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Proton Transfer in Time-of-Flight Secondary Ion Mass Spectrometry Studies of Frozen-Hydrated Dipalmitoylphosphatidylcholine

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A frozen water matrix, as found in freeze-fractured frozen-hydrated cellular samples, enhances the ionization of phosphatidylcholine lipids with static time-of-flight secondary ion mass spectrometry (TOF-SIMS). Isotopic profiles of the phosphocholine ion from deuterated forms of dipalmitoylphosphatidylcholine (DPPC) have been examined under various sample preparation conditions to show that ionization occurs through protonation from the matrix and is enhanced by the water present in freeze-fractured samples. The ionization of DPPC results in positively charged fragment ions, primarily phosphocholine, with a *m*/*z* of 184. Other ions include the M + H ion (*m*/*z* 735) and an ion representing the abstraction of the two palmitoyl fatty acid groups (*m*/*z* 224). Freeze-fracture techniques have been used to prepare frozen aqueous samples such as liposomes and cells to expose their membranes for static TOF-SIMS imaging. Due to the importance of surface water during SIMS analyses, sources of gas-phase water resulting from freeze-fracture were examined. Under proper fracturing conditions, water vapor, resulting from water in the sample and water condensed onto the outside of the sample, is released into the vacuum but does not condense back onto the surface. Combining the demonstrated enhancement of phosphatidylcholine lipid signal from water with the freeze-fracture preparation techniques described herein demonstrates potential advantages of studying biological samples in a frozen-hydrated state.

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

One of the most common positively charged phospholipid ions observed in static SIMS, phosphocholine ion, has been shown to form from phosphatidylcholine and sphingomyelin.[11−13] As a result, the phosphocholine fragment ion is important in studying these phospholipids in membranes. Since phosphatidylcholine has a large variety of tail groups in cell membranes, detection of molecular ions is difficult. On the other hand, detection of phosphocholine is relatively easy since it results from ionization of any lipid molecules containing the phosphatidylcholine head group. Although a mechanism for the ionization of phosphocholine in TOF-SIMS has not previously been described, a mechanism has been proposed for the formation of this ion in fast-atom bombardment mass spectrometry (FAB-MS).[14] This mechanism involves a cleavage of phosphocholine from the glycerol backbone and an intramolecular abstraction of a proton from the backbone resulting in a positive ion with a mass-to-charge ratio (*m*/*z*) of +184.

Several groups have used phosphocholine as a marker for phosphatidylcholine-containing lipids in TOF-SIMS images of biological samples.[1,2,10,11,13,15] Todd et al. have shown the phos-

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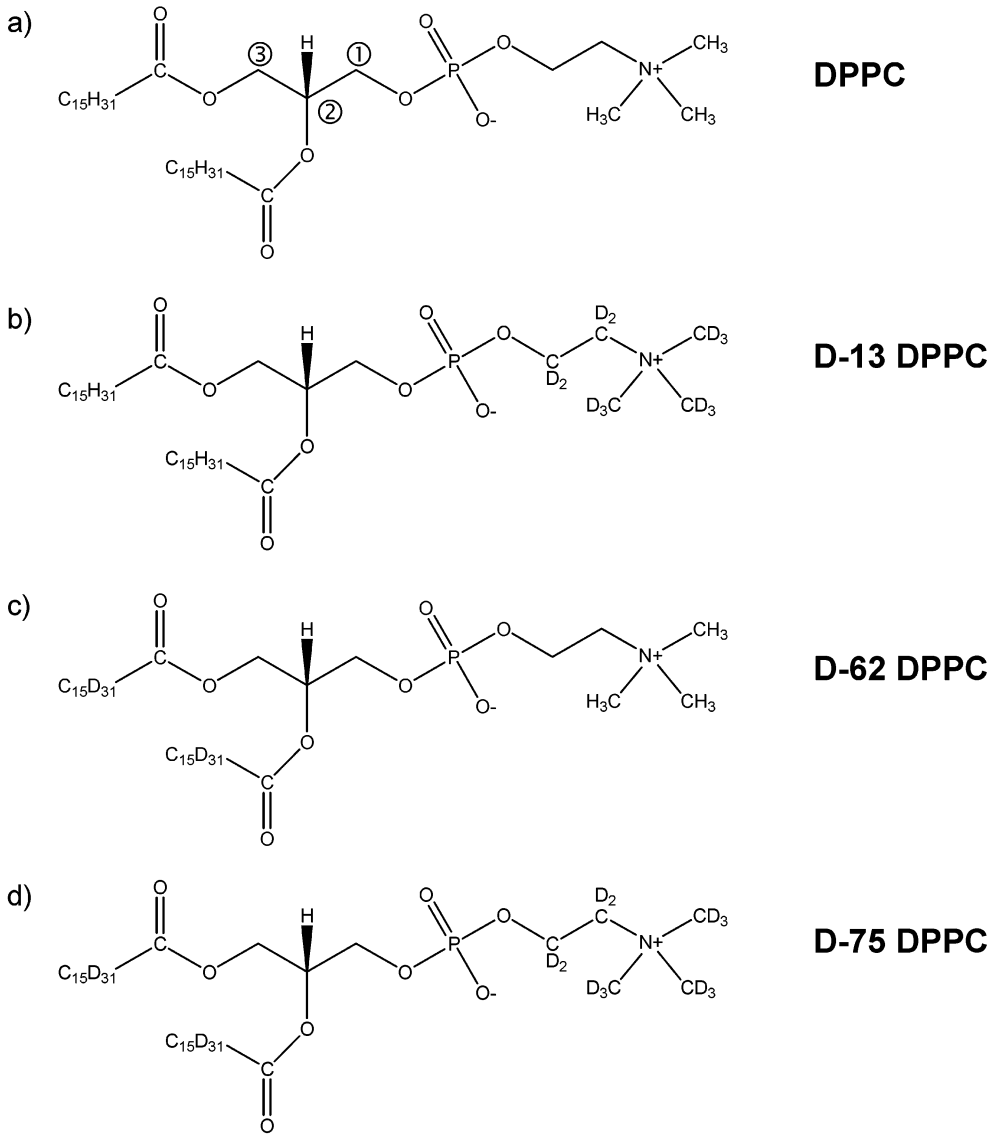

Figure 1. Structures of (a) dipalmitoylphosphatidylcholine (DPPC), (b) D-13 DPPC, (c) D-62 DPPC, and (d) D-75 DPPC. The glycerol carbon numbers are denoted in (a).

phocholine ion, resulting from the ionization of phosphatidylcholine lipids and sphingomyelin, as a predominant ion in freeze-dried brain slices.[11] The same ion has been imaged in freeze-dried red blood cells and varies in intensity between different samples depending upon the sample preparation.[1] Cannon et al. have imaged the location of dipalmitoylphosphatidylcholine (DPPC) in freeze-fractured frozen-hydrated liposomes using TOF-SIMS and have even imaged instances of lipid exchange between liposomes of unique lipid composition.[10] More recently, the phosphocholine ion was imaged across surfaces of single freeze-fractured rat pheochromocytoma cell membranes.[5]

The conditions of freeze-fracture are critically important in obtaining TOF-SIMS images from frozen samples.[10] Since static TOF-SIMS is a surface-specific technique, sublimation and condensation of water at the surface significantly alters the resultant images. Specifically, studies of phosphatidylcholine liposomes have shown that the temperature at which samples are fractured is important in balancing the flux of water at the surface.[10] When fractures occur at relatively high temperatures, water is rapidly sublimed, resulting in homogeneous images of solutes from the sublimed solution and other species that have diffused across the surface. On the other hand, when fractures occur at relatively low temperatures, water is condensed on the surface, resulting in homogeneous images of condensed water instead of the underlying object of interest. Even with the optimized fracture conditions, the flux of water released during the fracture and the resulting effects in the surface spectra have not been well understood for these analyses.

In this paper, we compare spectra from thin films and freeze-fractured samples to demonstrate substantial enhancement of DPPC signal in frozen water suspensions. Deuterated phosphatidylcholine standards and deuterated water matrixes are used to determine that protonation of phosphocholine in TOF-SIMS occurs largely from extramolecular proton sources, supporting the matrix-enhancing effect of a frozen water matrix. Using residual gas analysis during freeze-fracture of a deuterated sample, we show that both sample water and water condensed onto the outside surface of the sample are released during freeze-fracture. In addition, we demonstrate other sources of water that emerge during sample preparation and analysis and the effects this water

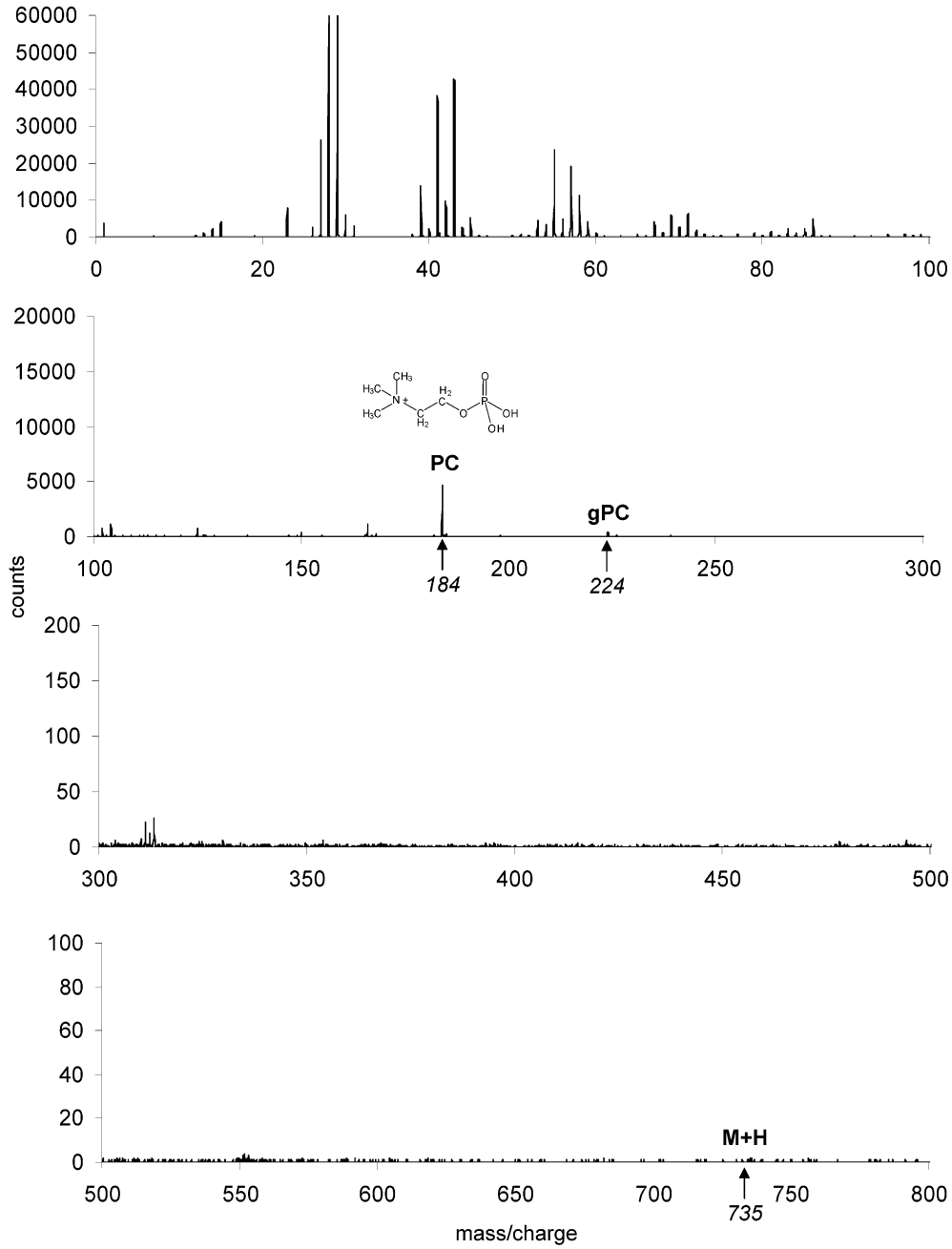

Figure 2. Spectrum of an anhydrous DPPC thin film using 1.0 × 10^{12} Ga+ ions/cm^2 with 25-kV beam voltage.

has on the resulting TOF-SIMS spectra. Most importantly, since freeze-fracture techniques provide the ability to analyze biological samples in a frozen-hydrated state, enhanced phosphatidylcholine lipid signal is available for improved TOF-SIMS images of cell membranes.

EXPERIMENTAL SECTION

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

Freeze-Fractured Sample Preparation. DPPC suspensions were prepared by sonicating and freezing in either H_2O (Millipore, Bedford, MA; MilliQ Synthesis purified) or D_2O (Sigma-Aldrich, St. Louis, MO; 42,345-9, >99.96% isotopic purity). H_2O samples were stored in liquid nitrogen prior to use, and D_2O samples were analyzed immediately after freezing to limit hydrogen exchange with the environment. The details of sample introduction and freeze-fracture in ultrahigh vacuum were previously described.[10] Briefly, the sample, sandwiched between two pieces of silicon, was held in a cold stage (Kurt J. Lesker, Pittsburgh, PA) at −196 °C and slowly warmed to −105 °C. Next, the top piece of silicon was removed with a cold knife, exposing a fresh surface of the aqueous sample for analysis. A residual gas analyzer (RGA) using electron impact ionization, quadrupole mass analysis, and electron multiplier detection (VacCheck100, MKS Industries, Branford, CT), was used to monitor vacuum gases during the sample preparation and fracture. Water (m/z 18) and D_2O (m/z 20) were monitored over time during the freeze-fracture experiments using

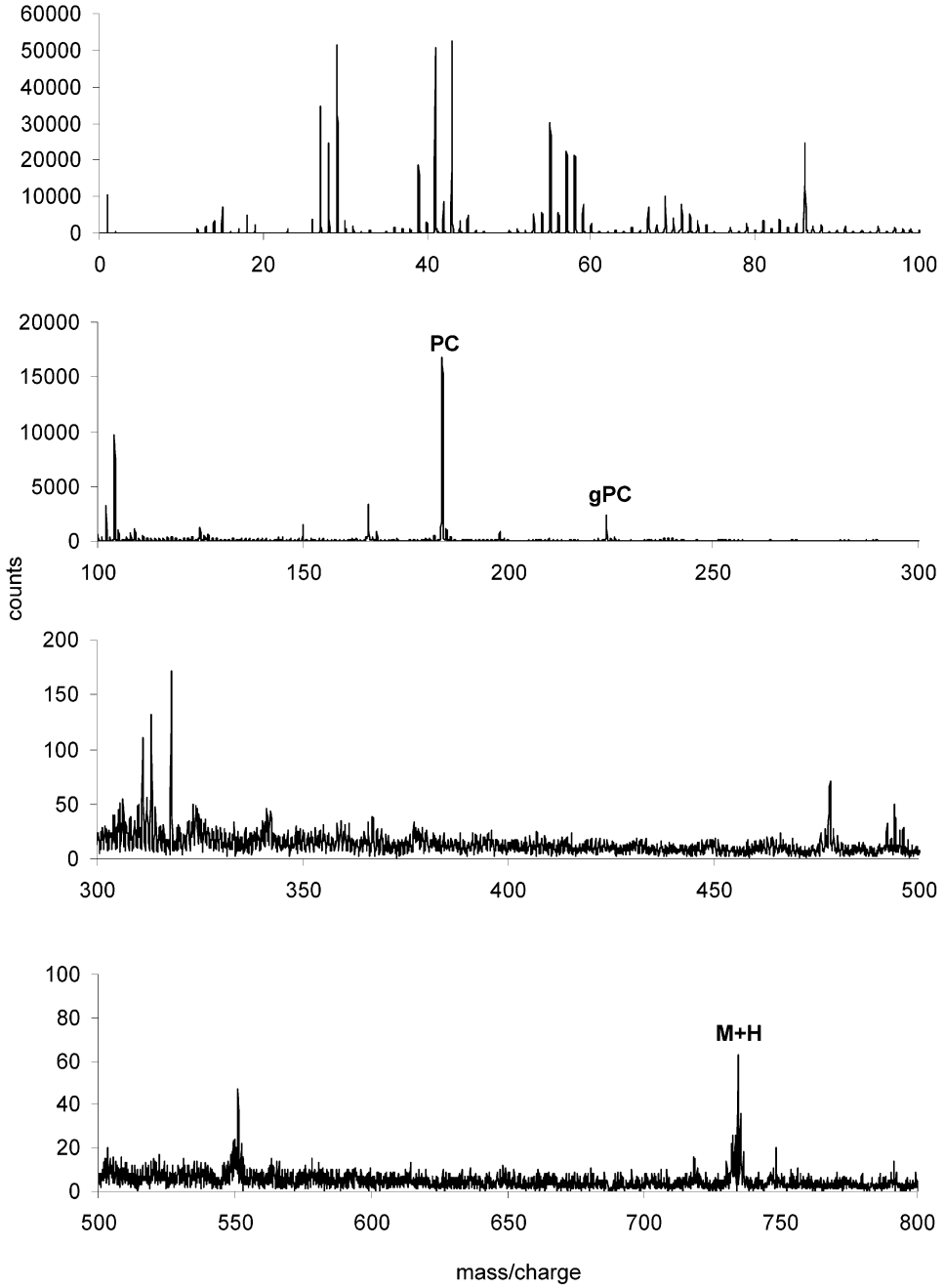

Figure 3. Spectrum of frozen-hydrated DPPC using 1.0×10^{12} Ga+ ions/cm^2 with 25-kV beam voltage.

Spectra software, included with the RGA. Following freeze-fracture, the sample was cooled to −196 °C and placed into the cold sample analysis stage.

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

RESULTS AND DISCUSSION

Water Matrix Enhancement of DPPC Ionization. Prior to SIMS imaging experiments, it is desirable to acquire standard spectra of the substances of interest to determine unique peaks and possible fragment structures that occur during ionization. DPPC has a molecular mass of 734 Da and a structure as shown

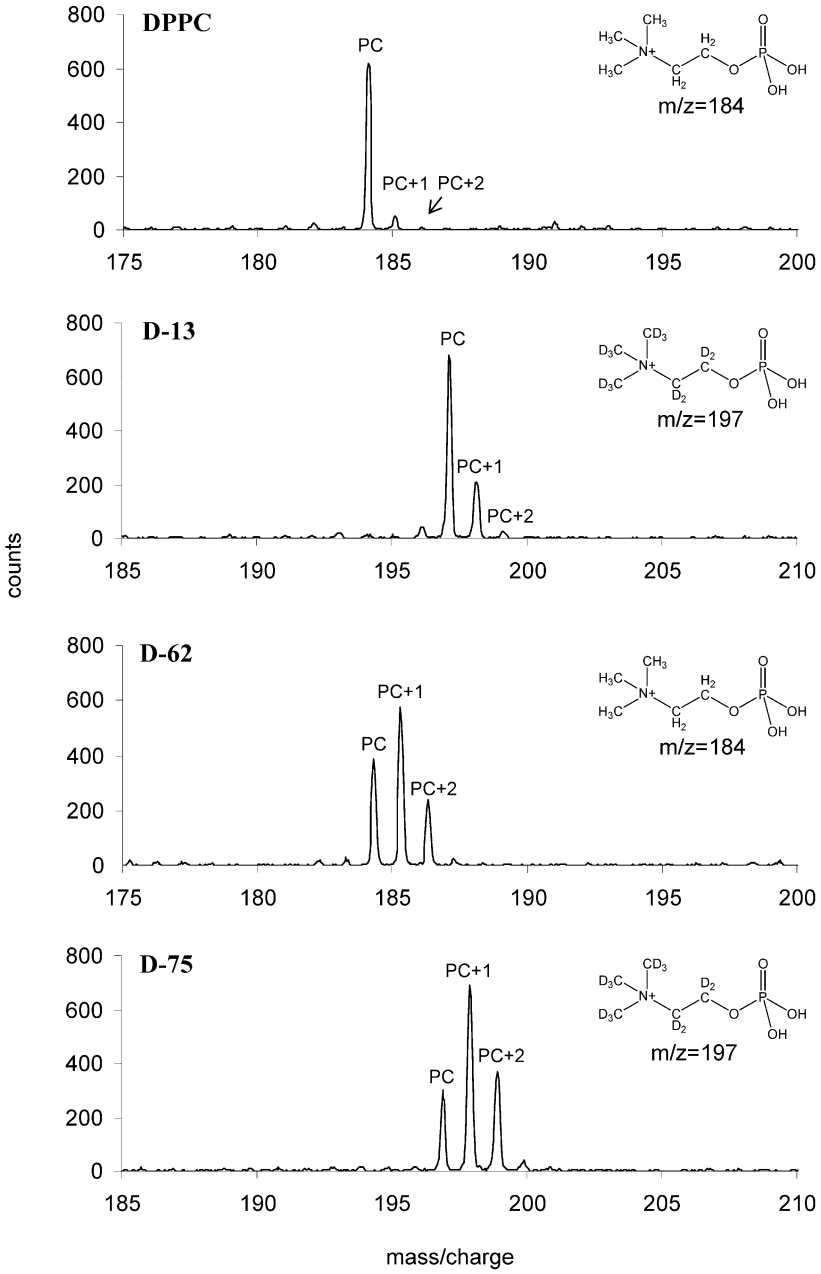

Figure 4. Spectra of four anhydrous thin films of stable isotopes of DPPC, each using 1 × 10^{12} Ga+ ions/cm^2, with 25-kV beam voltage. Structures of the head group ion are shown for each spectrum.

in Figure 1a. A typical positive ion mass spectrum of an anhydrous thin film of this lipid from 0 to 800 m/z is shown in Figure 2. As expected, phosphocholine ion signal is observed at m/z 184. The head group-containing fragment at m/z 224 represents DPPC after abstraction of the palmitoyl tail groups of the molecule. A head group fragment at m/z 166 and the choline ion at m/z 86 are also abundant. Hydrocarbon fragments of the molecule dominate the rest of the spectrum. Molecular or protonated DPPC ions are not observed in the thin-film experiment under these experimental conditions.

To more closely represent freeze-fractured cell samples, freeze-fractured samples of DPPC in H_2O have been analyzed using the same primary ion, beam energy, and dosage conditions used in the thin-film experiments. As shown in Figure 3, signal of higher mass ions is surprisingly high compared to that obtained from thin-film DPPC, even yielding signal from the protonated ion $[M + H]^+$ of the lipid (m/z 735). In addition to the DPPC and hydrocarbon signal, water clusters are also present in lesser amounts throughout the spectrum. The presence of the molecular ion signal likely arises from matrix effects similar to those observed with other techniques such as matrix-assisted laser desorption or matrix-enhanced SIMS.[16−18] The matrix may increase molecular ion intensity by providing a higher abundance of available protons for ionization or by absorbing excess energy. It is important to understand the mechanism that allows observa-

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tion of higher mass ions because increased signal and a larger mass range would improve the sensitivity and spatial resolution of images obtained from biological samples.

Sources of Protons for DPPC Ionization in Anhydrous Films. To investigate the source of protons for DPPC ionization, different isotopes of the molecule and the matrix were investigated. First, three stable isotopic forms of DPPC were analyzed in anhydrous thin films. The D-13 form of DPPC (Figure 1b) has the 13 hydrogen atoms of the head group substituted with deuterium. In Figure 1c, the D-62 form of DPPC has all of the hydrogens on the tail groups substituted with deuterium. Finally, in Figure 1d, the D-75 form of DPPC has all of the head group and tail group hydrogens substituted with deuterium. It is noteworthy that five hydrogen atoms are present in the glycerol backbone of all four structures.

It is interesting to compare the ionization mechanism associated with the solid film samples reported here to the FAB ionization mechanism where the target molecule is dissolved in liquid glycerol. As shown in Figure 1a, the hydrogen atom on the 2-carbon acts as a hydrogen source in the FAB ionization mechanism.[14] If the same mechanism occurs during ionization from the film, no other sources of hydrogen and deuterium should contribute to the phosphocholine peak. As a result, the intensity of the m/z 184 (PC) peak versus the isotope peaks at m/z 185 (PC + 1 amu) and m/z 186 (PC + 2) should be representative of the natural isotopic abundances of DPPC regardless of the surrounding matrix.

As shown by the spectra in Figure 4, there are clear deviations from this distribution. As expected, in the spectrum of DPPC (Figure 4a), the majority of signal is detected at 184 m/z, representing the PC m/z, and peaks present at m/z 185 and 186 are present in their natural isotopic abundance. In the D-13 DPPC spectrum, the m/z 184 peak profile is shifted 13 amu to m/z 197 due to the deuterium addition on the head group. In addition, there is a greater abundance of signal corresponding to PC + 1 m/z and PC + 2 m/z due to the presence of deuterium on the phosphate group instead of hydrogen. This addition of deuterium occurs either in sample preparation or during SIMS ionization. Due to the relatively low abundance of free deuterium ions in chloroform and in dried film samples under ultrahigh vacuum, these proton exchanges most likely occur between fragments of lipid in the gas phase during ionization.

Additional evidence of deuterium addition is shown in the D-62 and D-75 DPPC spectra. When the palmitoyl (C16) tail groups are completely saturated with deuterium, there is an even greater abundance of the PC + 1 and PC + 2 ions (m/z 185 and 186). Furthermore, saturation of the head group and tail groups with deuterium in D-75 DPPC leads to the greatest amount of deuterium addition, resulting in relatively little PC ion (m/z 197) and large amounts of PC + 1 and PC + 2 ions. Since the degree of isotope substitution directly affects the peak profiles, it is likely that protons from the substrate or adjacent lipid molecules contribute to ionization of DPPC in SIMS of dried thin films.

Sources of Protons for DPPC Ionization in Frozen-Hydrated Samples. Static TOF-SIMS analyses of cells are often performed in a frozen water matrix, which could act as a source of protons for ionization. To investigate these proton sources, spectra of frozen suspensions of DPPC have been collected using

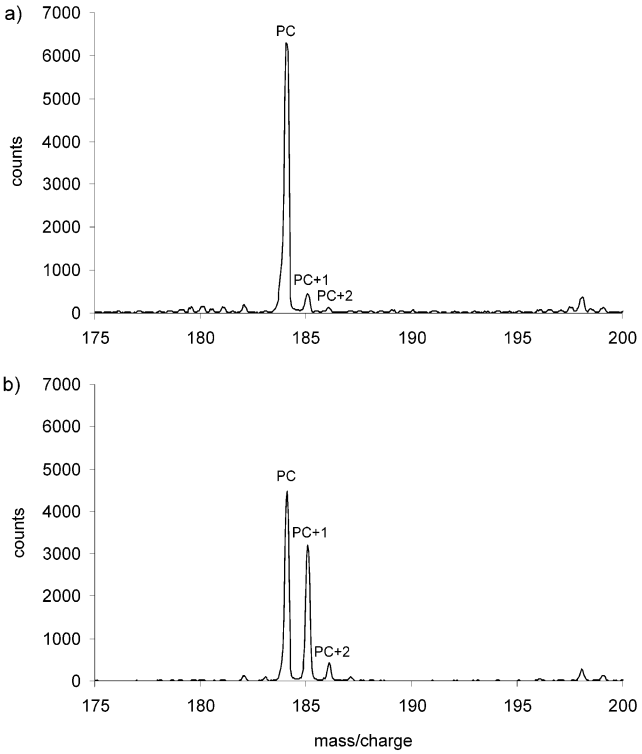

Figure 5. (a) Spectrum of freeze-fractured DPPC frozen in H_2O and (b) spectrum of freeze-fractured DPPC frozen in D_2O. (a) and (b) used 1 × 10^{11} In^+ ions/cm^2 with 15-kV beam voltage.

D_2O and H_2O as the matrixes. The important phosphocholine region of a spectrum of freeze-fractured DPPC in H_2O is shown in Figure 5a. The same spectral region of freeze-fractured DPPC in D_2O is shown in Figure 5b. The intense PC + 1 peak (m/z 185) and, more significantly, the PC + 2 (m/z 186) peak, which cannot form through simple aqueous proton exchange, are clear evidence supporting the transfer of deuterium from the aqueous matrix during phosphocholine formation. It is noteworthy that if an ionized molecule is completely surrounded by D_2O, the majority of the signal would be in the PC + 1 and PC + 2 peaks. Although there is a change in the peak profile that implies deuterium addition from the matrix, there is not a complete shift of the peaks. This is likely a result of the DPPC aggregating in the aqueous solution and partially acting as its own matrix, providing hydrogen from intramolecular sources and adjacent molecules.

Conversely, the spectrum of D-75 DPPC frozen in H_2O should contain peaks corresponding to the PC ion plus 13 m/z (m/z 197) to account for the 13 deuterium atoms in the head group ion. Unfortunately, isobaric interference of the $[H_2O]_{11}^+$ cluster ions occurs in the peaks of interest at m/z 197, 198, and 199, so another phosphocholine-containing peak is required for this comparison. In Figure 6, a fragment that contains phosphocholine and part of the glycerol backbone (gPC), was compared for (a) DPPC in H_2O, (b) DPPC in D_2O, and (c) D-75 DPPC in H_2O. As expected, DPPC in H_2O produces a profile of gPC (m/z 224), gPC + 1 (m/z 225), and gPC + 2 (m/z 226) resembling the natural isotope abundances of these peaks (Figure 6a). The spectrum of DPPC in D_2O (Figure 6b) contains more intense gPC + 1 and gPC + 2 peaks implying the addition of matrix deuterium to the fragment. The spectrum of D-75 DPPC in H_2O, obtained to investigate the addition of hydrogens to a deuterated head group ion, is shown in Figure

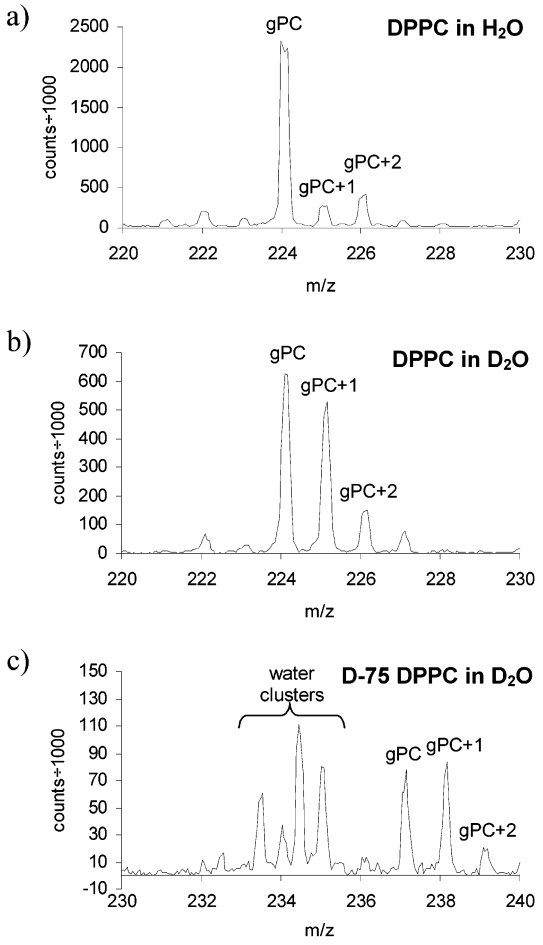

Figure 6. (a) Spectrum of freeze-fractured DPPC frozen in H_2O, (b) spectrum of freeze-fractured DPPC frozen in D_2O, and (c) spectrum of freeze-fractured D-75 DPPC in H_2O. 1×10^{11} In^+ ions/cm^2 with 15-kV beam voltage was used in each experiment.

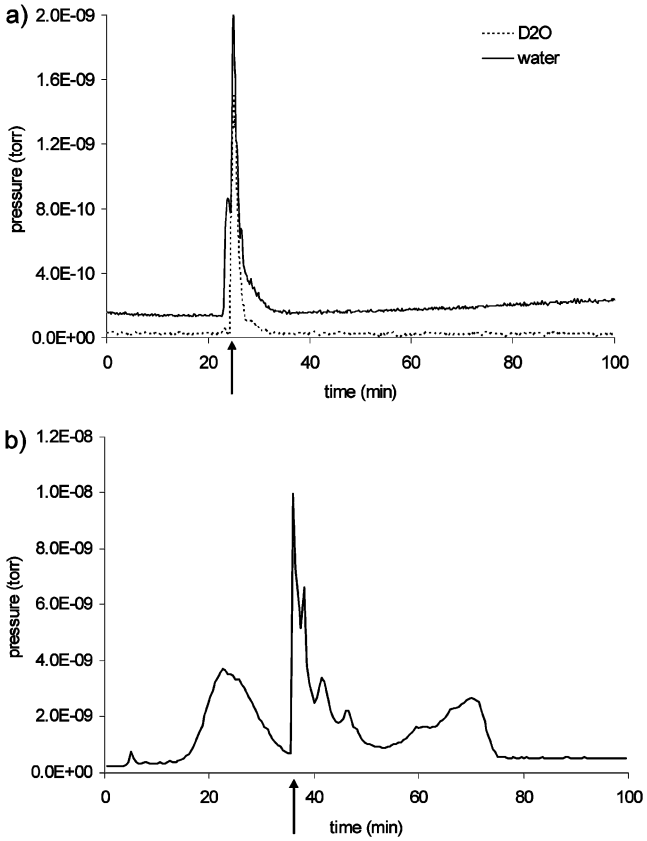

Figure 7. Residual gas analysis traces of the partial pressures of H_2O and D_2O in the sample preparation chamber during two freeze-fractures. The time of fracture is indicated by the arrow. (a) Successful freeze fracture at −106 °C with little effects of water on the sample; b) unsuccessful fracture at −103 °C demonstrating several sources of water as described in the text. For this case, the Si shard fell onto a warm part of the chamber and subsequently contributed to the background water pressure. At this point, the sample was much cooler and this adventitious source of water condensed on top of the sample.

6c. The spectrum contains a higher ratio of gPC to gPC + 1 than what was observed in anhydrous D-75 DPPC, but it is not entirely gPC. Again, this is evidence of H_2O matrix proton addition; however, gPC + 1 and gPC + 2 are also observed. As explained in the previous example, the D-75 DPPC aggregates in solution and is probably acting as its own matrix in addition to H_2O.

Monitoring Water Flux during Sample Freeze-Fracture. The observed matrix effect of water on the ionization of phosphatidylcholine suggests that environmental water originating from either the transfer procedure or from the residual gas in the vacuum system may condense on samples to provide protons for ionization. To reveal the extent of this effect, deuterated water has been employed to distinguish whether environmental water or water from the sample itself participates in the ionization process during TOF-SIMS of freeze-fractured DPPC samples. In the experiment, DPPC samples are suspended in D_2O and fractured prior to analysis in the sample preparation chamber. A residual gas analyzer in the preparation chamber is used to monitor water flux from the sample during the relatively violent process of freeze-fracture. Ideally, the preparation chamber is pumped to less than 10^{-8} Torr prior to freeze-fracture. After the sample is warmed to −105 °C, the silicon shard covering the frozen D_2O sample is removed with a cold knife resulting in a large pressure spike in the chamber. Residual gas analysis is used to track the partial pressures of H_2O and D_2O during a successful freeze-fracture of DPPC in a D_2O matrix (Figure 7a). Since the sample matrix is D_2O, the H_2O pressure spike must originate from water that condenses onto the sample while freezing and transporting to the instrument. The pressure of H_2O increases by a factor of 10 relative to baseline, whereas, the pressure of D_2O increases by a factor of 100. Hence, we conclude that most of the pressure burst consists of solvent molecules.

Within minutes, this pressure is reduced to baseline pressures by a cryogenically cooled shroud and a turbomolecular pump (described in detail by Cannon et al.[10]). During this time, water condenses or sublimes from the surface of the sample, depending upon the pressure and the temperature. For a successful fracture, the surface remains at equilibrium and does not significantly condense or sublime water, which would perturb the molecular structure of the surface. For unsuccessful fractures, pressures do not quickly return to baseline, as shown in Figure 7b, and poor TOF-SIMS spectra are obtained. For this particular example, the stage was held at a temperature above −105 °C during fracture and water from the sample sublimed after fracture. As the stage was cooled toward −190 °C, water condensed onto the sample and the TOF-SIMS spectra were characteristic of the water-covered surface. This example illustrates that water can be either

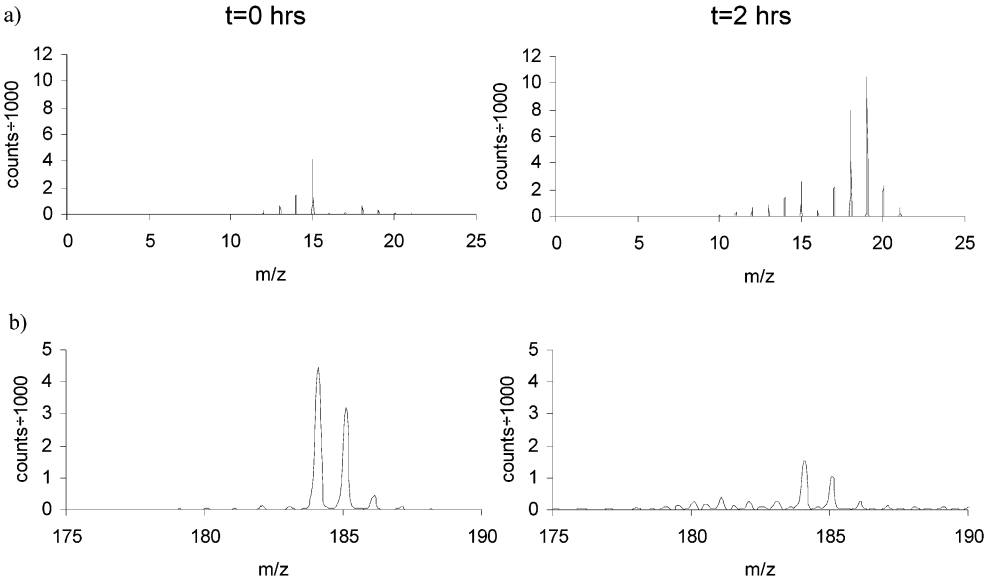

Figure 8. (a) Spectra of the water region of a freeze-fractured sample at the beginning of the experiment and after 2 h; (b) spectra of the phosphocholine head group region of a freeze-fractured sample at the beginning of the experiment and after 2 h. Spectra were acquired with 1×10^{11} In+ ions/cm^2 with 15-kV beam voltage.

helpful or detrimental to static SIMS experiments. By monitoring the pressure spike and its subsequent return to baseline behavior, it is possible to assess whether a fractured sample will be a good candidate for SIMS analysis.

After a successful freeze-fracture, the sample is kept at liquid nitrogen temperatures at approximately 5×10^{-9}–5×10^{-8} Torr throughout the subsequent TOF-SIMS analysis. Consequently, it is possible for water to condense onto the surface of the sample. To investigate the effects of possible condensation of endogenous water, spectra were collected from a freeze-fractured DPPC sample for over 2 h with an approximate 1×10^{-8} Torr working pressure. The water and phosphocholine regions of spectra are shown in Figure 8. The water signal increases over time, whereas the phosphocholine signal decreases over time, indicating that the surface is being covered by water during the analysis.

In summary, while water enhances ionization of DPPC in frozen aqueous samples, water that condenses onto the sample after freeze-fracture inhibits phosphocholine signal. Therefore, for optimal DPPC signal, it is necessary to monitor pressures in the instrument throughout the experiment and to monitor the signal of water in spectra during TOF-SIMS analysis.

SUMMARY

Freeze-fracture techniques have been used to prepare aqueous samples for TOF-SIMS analyses. More high-mass ion signal is obtained from frozen-hydrated DPPC than the corresponding dried thin-film samples. This matrix-enhancing effect is possibly due to the abundant proton sources in the matrix of frozen aqueous samples. TOF-SIMS analyses of different isotopes of DPPC and DPPC in D_2O have shown that protons leading to the formation of the phosphocholine ion are obtained from the region surrounding the molecule in addition to the intramolecular sources previously shown in FAB-MS. The monitoring of H_2O and D_2O with a residual gas analyzer has allowed the optimization of conditions for freeze-fracture TOF-SIMS experiments. Also, spectral analysis has demonstrated that the condensation of water onto freeze-fractured samples during TOF-SIMS analysis affects the ionization of DPPC, so monitoring the water signal is crucial during analysis. Overall, freeze-fracture preparation techniques have made it possible to analyze biological samples in their native frozen-hydrated state and to enhance the signal of DPPC for improved TOF-SIMS imaging experiments.

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