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Selective Sterol-Phospholipid Associations in Fluid Bilayers

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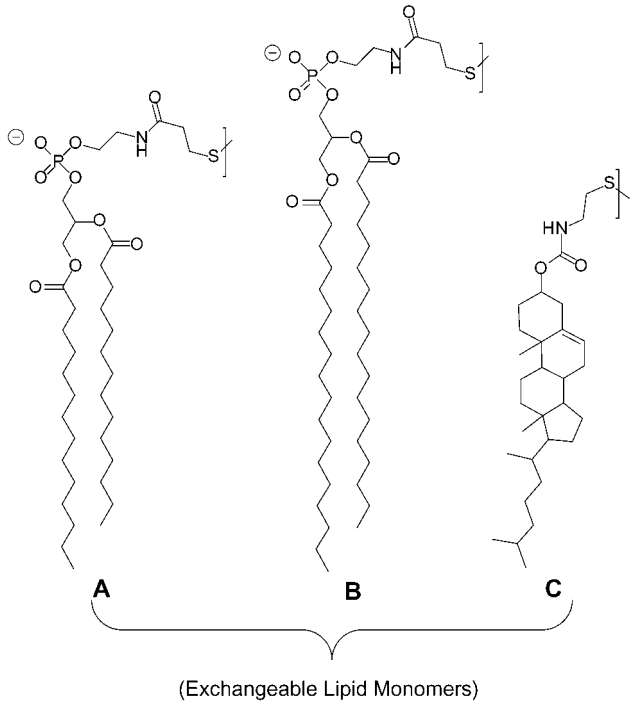
Abstract: The nearest-neighbor preferences of three exchangeable lipid monomers (two phospholipids that differ in length, **A** and **B**, and a derivative of cholesterol, **C**) have been quantified in the fluid bilayer state by use of the nearest-neighbor recognition method (Davidson, S. K. M.; Regen, S. L. *Chem. Rev.* **1997**, *97*, 1269). Thus, an analysis of the equilibrium dimer distributions has shown that (i) the sterol favors both phospholipids as nearest neighbors relative to other sterol molecules, (ii) that this recognition is selective (i.e., the sterol favors the longer phospholipid as a nearest neighbor over the shorter one, especially when the sterol concentrations in the bilayer is high (e.g., 40 mol %), and (iii) the phospholipids, themselves, are unable to recognize each other. Taken together, these findings indicate that the probable mechanism by which cholesterol induces homoassociation of **A** and **B** in analogous bilayers is one in which the sterol "pulls" two or more of the longer phospholipid monomers (**B**) out of a "sea" of randomly mixed **A** and **B**. These findings also lend support for the notion of cholesterol−phospholipid complexation in fluid bilayers. The biological implications of these findings are briefly discussed.

Introduction

The need for clarifying the two-dimensional structure of biological membranes is becoming increasingly evident. In particular, the notion that transient microheterogeneities in mammalian cell membranes control many important cellular processes such as signal transduction, membrane fusion, and membrane trafficking is becoming widely accepted.[1−11] Recently, evidence has begun to emerge which suggests that microheterogeneities may also be responsible for producing the diseased state. For example, it now appears likely that certain regions of cellular membranes, which are rich in cholesterol and highly saturated (high melting) lipids, play a critical role in the production of HIV-1 viral particles, and the ability of these particles to infect healthy cells.[12] Although a considerable amount of effort has been made in trying to define how lipids and proteins organize in fluid bilayers, this remains as one of the most poorly understood areas of modern structural biology.[13−15]

To clarify the fundamental relationships that exist between the structure of lipids and their mixing behavior, we have been



exploiting a technique that takes "molecular level snapshots" of membrane organization. In essence, this nearest-neighbor

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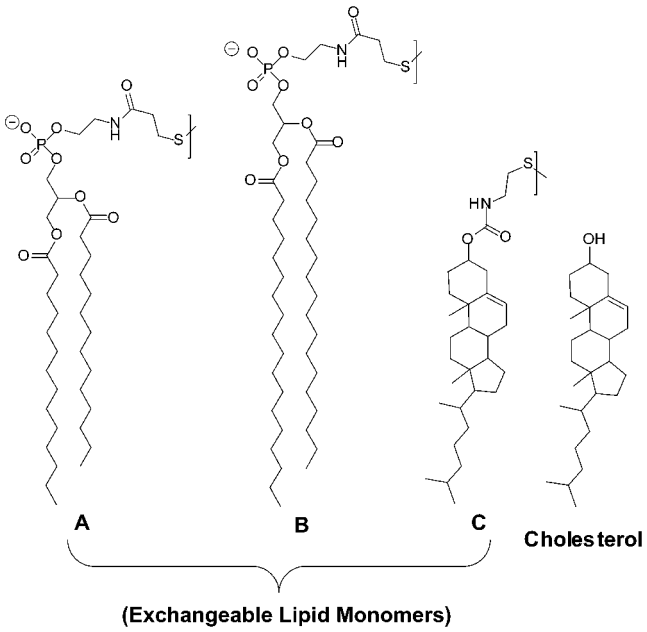
recognition (NNR) method detects and measures the thermodynamic tendency of lipids to become nearest neighbors in liposomal membranes.[16−22] Experimentally, two lipids of interest are first converted into exchangeable dimers and allowed to undergo monomer interchange via thiolate−disulfide displacement. Resulting equilibrium dimer distributions are then analyzed as formal, noncovalent bonds between pairs of adjacent lipids. Thus, a bilayer that is composed of dimers made from *A* and *B* monomers may be treated as an equilibrium mixture of homodimers (*AA* and *BB*) and heterodimer (*AB*) according to eqs 1 and 2, where *K* is the corresponding equilibrium constant. If *A* and *B* are randomly distributed, then the observed dimer distribution is statistical. In other words, if an equimolar amount of *A* and *B* were present, the mole ratio of *AA*/*AB*/*BB* would be 1/2/1, and the equilibrium constant would be equal to 4. If a thermodynamic preference for heteroassociations exists, this would be reflected by a value of *K* that is greater than 4. In contrast, favored homoassociations would be indicated by a value of *K* that is less than 4. It should be noted that although the NNR method involves the use of exchangeable dimers, it provides thermodynamic information that relates to nearest-neighbor interactions between *individual* lipid monomers.[22]

$$AA + BB \overset{K}{\rightleftharpoons} 2AB \quad (1)$$

$$K = [AB]^2/([AA][BB]) \quad (2)$$

In previous work, we have shown that the exchangeable phospholipid monomers **A** and **B** (bearing myristoyl and stearoyl groups, respectively) mix, ideally, in fluid bilayers.[16−19] We have also shown that the addition of cholesterol leads to nonideal mixing, such that homoassociations are thermodynamically favored.[19] A fundamental issue that we have long sought to clarify is the mechanism by which cholesterol induces such recognition. One possibility is that by condensing the fluid bilayer, cholesterol increases the attractive van der Waals forces, especially between the longer phospholipid molecules, **B**.[23] In a sense, this type of recognition would mimic the nearest-neighbor recognition that has been found in the gel-fluid coexistence region of membranes composed of **A** and **B**.[16,17] A second, and more intriguing, mechanism is one in which the sterol associates, selectively, with **A** or **B**, thereby removing it from a "sea" of randomly mixed phospholipids. To date, we have been unable to distinguish between these two scenarios.

The primary aim of the work that is described herein was to test the hypothesis that selective sterol−phospholipid association can exist in fluid bilayers, and that this selectivity can provide a driving force for phospholipid segregation. A secondary aim was to demonstrate the feasibility of probing the mixing behavior of *three* exchangeable lipids, simultaneously, thereby expanding the scope of the NNR method. For this purpose, an exchangeable form of cholesterol, **C**, was chosen for study. Previous experiments have shown that **C** has a condensing effect on fluid phospholipid monolayers, which is very similar to that of cholesterol.[24]



Experimental Section

Nearest-Neighbor Recognition Analysis. In a typical liposome preparation, a test tube was charged with a chloroform solution that contained 0.15 μmol of **AA**, 0.15 μmol of **BB**, 0.6 μmol of **AC**, and 0.6 μmol of **BC**, which corresponds to 1.5 μmol of dimer or 3.0 μmol of exchangeable monomer and 40 mol % exchangeable sterol. The chloroform was then evaporated by passing a stream of argon over the solution. The lipid mixture was then dissolved in 150 μL of chloroform and diluted with 400 μL of diisopropyl ether. Subsequent addition of 50 μL of 3.3 mM borate buffer (47 mM NaCl and 0.7 mM NaN_3, pH 7.4) produced an emulsion. After the emulsion was sonicated for 3 min, using a mild (bath-type) sonicator, the organic phase was removed by gentle evaporation at 60 °C, resulting in a white gel at the bottom of the test tube. The gel was then collapsed by vigorous vortex mixing for 5 min, and 3.0 mL of additional buffer (10 mM borate, 140 mM NaCl, and 2.0 mM NaN_3, pH 7.4) was added dropwise with vortex mixing. The dispersion was then degassed with an aspirator for 20 min, and the residual traces of organic solvent removed by dialysis (Spectra/Por Membrane, MWCO 6000−8000) under an argon atmosphere, using two 200-mL portions of degassed 10 mM borate buffer (pH 7.4) over the course of 18 h. Vesicles formed under these conditions were typically 10 000 Å in diameter (dynamic light scattering).

Thiolate−disulfide interchange reactions were initiated, after dispersions had equilibrated at the desired temperature, by increasing the pH to 8.5 (addition of 20 μL of 0.15 M NaOH) and injecting 83 μL of an aqueous solution of 26 mM dithiothreitol with brief vortex mixing. All dispersions were maintained under an argon atmosphere throughout the course of the interchange reactions. Aliquots (0.45 μL) were withdrawn as a function of time and quenched with 0.12 mL of 0.01 M HCl (final pH 5.0). After removal of water under reduced pressure, the residue was triturated with 2 mL of chloroform and centrifuged. The chloroform was then removed under reduced pressure to yield a clear film, which was, subsequently, dissolved in 5 μL of chloroform and 95 μL of the mobile phase, which was used for HPLC analysis. Under the conditions used, the extent of reduction of the lipid dimers by dithiothreitol was less than ca. 10%.

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Chart 1

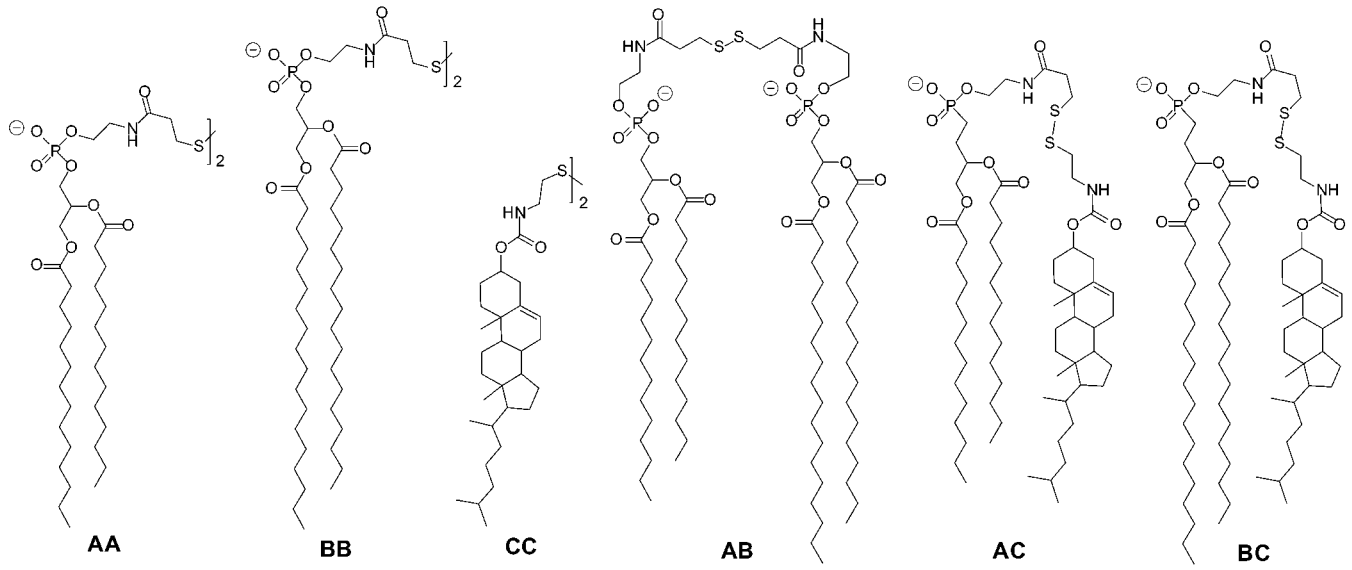

Table 1. Equilibrium Dimer Distributions at 60 °C

sterol content[a] (mol %)	equilibrium mole fractions[b]					
	AA	**BB**	**CC**	**AC**	**BC**	**AB**
0[c]	0.250	0.250				0.500
16	0.176 ± 0.017	0.177 ± 0.016	0.023 ± 0.002	0.136 ± 0.008	0.131 ± 0.006	0.357 ± 0.019
24	0.141 ± 0.006	0.137 ± 0.004	0.052 ± 0.003	0.184 ± 0.002	0.193 ± 0.006	0.293 ± 0.016
33	0.115 ± 0.006	0.106 ± 0.004	0.095 ± 0.002	0.221 ± 0.004	0.244 ± 0.001	0.219 ± 0.003
40	0.089 ± 0005	0.079 ± 0.004	0.136 ± 0.004	0.247 ± 0.006	0.272 ± 0.002	0.177 ± 0.001

[a] The sterol content that is listed as mol % reflects the quantity of sterol monomer units that are present in the membrane, where each dimer (**AA**, **BB**, **CC**, **AC**, **BC**, and **AB**) counts as two lipids. [b] Each equilibrium mole fraction listed corresponds to an average value derived from two independent vesicles experiments, i.e., one batch of vesicles that was prepared from a suitable mixture of **AA**/**BB**/**AC**/**BC**, and one that was prepared from a mixture of **AB**/**AC**/**BC**. [c] Equilibrium distribution of **AA**/**AB**/**BB** in the absence of exchangeable sterol; taken from ref 16.

Analysis of Dimer Distributions by High-Performance Liquid Chromatography. Mixtures of lipid dimers were analyzed by HPLC using a Beckman Ultrasphere C18 reverse phase column (4.6 × 250 mm, 5 μm particle size). The mobile phase that was used was composed of 7 mM tetrabutylammonium acetate (TBA) in denatured ethanol/water/hexane (82/12/6, v/v/v). The flow rate was 0.9 mL/min, and the column temperature was maintained at 31.0 °C. Peaks were monitored at 205 nm by use of a Waters 996 photodiode array detector. Data were collected and processed using a Millennium workstation (Waters Corp.). Retention times for **AA**, **BB**, **CC**, **AB**, **AC**, and **BC** were 6.3, 39.7, 73.3, 14.3, 17.3, and 49.8 min, respectively.

Results

Nearest-Neighbor Recognition Measurements. The syntheses of the six lipid dimers that were used in this investigation, **AA**, **BB**, **CC**, **AB**, **AC**, and **BC**, have previously been reported (Chart 1).[17,18,24] To examine the mixing behavior of each exchangeable monomer (i.e., **A**, **B**, and **C**) as a function of sterol content, vesicles were prepared from varying mixtures of **AA**/**BB**/**AC**/**BC**, and also from corresponding mixtures of **AB**/**AC**/**BC** *having the same mole percentages of exchangeable monomers*, **A**, **B**, and **C**. The reason for carrying out thiolate−disulfide interchange reactions from these two separate preparations was to ensure that final product mixtures were thermodynamically controlled. Specifically, convergence of the two sets of data with time rules out the possibility that the product mixtures are controlled by the kinetics of the thiolate−disulfide interchange reaction. Thus, for each sterol concentration that was investigated, vesicles were prepared using (i) an equimolar mixture of **AA** and **BB**, along with varying percentages of an equimolar mixture of **AC** and **BC**, and (ii) **AB** plus varying percentages of an equimolar mixture of **AC** and **BC**. All monomer interchange reactions were initiated at 60 °C in order to maintain the fluid phase; the gel to liquid-crystalline phase transition temperatures for **AA**, **AB**, and **BB** are 22.7, 33.9, and 55.4 °C, respectively.[16,17]

In Table 1 are shown the dimer distributions that were obtained with four different sterol concentrations. For purposes of comparison, the dimer distribution that was previously obtained in the absence of sterol is also listed. In every case, equilibrium was reached within 3 h, as indicated by dimer distributions that become constant with time, and by the convergence of the data obtained from the two sets of experiments. Equilibrium mole fractions that appear in Table 1 were obtained by averaging dimer distributions, which were determined from at least three different reaction times, for vesicles made from **AA**/**BB**/**AC**/**BC**, and for those made from **AB**/**AC**/**BC**. The sterol contents that are listed represent the mole percentage of the **C** that is present, where each mole of lipid dimer has been counted as 2 mol of lipid.

For these systems, three independent equilibria define the mixing behavior of **A**, **B**, and **C**. Thus, the equilibrium that is established for **AA**, **BB**, and **AB** defines an equilibrium constant, K_1, that characterizes the interactions between the exchangeable phospholipids **A** and **B** (eqs 3 and 4). An analogous equilibrium constant, K_2, for **BB**, **CC**, and **BC** characterizes the interactions between the longer (stearoyl-bearing) phospholipid, **B**, and the

Table 2. Equilibrium Constants as a Function of Sterol Content[a]

sterol content (mol %)	K_1	K_2	K_3
0	4.0		
16	4.1 ± 1.2	4.2 ± 1.1	4.6 ± 1.4
24	4.4 ± 0.8	5.2 ± 0.8	4.6 ± 0.6
33	3.9 ± 0.5	5.9 ± 0.4	4.5 ± 0.5
40	4.5 ± 0.5	6.9 ± 0.7	5.0 ± 0.7

[a] Calculated from the data in Table 1.

exchangeble sterol, **C** (eqs 5 and 6). Similarly, K_3 defines the interactions between the shorter (myristoyl-bearing) phospholipid, **A** with **C** (eqs 7 and 8). Specific values of K_1, K_2, and K_3, that have been calculated from the data that are listed in Table 1 are shown in Table 2.[25]

$$\mathbf{AA} + \mathbf{BB} \overset{K_1}{\rightleftharpoons} 2\mathbf{AB} \tag{3}$$

$$K_1 = [\mathbf{AB}]^2/([\mathbf{AA}][\mathbf{BB}]) \tag{4}$$

$$\mathbf{BB} + \mathbf{CC} \overset{K_2}{\rightleftharpoons} 2\mathbf{BC} \tag{5}$$

$$K_2 = [\mathbf{BC}]^2/([\mathbf{BB}][\mathbf{CC}]) \tag{6}$$

$$\mathbf{AA} + \mathbf{CC} \overset{K_3}{\rightleftharpoons} 2\mathbf{AC} \tag{7}$$

$$K_3 = [\mathbf{AC}]^2/([\mathbf{AA}][\mathbf{CC}]) \tag{8}$$

Discussion

Inspection of the K_1 values listed in Table 2 reveals that the two exchangeable phospholipids, **A** and **B**, mix ideally at all sterol concentrations. In contrast, K_2 values show that the longer phospholipid, **B**, and cholesterol become thermodynamically favored nearest neighbors, especially when the membrane becomes rich in sterol; that is, K_2 increases well beyond 4.0 at the high sterol concentrations. In addition, there is also a tendency for the shorter phospholipid, **A**, and the sterol to also become favored nearest neighbors, but this preference is relatively modest and apparent only at the highest sterol concentration examined. Thus, this exchangeable sterol shows a preference for becoming a nearest neighbor of **B** relative to **A**; that is, *sterol−phospholipid association within these bilayers is selective*.

Recent monolayer studies have led to the proposal that phospholipids and cholesterol form "complexes" at the air−water interface, having a stoichiometry of two phospholipids per sterol.[26−29] The present findings are consistent with this proposal and also with the suggestion that such complexes are likely to be favored in the fluid bilayer state.[26] If 2/1 phospholipid/sterol complexes do exist, then the stronger association between **B** and **C**, relative to **A** and **C**, may be accounted for in terms of greater hydrophobic interactions. Specifically, two molecules of the longer phospholipid, **B**, when associated with the sterol, would provide greater stability to the complex due to stronger hydrophobic interactions between the acyl chains.

Whether discrete sterol−phospholipid complexes are actually formed within these bilayers or whether there is a continuity of preferred phospholipid/sterol nearest-neighbor arrangements (e.g., 2/1) due to optimization in bilayer packing is an issue that remains to be clarified. Regardless of this uncertainty, our results provide strong inferential evidence that the role of cholesterol in promoting the nonideal mixing of **A** and **B** in analogous membranes is to "pull" two or more molecules of **B** out of a "sea" of randomly mixed **A** and **B**. Given the observed increase in nonideality and selectivity for the sterol−phospholipid interactions among **A**, **B**, and **C** with increasing sterol concentrations, it is tempting to speculate that such changes are a consequence of moving from the liquid-disordered to the liquid-ordered phase.[23]

What are the biological implications that may be drawn from these results? The present findings show that a sterol can favor C-18 over C-14 phospholipids as nearest neighbors, and that this preference depends, significantly, on the sterol content that is present within the bilayer. If cholesterol behaves similarly in mammalian membranes, then the existence of functional clusters composed of cholesterol and highly saturated (higher melting) lipids should also be sensitive to the overall cholesterol content. The fact that methyl-β-cyclodextrin (an agent that is known to remove cholesterol from cell membranes) has been found to reduce the infectivity of HIV-1 particles supports not only this premise but also the notion that cholesterol-rich phospholipid clusters are necessary for infection.[12] It may well be that cholesterol levels play a similar role in modulating the transmission of other enveloped viruses. In a broader context, the present results suggest that cholesterol concentrations may play an important role in cellular function by controlling the density (and possibly the size) of lipid microdomains. The fact that mammalian cells contain phospholipids having, predominantly, acyl chains of C16 and greater in length, together with our finding that the interactions between an exchangeable form of cholesterol and an exchangeable C14-based phospholipid are relatively weak, also raises the intriguing possibility that one important role of cholesterol in biological membranes is to control the length of the phospholipids that are retained in the membrane.[13]

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(25) A fourth equilibrium (**AB** + **CC** $\overset{K_4}{\rightarrow}$ **AC** + **BC**) may also be considered, where **AB** and **CC** are in equilibrium with **AC** and **BC** such that $K_4 = ([\mathbf{AC}][\mathbf{BC}])/([\mathbf{AB}][\mathbf{CC}])$. However, this equilibrium is not independent of eqs 4, 6, and 8; that is, $K_4 = [K_2K_3/K_1]^{1/2}$.

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