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Relative ion yields in mammalian cell components using C_{60} SIMS

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Time of flight secondary ion mass spectrometry has been used to better understand the influence of molecular environment on the relative ion yields of membrane lipid molecules found in high abundance in a model mammalian cell line, RAW264.7. Control lipid mixtures were prepared to simulate lipid–lipid interactions in the inner and outer leaflet of cell membranes. Compared with its pure film, the molecular ion yields of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine are suppressed when mixed with 2-dipalmitoyl-*sn*-glycero-3-phosphocholine. In the mixture, proton competition between 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, and 2-dipalmitoyl-*sn*-glycero-3-phosphocholine led to lower ionization efficiency. The possible mechanism for ion suppression was also investigated with ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy. The formation of a hydroxyl bond in lipid mixtures confirms the mechanism involving proton exchange with the surrounding environment. Similar effects were observed for lipid mixtures mimicking the composition of the inner leaflet of cell membranes. The secondary molecular ion yield of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine was observed to be enhanced in the presence of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine. Copyright © 2012 John Wiley & Sons, Ltd.

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Introduction

Lipids comprise major structural and metabolic components of cells and have essential functions in the formation of cell membranes. Biological membranes contain hundreds of chemically distinct lipid components that form the actual bilayer membrane. Phospholipids constitute a major class of lipids present in cellular membranes and are asymmetrically distributed across the membrane bilayer. Phosphatidylcholine is in higher abundance in the outer leaflet, whereas aminophospholipids such as phosphatidylserine and phosphatidylethanolamine are found in high concentration on the inner leaflet.[1]

Lipids play an important role in human disease. The main goal of the Lipid Metabolites and Pathways Strategy consortium is to improve the understanding of lipid metabolism and the active role that lipids play in human disease.[2] The mouse macrophage is a useful model system for examining the role of lipids in several processes. The RAW264.7 is a mouse macrophage-like cell line derived from tumors induced in male BALB/c mice by the Abelson murine leukemia virus. In a recent lipidomic study, it has been shown that the inflammatory stimulation of RAW264.7 resulted in a large increase in cellular spingolipids, which play a key role in degradation of intracellular components in membrane vacuoles.[3] Therefore, it is important to assess the interaction of lipids with each other and with other membrane components to better understand the cell membrane functions.

Lipid–lipid interactions within cellular and model membranes have been investigated with many techniques: fluorescence microscopy,[4,5] solid-state nuclear magnetic resonance (NMR),[6,7] X-ray diffraction,[8,9] and mass spectrometry. Time of flight secondary ion mass spectrometry (TOF-SIMS) is an attractive alternative to the traditional techniques used for studying lipid domains owing to its molecule-specific imaging ability, high mass resolution, and quasi-parallel ion detection. Although TOF-SIMS offers a great potential for biological applications, the dependence of its results on biological matrices is still not well investigated. Matrix effects play a significant role in the data interpretation process as one compound might strongly enhance/suppress the detection of another. Sostarecz *et al.*[10] demonstrated the influence of the molecular environment on the analysis of phospholipids. The addition of cholesterol to the Langmuir–Blodgett film of phospholipid 2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) led to an increase in the phosphocholine fragment signal because of its proton-donating nature. Conversely, its addition masked the presence of the corresponding head group of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (DPPE). Jones *et al.* have shown that haloperidol drug molecule ion intensity was enhanced in the presence of cholesterol, whereas it was suppressed in the phosphatidylcholine containing lipid environment.[11] Moreover, salt in biological material also introduces a matrix effect by suppressing the signal and generating new ionic species.

It has been demonstrated that the SIMS depth profile of a neat freeze-dried tissue is dominated by sodium and potassium signals, although the initial spectrum showed surface features of lipids, amino acids, and other biomolecules.[12,13] The organic ion suppression effect was also evident from the study on salt-doped arginine films.[14] The loss of negative chloride ion

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intensity in the negative ion spectrum during depth profiling suggests that the chloride anion ion paired with the positive organic secondary ions, which leads to suppression of the organic signals.

In this report, we investigate the potential ion suppression/enhancement effects on several lipids found in high abundance in a model mammalian cell line, RAW264.7. To mimic the inner and outer leaflet of the cellular membrane, we prepared lipid mixtures of the main lipid components in these leaflets. NMR spectra of the lipids and lipid mixtures were obtained to better understand of the influence of molecular environment on secondary ion yield.

Experimental

RAW264.7 cells were grown on silicon shards in a Petri dish using Dulbecco's modified Eagle's medium (Sigma Aldrich, UK), which is a mixture of inorganic salts, essential and nonessential amino acids, vitamins, and other growth media. Si shards were then washed with ammonium formate (0.15 M, pH 7.4) and residual media were removed by passing over a gentle stream of nitrogen gas. To preserve the cell structure, we plunged Si shards in liquid nitrogen–cooled propane and stored under liquid nitrogen. Samples were analyzed in a precooled analysis stage, which was held at approximately 90 K based on the thermocouple measurements to maintain their frozen-hydrated state.

For the lipid–lipid interaction study, five commercially available glycerolipids (Avanti Lipids, Sigma, Chiron), DPPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS), were selected for analysis. All lipids and chloroform (Sigma-Aldrich, MO, USA) were obtained and used without further purification. The SIMS information was obtained from the single lipids as well as the 1:1 mixed lipid films. Films were prepared by spin coating 1.5 mM, 50-μl aliquots of lipid solution onto 5 mm^2 silicon shards at a spin rate of 3000 rpm for 60 s.

Experiments were performed using a BioToF TOF-SIMS, described in detail elsewhere.[15] The spectrometer was equipped with a 40-keV C_{60}^+ primary ion source (Ionoptika Ltd, Southampton, UK). Each lipid spectrum was taken from an area of 300 × 300 μm using 1×10^{11} primary ions/cm^2. ^1H and ^{13}C NMR spectra of the lipids and mixtures were obtained by a Bruker DRX-400 NMR spectrometer. Deuterated chloroform ($CDCl_3$) was used as the solvent, and proton signals were referenced to tetramethylsilane at 0 ppm as the internal standard.

Result and Discussion

The molecular ion distributions for frozen-hydrated RAW cells obtained at the single cell level and the positive ion SIMS spectrum of frozen-hydrated RAW264.7 cells are shown in Figure 1. This spectrum represents the composition of the outer cell membrane of several RAW264.7 cells. In this spectrum, the peaks at 86.10, 184.07, and 224.10 indicate the presence of phosphocholine head group fragment ions. The inset in Figure 1 shows the enlarged view of the high mass region (m/z = 600–900). Although biological membranes contain hundreds of chemically distinct lipid components that form the actual bilayer membrane, the most intense peaks observed in the high mass region belong

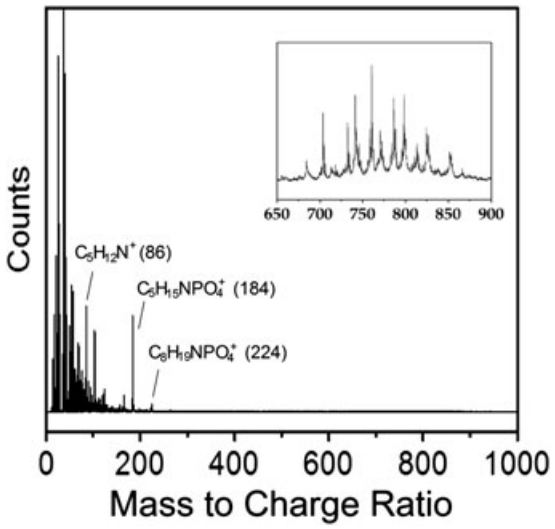

Figure 1. Mass spectrum of RAW264.7 cells.

to phosphocholine type lipid molecular ions and corresponding salt adducts. It is well known from the literature that matrix effects play a significant role in data interpretation process as one compound might strongly enhance/suppress the detection of another.[10–14] Therefore, the absence of peaks corresponding to other lipid types does not mean they are not present. Hence, understanding the relationship between various lipids found in cellular membrane is crucial for a proper identification of molecules within a system.

To understand the interaction of phosphocholine lipids found in different parts of RAW264.7 cell membrane, we prepared model lipid mixtures. Although the influence of salt and frozen-hydrated matrix on secondary ion yields of peaks observed at high mass is known, the main scope of this report is to study the interaction of phosphocholine lipids found in high abundance in a cell membrane. To mimic the outer leaflet of the cell membrane, we prepared 1:1 mixtures of DPPC, DOPC, and POPC and for the inner leaflet POPE and POPS lipids, and lipid mixtures were studied. Potential ion suppression or ion enhancement effects were examined by comparing relative secondary ion yields for the protonated species, $[M+H]^+$, of single lipids and mixtures. Five areas from each sample were selected, and positive ion SIMS spectra were obtained. Relative secondary ion yields shown in Figure 2 were determined for $[M+H]^+$ by calculating the integral areas of the peaks at m/z 734.58, 760.59, and 786.60 for the lipids DPPC, POPC, and DOPC, respectively. Assuming that the secondary ion yield is proportional to the surface concentration, values obtained from mixtures were doubled.

The comparison of the average relative secondary ion yields is given in Figures 2a–2c. Compared with its pure film, DOPC and POPC $[M+H]^+$ ion yields decrease when mixed with DPPC. Therefore, the molecular environment has a profound effect on the formation of molecular ions as it affects the secondary ion yields of DOPC and POPC in the presence of DPPC. Similarly, the $[M+H]^+$ ion yield of DPPC also decreases when mixed with DOPC and POPC. Protons from the adjacent lipid molecules contribute to the ionization of phosphocholine lipids. Fast atom bombardment (FAB) studies suggest that the formation of the molecular ion is achieved by proton transfer from the matrix.[16]

The fragmentation scheme of DPPC is shown in Figure 3. Phosphocholine (m/z = 184.07) formation is achieved by the

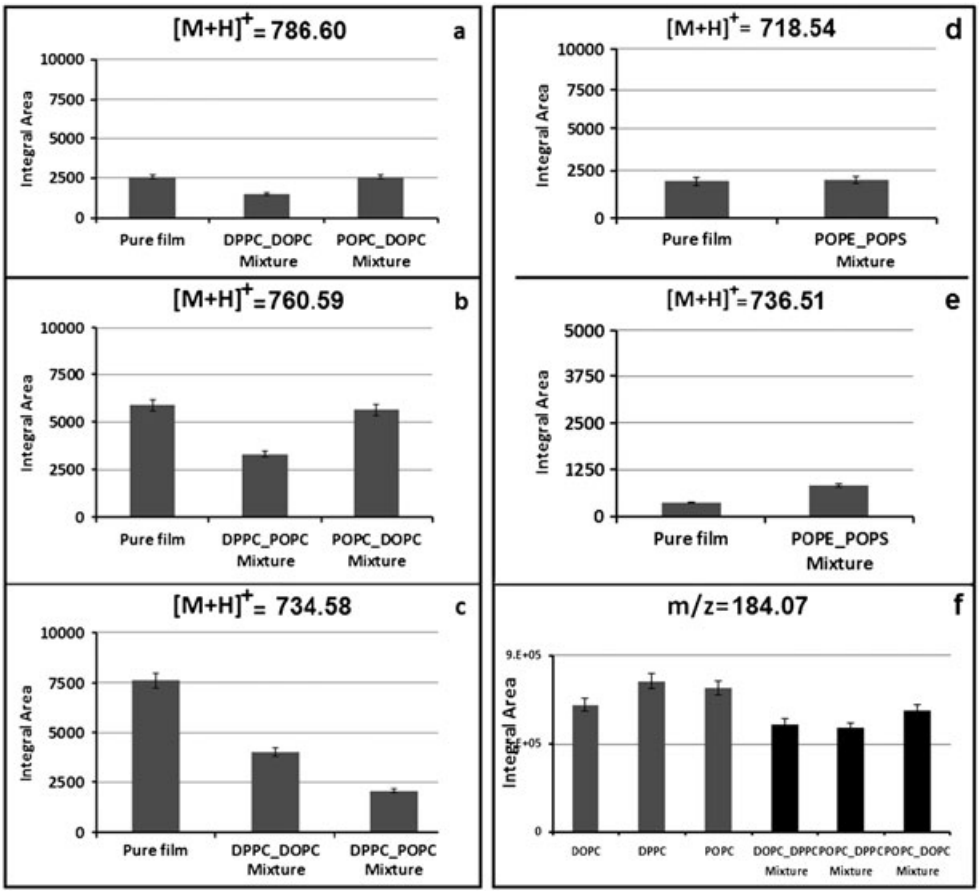

Figure 2. Relative secondary ion yields of the [M + H] + ions at *m/z* (a) 786.60 (DOPC), (b) 760.59 (POPC) and (c) 734.58 (DPPC), (d) 718.54 (POPE), (e) 736.51 (POPS), and (f) 184.07 for the lipids and the mixtures.

addition of a proton from the DPPC molecule itself by intramolecular rearrangement (2) or from the molecular environment (3). Therefore, like DPPC, DOPC and POPC require protons for charge balancing during fragmentation. Competition for proton in these lipid mixtures might lead to the preferential donation of a proton from DOPC and POPC to DPPC. Similar behavior is also observed from the secondary ion yield data of the *m/z* 184.07 peak of pure lipids and lipid mixtures shown in Figure 2f. There is less fragmentation when lipids are in the presence of other lipids. The effect of the molecular environment is also evident from Figure 2f because the degree of fragmentation of lipids is affected in the mixed forms.

Although NMR and TOF-SIMS provide different types of information, further evidence of this transfer mechanism was also evaluated with ^{1}H and ^{13}C NMR studies. ^{1}H and ^{13}C NMR spectra of a DOPC and DOPC_DPPC mixture are shown in Figure 4. Proton NMR results reveal the formation of a peak at 4 ppm on mixing, which is assigned to the hydrogen present in R-OH. This peak is also observed for POPC_DPPC and POPC_DOPC lipid mixtures (data not shown). It is apparent from the ^{13}C NMR data that the hydroxylic moiety is not attached to the fatty acid chain. Therefore, a possible source for the hydroxyl bond formation is the phosphocholine head group.

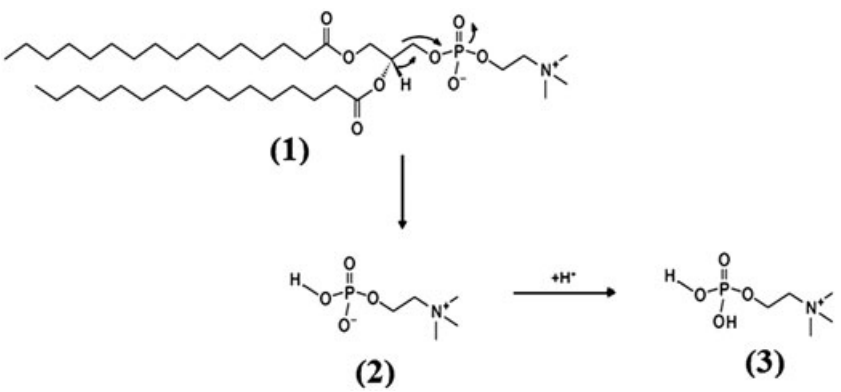

Figure 3. Fragmentation scheme of DPPC adapted from reference 16.

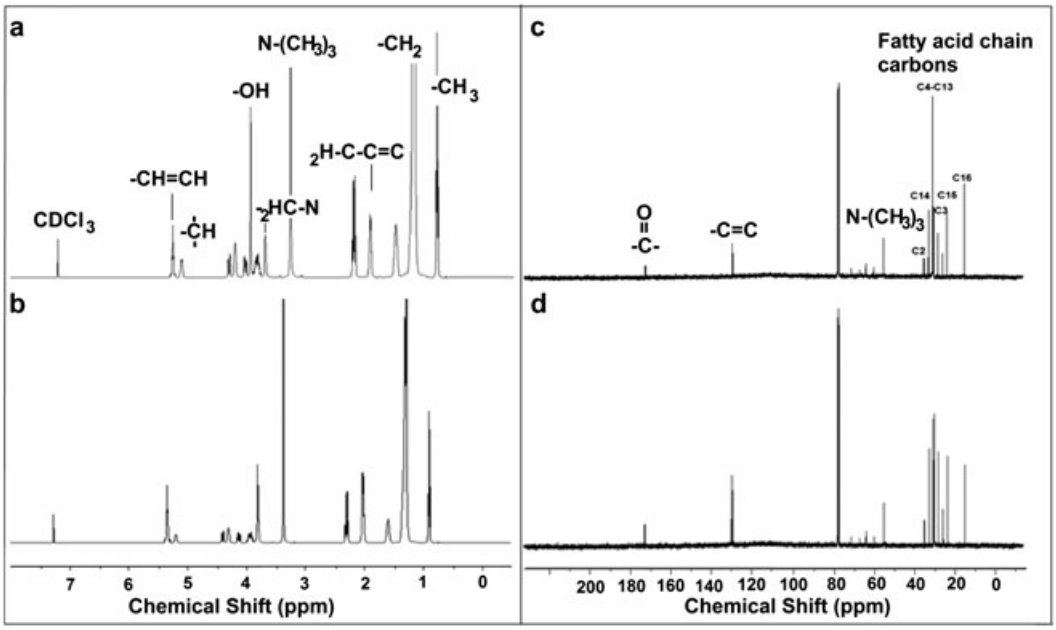

Figure 4. ^{1}H NMR spectrum of (a) DOPC_DPPC mixture, (b) DOPC and ^{13}C NMR spectrum of (c) DOPC_DPPC mixture, and (d) DOPC.

The average secondary ion yields of a lipid mixture representing the inner leaflet of the cell membrane are given in Figure 2. Secondary ion yields for the $[M+H]^+$ were determined by calculating the integral areas of the peaks at *m/z* 718.54 and 736.51 for the lipids POPE and POPS, respectively. Although the POPE molecular ion intensity exhibits a slight increase when mixed with POPS, statistically this increase is within error bar limits. Similarly, molecular ion yields of POPS tend to increase when mixed with POPE. Although POPE and PC head groups appear to ionize by similar mechanisms, ^{1}H and ^{13}C NMR results for these systems do not provide useful information regarding the proton exchange behavior.

Conclusion

Although it is difficult to extract significant molecular information from a single cell because of the amount of material available and the requirement that the ion beam be focused to a much smaller spot, in this study we are able to obtain the molecular ion distributions of frozen-hydrated RAW264.7 cells at the single cell level. Lipid–lipid interactions are found to influence secondary ion yields of the phosphocholine lipids found in high abundance in the cell membrane. NMR studies also confirm the effect of molecular environment on observed lipid distributions. The results presented here strongly suggest that careful data interpretation is needed when analyzing biological systems, and quantification of the lipid distribution requires sophisticated mass spectrometry. Therefore, imaging of single cells continues to be a challenging but potentially a killer application for SIMS.

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