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Phosphatidylethanolamine-Induced Cholesterol Domains Chemically Identified with Mass Spectrometric Imaging

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Cholesterol (CH) domains in cellular membranes are currently of interest due to their proposed participation in transport and signaling;[1−3] thus the content and formation mechanisms of these domains are of significant interest.[1−6] Cholesterol domains are predicted to exist in both the inner and outer leaflets of the plasma membrane[1,4−7] and to exist in the liquid-ordered (l_o) phase.[2−9] Lipids in the l_o phase are tightly packed but retain a high degree of lateral mobility.[3−6,8] The lipid distribution in l_o domains has mainly been inferred from low-temperature detergent solubility studies.[2,6−8] Although information is available on the l_o phase for lipid fractions similar to the outer bilayer leaflet, little is known about its formation in the phosphatidylethanolamine-rich inner leaflet.[1,3−6,10] However, the relationship between the two leaflets is essential to understanding transport and signaling in these complex structures.

Cholesterol domains may be investigated using simplified model membrane systems, such as Langmuir−Blodgett (LB) films. Supported LB films have been shown to maintain the same lateral distribution of lipids observed at the air−water interface.[11] Phospholipid and CH LB monolayers have been investigated with fluorescence microscopy[12,13] and atomic force microscopy (AFM).[12,14] These studies have identified the existence of different lipid phases[12−14] but have not chemically confirmed their specific content. To surmise the location of CH, stepwise experiments with varying amounts of CH must be performed. Recently, it has been shown that time-of-flight secondary ion mass spectrometry (TOF-SIMS) is capable of chemically imaging and identifying lipid domains.[11,15] In this work, we employ this unique form of imaging to show that dipalmitoylphosphatidylethanolamine (PE) disrupts a single homogeneous liquid phase dipalmitoylphosphatidylcholine (PC)/CH system and forms micrometer-size domains of phospholipid and CH. This result is significant because it demonstrates the likely organization of CH domains within *both* leaflets of the plasma membrane bilayer.

The LB films investigated at 3 mN/m were 2:1 PC/CH, 2:1 PE/CH, and 2:1 PC/CH with 40 mol % PE (PC/CH/PE). Although higher pressures are believed to mimic biological membrane densities, lower pressures are required to investigate liquid miscibility.[16] The TOF-SIMS spectrum of a PC/CH/PE LB film is shown in Figure 1 with the representative peaks for all of the films labeled. The phosphocholine ion at mass-to-charge ratio (m/z) 184, $(C_5H_{15}NPO_4)^+$, is characteristic of the PC headgroup (HG) and the fragment ion at m/z 552, $(C_{35}H_{67}O_4)^+$, is the characteristic tailgroup (TG) fragment for PE and PC. Ions at m/z 124 and 142 for the PE HG are masked by the low-mass CH fragments.[17] Cholesterol is identified by two ions at m/z 369, $(M-OH)^+$, and 385, $(M-H)^+$. Figure 2 contains the total ion, molecule-specific, and AFM images of the three different films. The TOF-SIMS images of the PC/CH film identify a homogeneous film of phospholipid/CH. The PE/CH film has CH segregating into micrometer-size domains, as does the PC/CH/PE film. An explanation of the lateral organization of the three different lipid films is given below.

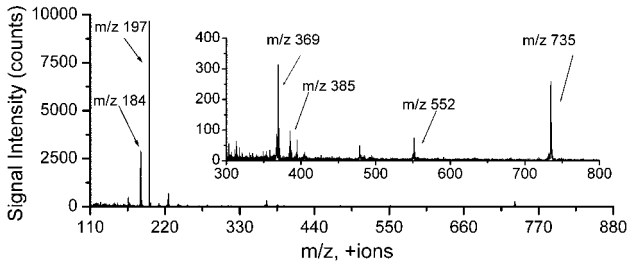

Figure 1. A mass spectrum of a PC/CH/PE LB film. The characteristic ions are Au^+ at m/z 197, phosphocholine at m/z 184, PC protonated molecular ion at m/z 735, phospholipid tailgroup at m/z 552, and CH at m/z 369 and 385. LB monolayers were made at 7 Å^2/molecule/min and deposited onto acid-terminated SAMs on Au at 3 mm/min at a surface pressure of 3 mN/m. A 15 keV Ga^+ primary ion beam was used with a primary ion dose of less than 10^{12} ions/cm^2. Sample preparation and analysis was done at room temperature.

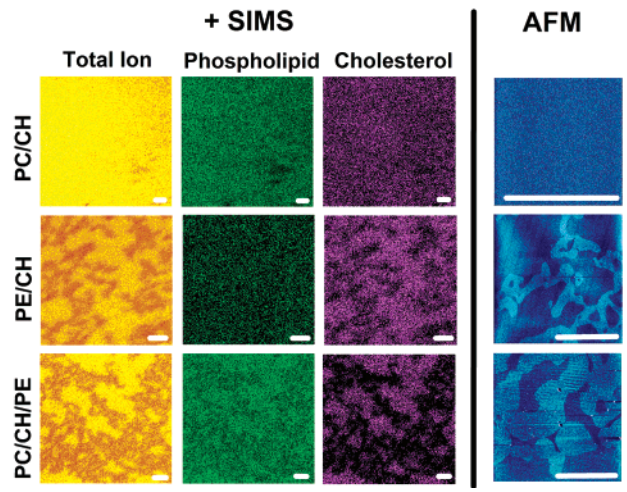

Figure 2. The three phospholipid/CH LB films were imaged by TOF-SIMS and AFM. The bars represent 40 μm (SIMS) and 25 μm (AFM). SIMS images are 256 pixels × 256 pixels with approximately 17 shots/pixel. Phospholipid signal is represented in green by phosphocholine at m/z 184 and phospholipid tailgroup at m/z 552. CH is represented in purple by m/z 369 and 385. Note that m/z 184 is not present in the PE/CH LB film. AFM images are 512 pixels × 512 pixels with a scan rate of 1 Hz. SIMS lateral image resolution is ∼200 nm. Domain existence and content is qualitatively reproducible from spot to spot for each of the LB films.

The result of the addition of CH to PC is represented in Figure 3a by isotherms of PC, CH, and a 2:1 PC/CH mixture. A surface pressure−area isotherm of PC contains three phases: LC, LC/LE, and LE.[18] The LC phase corresponds to a highly ordered liquid condensed phase, and the LE phase corresponds to a highly disordered liquid expanded phase. A 2:1 PC/CH mixture results in

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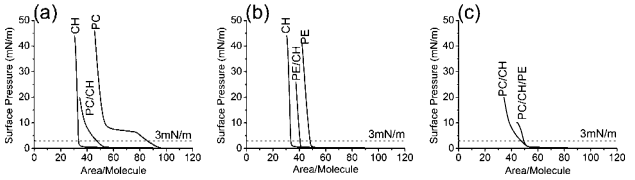

Figure 3. Pressure-area isotherms for (a) PC, CH, and 2:1 PC/CH, (b) PE, CH, and 2:1 PE/CH, and (c) 2:1 PC/CH and 2:1 PC/CH with 40 mol % PE.

the abolishment of the LC/LE phase with the actual molecular area at 3 mN/m 30% lower than that predicted for two noninteracting lipids. A decrease in molecular area due to the addition of CH is ascribed to the "condensing effect" of CH.[19] It is known from nearest-neighbor recognition[9] and molecular dynamics studies[20] that CH induces order in the phospholipid molecules. A mixture of PC and CH in a 2:1 mole ratio is known to form a single homogeneous liquid phase,[5,14] and the 2:1 PC/CH film at 3 mN/m imaged by TOF-SIMS is homogeneous with PC and CH throughout the film (Figure 2). Mixtures of PC and CH are thought to form "condensed complexes" that are believed to be responsible for the condensing effect of cholesterol and are therefore linked to the l_o phase of bilayers.[21,22]

The two phospholipids, PC and PE, differ by the size and chemical nature of their HGs. Both HGs have a positive charge on the nitrogen but the PC HG is larger due to the three methyl groups at the N-terminus. The dynamics of this difference can be visualized in Figure 3b. A 2:1 mixture of PE/CH has an average area per molecule <10% lower than that predicted for a noninteracting mixture. Cholesterol has a different effect on PE than PC due to strong inter-HG hydrogen bonding and electrostatic interactions between PE molecules; thus PE−PE interactions are stronger than PE−CH interactions.[8,10] The TOF-SIMS image of a PE/CH film at 3 mN/m shows that the amount of CH required to form a single homogeneous liquid phase with PC is not sufficient for PE. The formation of CH micrometer-size domains is evident in the PE/CH film. The strong PE−PE interactions cause CH to minimize lipid mixing and result in CH-rich domains with the PE present in two liquid phases. This is similar to that seen for PC in PC/CH monolayers of less than 30 mol % CH where the PC can be found in a LE and a "liquid ordered" phase.[12] For PE/CH, the CH acts as a spacer and creates a similar liquid ordered phase of PE/CH in LC phase PE. Also, PE should form condensed complexes with CH analogous with dilauroylphosphatidylethanolamine.[21] The condensed complex is probably the CH-rich phase as only two liquid phases are observed.[16]

The isotherm for a film of 2:1 PC/CH with 40 mol % PE is shown in Figure 3c. The ternary system is at a slightly higher area per molecule at 3 mN/m than PC/CH; therefore the condensing effect of CH is reduced. The inclusion of 40 mol % PE to 2:1 PC/CH disrupts the interaction between CH and PC resulting in the formation of CH-rich domains. Both *m*/*z* 184 and 552 are found throughout the analyzed area of the PC/CH/PE film as seen in Figure 2. Unfortunately, PE-specific HG peaks are masked by the addition of cholesterol,[17] so although PE is certainly present its location cannot be specifically determined. From lipid bilayer studies, it is known that PC and PE are almost ideally miscible,[23,24] and in a PC/CH/PE system, CH shows no preferential affinity for PC.[24] In the PC/CH/PE LB film, phospholipid is in multiple liquid phases, and the phospholipid−CH condensed complex may be the CH-rich phase. The coalescence of CH into domains appears to result from the nature of the phospholipid HGs. The formation of intermolecular hydrogen bonds between the NH_3^+ protons of the PE HG and the neighboring phosphate oxygens—including those on PC molecules—reduces the phospholipid−CH interactions. The CH domains in both of the PE-containing films are domains within domains, as confirmed by AFM and TOF-SIMS (Figure 2), supporting the theory of coalescing CH domains.[5,6] These results are particularly far-reaching because they represent the first chemical-specific imaging of CH−phospholipid domains in model membrane monolayer systems. This ability to discriminate chemical species has been used to demonstrate that CH domains form in a phosphatidylethanolamine-rich system—further evidence suggesting that CH is involved in interactions in the bilayer and could facilitate transport and signaling between the two leaflets of the plasma membrane.

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Supporting Information Available: The procedures for preparing and analyzing the LB model membranes. This material is available free of charge via the Internet at http://pubs.acs.org.

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