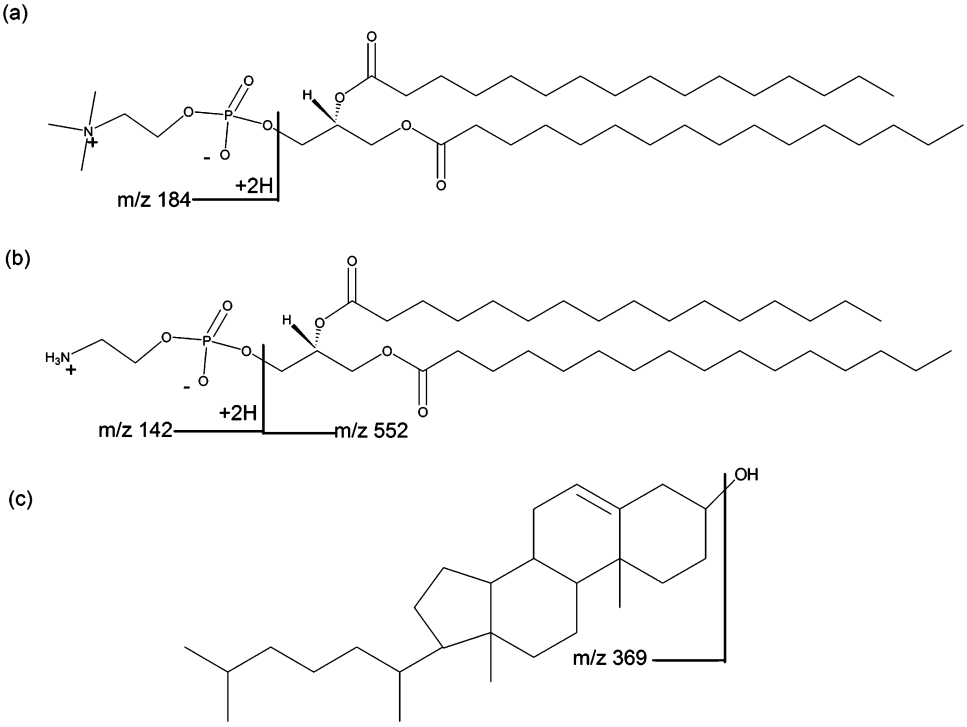

Figure 1. Structures and common fragments of (a) DPPC, (b) DPPE, and (c) cholesterol.

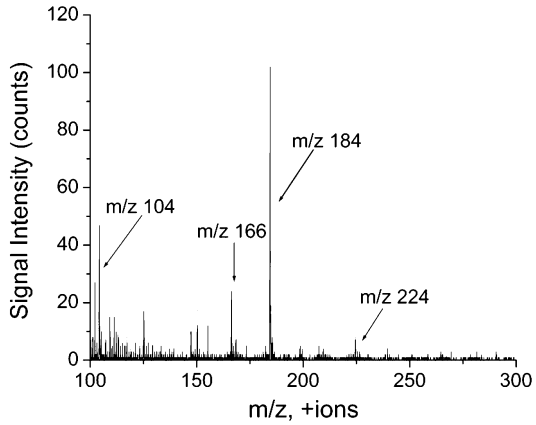

Figure 2. Mass spectrum of a DPPC film transferred at a surface pressure of 35 mN/m onto a SiO_2/Si substrate. The 15 keV Ga^+ ion dose is 10^{12} ions/cm^2 analyzed over an area of 200 μm × 200 μm.

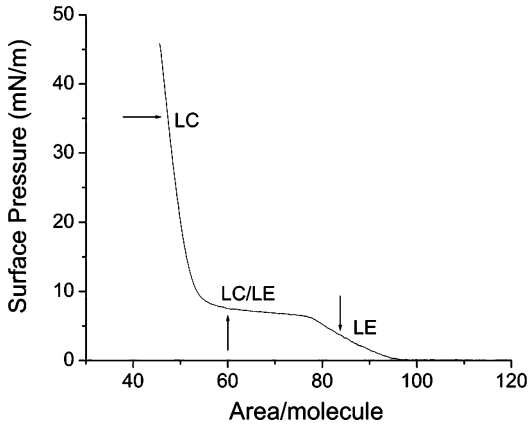

Figure 3. Plot of the surface pressure as a function of molecular area of DPPC at 24 °C. Films were transferred to a SiO_2/Si substrate in all three areas of the isotherm for film density studies as indicated by arrows.

1a along with its most abundant headgroup fragment ion, phosphocholine, at *m*/*z* 184 $((C_5H_{15}NPO_4)^+)$. The TOF-SIMS spectrum of an ordered DPPC LB film on a SiO_2/Si substrate is shown in Figure 2. Phosphocholine is clearly observed along with three other headgroup fragment ions at *m*/*z* 166 $((C_5H_{13}PO_3N)^+)$, *m*/*z* 104 $((C_5H_{14}NO)^+)$, and *m*/*z* 224 $(C_8H_{19}NPO_4)^+$). The headgroup ion peak at *m*/*z* 86 $((C_5H_{12}N)^+)$ and the protonated molecular ion at *m*/*z* 735 are not shown.

Monolayer films are applied with the LB technique to a SiO_2/Si substrate at varying molecular areas (Figure 3) as designated by arrows in the DPPC isotherm. An isotherm is a measure of the surface pressure in mN/m as a function of the molecular area. The molecules on the water surface interact as the area decreases (film density increases) and in the process form different phases. DPPC forms three phases termed LC, LC/LE, and LE.[26] The LC phase corresponds to a highly ordered liquid condensed phase and the LE phase corresponds to a highly disordered liquid expanded phase. The dependence of the ion peak area of phosphocholine on film density can be seen in Figure 4. Three different spots on each sample are analyzed, and the standard deviation is reported. The headgroup ion peak area is normalized to that of *m*/*z* 43 $(C_3H_7)^+$. Normalization to low mass hydrocarbons may be used for semiquantitative analysis of TOF-SIMS spectra.[27] The amount of phosphocholine is shown to decrease as the film density increases. Deviation from the mean is smallest in the LC phase indicating that the sample is most uniform in this ordered phase. The LC sample and the LE sample are statistically different at the 95% confidence level with a *t*-value of 3.12. The decrease in phosphocholine signal between the LE samples and LC samples may result from an increase in molecular interaction in the LC phase. Increased molecular interac-

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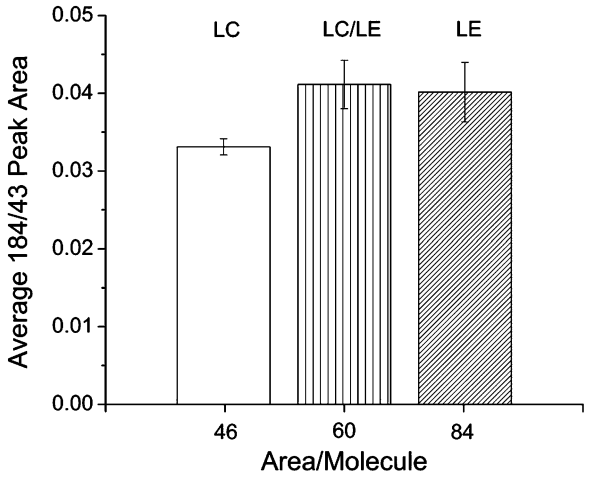

Figure 4. Average phosphocholine peak area (normalized to *m*/*z* 43) for varying densities of a DPPC LB film on SiO_2/Si. Spectra were acquired with 15 keV Ga^+ at an ion dose of 10^{12} ions/cm^2 analyzed over an area of 150 μm × 150 μm.

tion can make it more difficult for phosphocholine to be ejected from the LC sample.

Effect of Substrate and Cholesterol on Phosphocholine Yield. The molecular environment is also varied by changing the substrate to which the molecule is applied. The substrates include a SiO_2/Si substrate and two different SAMs on Au−*n*-hexadecanethiol (methyl-terminated SAM) and 16-mercaptohexadecanoic acid (acid-terminated SAM). SAMs were chosen as substrates for phospholipid monolayers to mimic a lipid bilayer in air using a method described by Meuse et al.[23] for the formation of hybrid bilayer membranes with methyl-terminated SAMs. As seen in Figure 5a−c, the yield (ion peak area/number of Ga^+ primary ions) of phosphocholine (*m*/*z* 184) increases as the substrate of the sample changes. The SAM samples (Figure 5b,c) yield more phosphocholine signal than the Si sample (Figure 5a) due to the Au substrate. Au is a better SIMS substrate than Si because heavier substrates have been shown to enhance ejection of particles.[28] Additionally, the use of SAMs as substrates for enhancing secondary ion emission in organic negative SIMS analysis has been reported by Van Stipdonk et al. with aminoethanethiol (AET) monolayers on Au substrates. In a sample of tetradecyl sulfate (TDS), the AET monolayer is shown to be important for the uptake of the TDS anion.[29] Notice that within the two SAM on Au samples (Figure 5b,c) the acid-terminated SAM sample produces 2-fold greater phosphocholine yield than the methyl-terminated SAM sample. The phosphocholine yield is increased even more (~3-fold) by the addition of cholesterol to the acid-terminated SAM sample as seen in Figure 5d. Besides aiding in the yield of phosphocholine, cholesterol may also be involved in yielding signal from the protonated DPPC ion at *m*/*z* 735 (Figure 5d).

Mechanism of Phosphocholine Formation. It is known from FAB-MS data that phosphocholine forms from the addition of two protons, one from the DPPC molecule itself (Figure 6a) and one from the molecular environment of the sample (Figure 6b).[11] Laser postionization and SIMS data of a DPPC film containing cholesterol on an acid-terminated SAM support the two sources of protons as seen with FAB-MS. Laser postionization (Figure 7a) results in a peak at *m*/*z* 183 ($(C_5H_{14}PO_4N)^+$). This fragment is desorbed as a neutral fragment at *m*/*z* 183 due to intramolecular proton rearrangement. In SIMS (Figure 7b), a peak is observed at *m*/*z* 184 for phosphocholine which

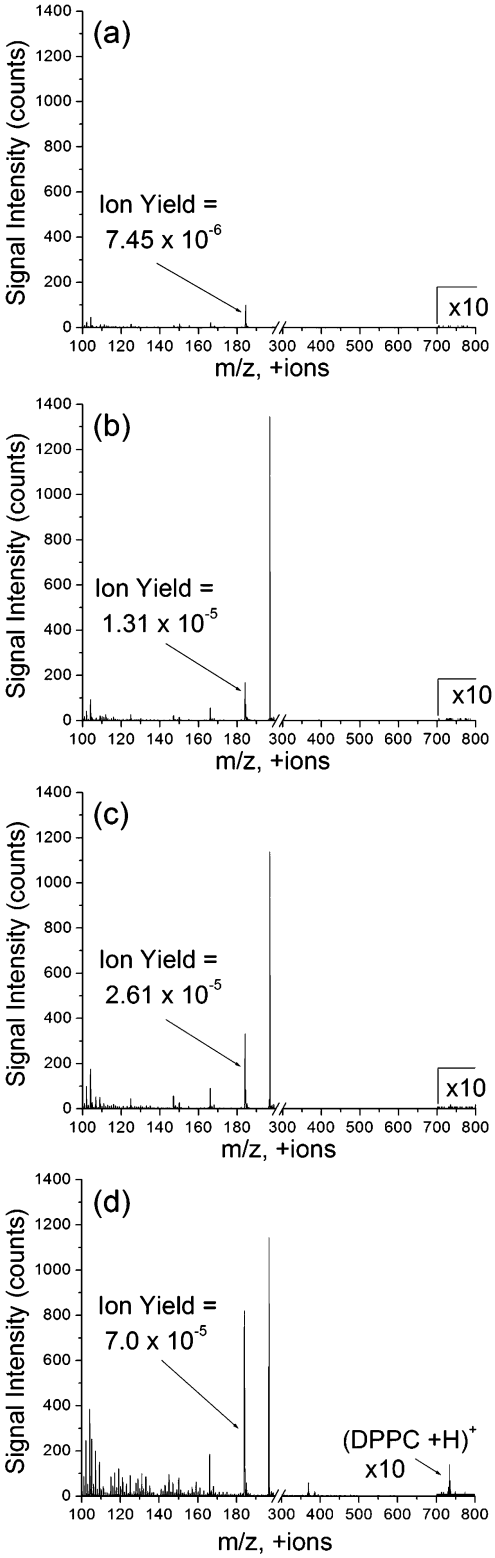

Figure 5. Positive ion spectra of four DPPC LB films (one including cholesterol) on three different substrates depicting the secondary ion yield of phosphocholine at *m*/*z* 184 and the appearance of the DPPC molecular ion at *m*/*z* 735. Samples include (a) DPPC on SiO_2/Si, (b) DPPC on methyl-terminated SAM on Au, (c) DPPC on acid-terminated SAM on Au, and (d) a mixture of cholesterol and DPPC on acid-terminated SAM on Au. Spectra were acquired with 15 keV Ga^+ at an ion dose of 10^{12} ions/cm^2 analyzed over an area of 200 μm × 200 μm.

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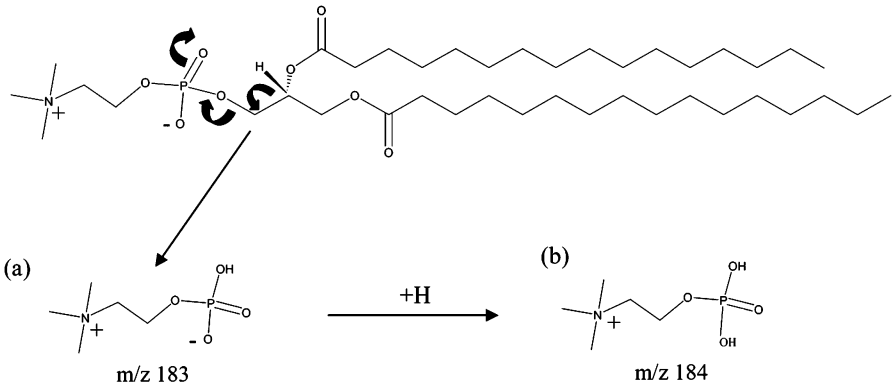

Figure 6. Mechanism for the formation of phosphocholine adapted from that which is reported in ref 11: (a) intramolecular rearrangement to form *m/z* 183 and (b) the addition of a proton from the molecular environment to form *m/z* 184.

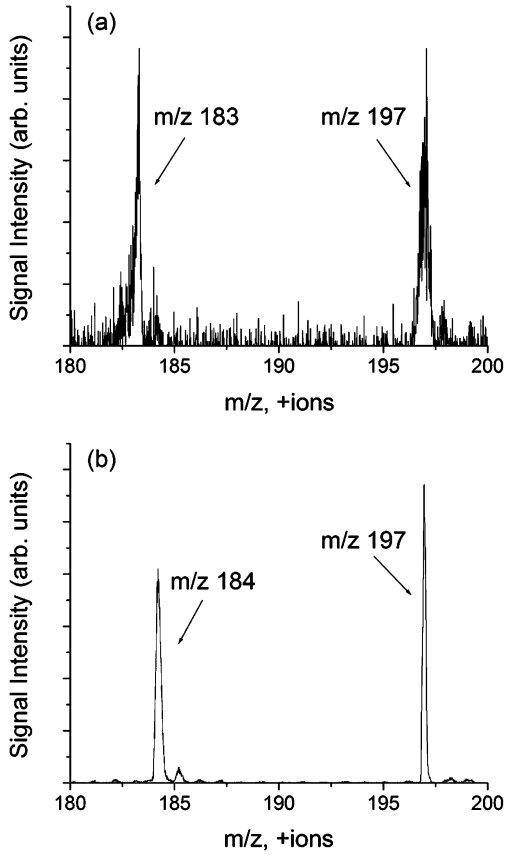

Figure 7. DPPC LB film with cholesterol on an acid-terminated SAM on Au: (a) laser postionization of the sample (800 nm, 150 fs laser pulses, 8.5×10^{12} W/cm^2 power density) and (b) positive SIMS spectrum of the sample. Absolute intensities are not comparable between spectra due to different detection modes as described in the text.

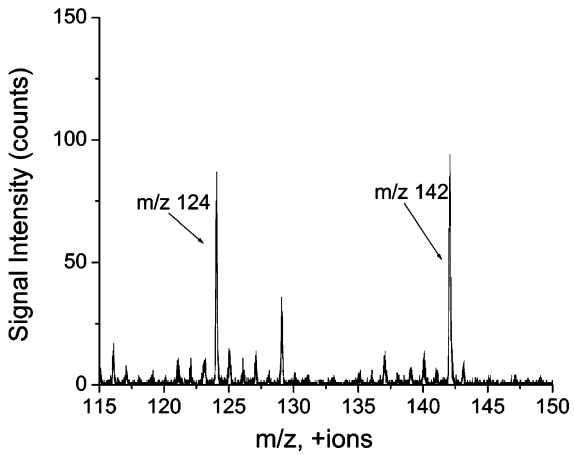

Figure 8. Positive SIMS spectrum of a DPPE LB film on an acid-terminated SAM on Au depicting the fingerprint region of the characteristic headgroup ion peaks at *m/z* 124 and *m/z* 142 of DPPE. Spectrum was acquired with 15 keV Ga^+ at an ion dose of 5×10^{10} ions/cm^2 at an area of 500 μm $\times$ 500 μm.

forms by the addition of a proton from the molecular environment to the neutral fragment. Note that this type of $(M + H)^+$ ion formation is typical for SIMS spectra with M representing an organic analyte molecule.

In this system several possibilities exist for the source of the transferred proton. For instance, the acid group of the SAM is located near the headgroup of DPPC therefore simulating the interactions between phospholipid headgroups in lipid bilayers. Furthermore, according to computer simulations, cholesterol in a DPPC bilayer spends equal amounts of time between the carbonyl and phosphate groups of DPPC.[30] The hydroxyl group of the cholesterol therefore forms a second possible proton donator. To distinguish in detail between these mechanisms, data on gas-phase proton affinities of the participating groups would be needed in addition to simulation results. From the SIMS spectra presented in Figure 5, it is apparent that both sources contribute to the observed signal of *m/z* 184.

LB films of lipids are valuable model membrane systems because ordered LB films of molecules found in cells can be reproducibly created under well-defined preset conditions. From the TOF-SIMS studies of these systems presented here, it is determined that phosphocholine in cells may also be formed by additional protons from adjacent lipid molecules and not just from a frozen water matrix as found by Roddy et al.[12] For the LB film studies described here, the hydroxyl group of the cholesterol and the acid group of the SAM, due to their close proximity to the phosphocholine headgroup, are shown to be a source for the additional protons needed to form phosphocholine.

Ability of Cholesterol To Mask the Phospholipid Signal. We have shown how the molecular environment can increase phospholipid signal in TOF-SIMS, and we will now describe how the environment can mask phospholipid signal. Investigation of SIMS analysis of a LB film of DPPE and cholesterol (structures and common fragment ions shown in parts b and c of Figure 11, respectively) demonstrates that the molecular environment can also mask the appearance of the signature phospholipid headgroup ion region of the phospholipid

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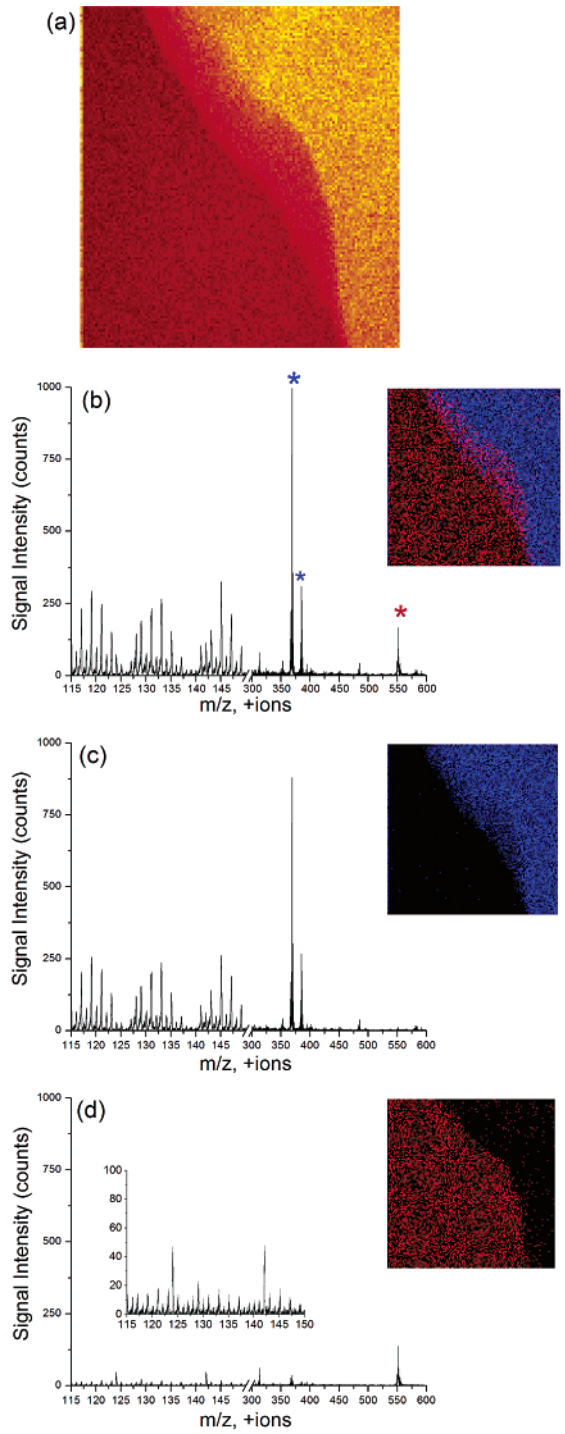

Figure 9. DPPE and cholesterol on an acid-terminated SAM on Au analyzed with 15 keV Ga^+ (5×10^{11} ions/cm^2; 284 μm $\times$ 284 μm field of view; 128 $\times$ 128 pixels). Mass spectra and images are as follows: (a) the total ion image; (b) the total ion spectrum (m/z 115−200 and m/z 300−600) is shown along with a molecule-specific image mapping cholesterol (m/z 369 $(M - OH)^+$ and m/z 385 $(M - H)^+$ in blue) and the DPPE tailgroup (m/z 552 $(C_{35}H_{67}O_4)^+$ in red); (c) the spectrum of the cholesterol side of the total ion image is shown along with the molecule-specific image of cholesterol (m/z 369 and m/z 385 in blue); (d) the spectrum of the DPPE side of the total ion image is shown along with the molecule specific image of the DPPE tailgroup (m/z 552 in red). Notice that the low mass region of (d) shows clearly the PE headgroup fingerprint region (two headgroup peaks at m/z 142 $((C_2H_9PO_4N)^+)$ and its dehydration product at m/z 124) whereas this fingerprint is masked in the total ion spectrum (b) by the low mass cholesterol fragment ions.

being studied. The headgroup fragment ion region of the mass spectrum of a LB film of DPPE on an acid-terminated SAM is shown in Figure 8. The two DPPE common PE headgroup fragment ions at m/z 142 $((C_2H_9PO_4N)^+)$ and its dehydration product at m/z 124 are clearly visible. This is the characteristic fingerprint of the PE headgroup fragment ions that can identify an unknown sample or a cell as containing PE.

The total ion image of a LB film of DPPE and cholesterol on an acid-terminated SAM is shown in Figure 9a along with the spectrum in Figure 9b. The fingerprint of the two common PE headgroup fragment ions (m/z 124 and m/z 142) is not as obviously represented as is the common phospholipid tailgroup fragment of DPPE at m/z 552 $((C_{35}H_{67}O_4)^+)$. In this simple one phospholipid system, m/z 552 is specific to DPPE, but in the analysis of a cellular system this tailgroup peak would not necessarily be specific to PE. Mapping the intensities of the tailgroup fragment ion (m/z 552 in red) and the cholesterol fragment ions (m/z 385 $(M - H)^+$ and m/z 369 $(M - OH)^+$ in blue) from the total ion spectrum results in a molecule-specific image depicting two distinct regions of the sample—one of DPPE and the other of cholesterol. These two regions result from the immiscibility of the two lipids when applied separately to the subphase. A mass spectrum obtained from the cholesterol side of the total ion image (Figure 9c) depicts strong cholesterol peaks at m/z 369 and m/z 385. The low mass range from m/z 115−150 is very crowded with peaks and resembles that of the total ion spectrum. If a mass spectrum is obtained from the DPPE side of the total ion image (Figure 9d), then it can be seen that the low mass region resembles that of the fingerprint of the PE headgroup fragment ions at m/z 124 and m/z 142 as shown in Figure 8.

Cholesterol has many low mass fragments, including small amounts of m/z 124 and m/z 142, therefore making the low mass region of the spectrum very crowded and in the process masking the fingerprint region of the PE headgroup fragment ions. This observation could only be made with the use of LB model membrane systems because they provide a unique way not only to observe the effects of the ionization of one molecule on another but also to observe them separately. If TOF-SIMS is used to investigate a cellular sample which contains both DPPE and cholesterol, then the headgroup fragment ion region of PE may not be obvious in the total ion spectrum. In the data observed in the TOF-SIMS analysis of red blood cells where multilayer densities of cells show mainly phospholipid tailgroup peaks,[20] the molecular environment of the cell may be masking the identity of the headgroup ion peak region.

Conclusion

TOF-SIMS analysis of phospholipid model membrane systems in the form of LB films provides valuable insight into the complexities surrounding TOF-SIMS investigations of cells. The molecular environment of the DPPC LB film has been shown to affect the yield of phosphocholine. Varying the film density has a lesser effect on the ion yield than changing the substrate or adding additional molecules to the film. The addition of proton-donating molecules such as cholesterol to the LB film aids in the ionization of the phosphocholine fragment. This increase in DPPC headgroup ion yield and the masking of DPPE headgroup ion fragments with the addition of cholesterol to the phospholipid film are two very important observations since cholesterol is found along with phospholipids in cells. These model membrane systems demonstrate the

influence of the molecular environment on the analysis of phopholipids and the importance of these studies in TOF-SIMS analysis of cellular systems.

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