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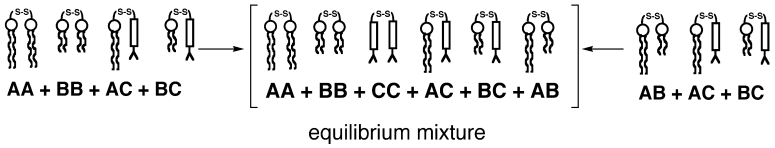

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Detection of Unusual Lipid Mixing in Cholesterol-Rich Phospholipid Bilayers: The Long and the Short of It

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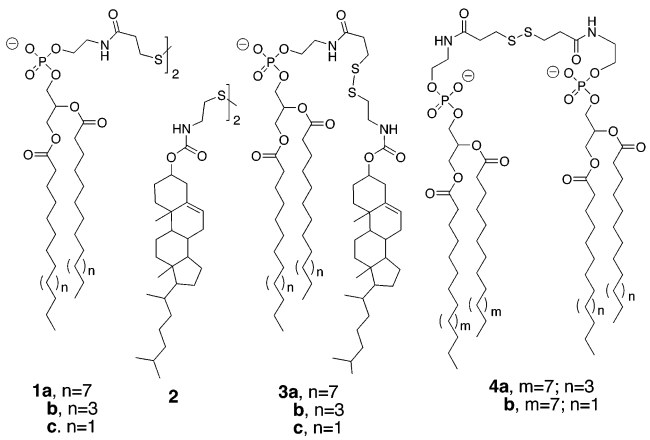
The notion that cholesterol combines with high melting sphingolipids to form transient clusters in biological membranes, and that these "rafts" play an important role in cellular function, is currently attracting broad attention.[1−5] In support of this view, there is a growing body of evidence indicating that cholesterol can form "condensed complexes" with certain high-melting phospholipids in monolayers, assembled at the air/water interface.[6] Our own work in this area has provided support for the existence of similar aggregates in the fluid bilayer state.[7] Here, we report our surprising discovery that favored sterol−phospholipid associations can be *reversed* in a bilayer, which contains relatively "long" (high-melting) and "short" (low-melting) phospholipids, when the sterol content is sufficiently high; that is, *like-lipids* now become favored nearest neighbors. A possible origin of this effect is briefly discussed.

The technique that we have used to study lipid mixing in fluid bilayers is the nearest-neighbor recognition (NNR) method.[8] In essence, NNR measurements take "molecular-level snapshots" of membrane organization by detecting and quantifying the thermodynamic tendency of lipids to become nearest neighbors. Experimentally, the lipids of interest are converted into disulfide-based dimers and allowed to undergo monomer interchange via thiolate-disulfide displacement reactions. Equilibrium dimer distributions are then analyzed as formal, noncovalent bonds between pairs of adjacent lipids. For example, if **A**, **B**, and **C** represent a long phospholipid, a short phospholipid, and cholesterol, respectively, then three independent equilibria define the mixing behavior of individual pairs of lipids in question.[7] Specifically, the nearest-neighbor preferences between **A** and **B** are given by the equilibrium constant, K_1, which governs the monomer interchange among **AA**, **BB**, and **AB** (eqs 1 and 2). Similarly, K_2 represents the nearest-neighbor preferences between **A** and **C** (eqs 3 and 4), and K_3 defines the mixing of **B** and **C** (eqs 5 and 6). When a pair of lipids mixes ideally, this is reflected by an equilibrium constant that equals 4.0.[7] When homoassociations are favored, the equilibrium constant is less than 4.0; a favored heteroassociation exhibits a value that is greater than 4.0.[7] As previously discussed, although the NNR method involves the use of exchangeable dimers, it provides thermodynamic information that relates to nearest-neighbor interactions between *individual* lipid monomers.[8]

Recent NNR measurements for bilayers made from **1a**, **1b**, and **2** have revealed a strong preference for the longer phospholipid to become a nearest neighbor of the sterol, especially at high sterol concentrations (Chart 1).[7] In an effort to determine the sensitivity of this recognition to chain-length mismatch, we chose to synthesize lauroyl analogues of **1b**, **3b**, and **4a** (i.e., **1c**, **3c**, and **4b**), thereby increasing the mismatch from four to six methylene units per acyl chain. Although we expected to detect significant differences, we were not prepared for a complete *crossover* from favored heteroassociations to favored homoassociations. This paper documents our findings.

Chart 1



$$\mathbf{AA} + \mathbf{BB} \overset{K_1}{\rightleftharpoons} 2\,\mathbf{AB} \tag{1}$$

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

$$\mathbf{AA} + \mathbf{CC} \overset{K_2}{\rightleftharpoons} 2\,\mathbf{AC} \tag{3}$$

$$K_2 = [\mathbf{AC}]^2/([\mathbf{AA}][\mathbf{CC}]) \tag{4}$$

$$\mathbf{BB} + \mathbf{CC} \overset{K_3}{\rightleftharpoons} 2\,\mathbf{BC} \tag{5}$$

$$K_3 = [\mathbf{BC}]^2/([\mathbf{BB}][\mathbf{CC}]) \tag{6}$$

Lipid dimers **1a**, **1c**, **2**, **3a**, and **4b** were prepared using methods previously described; lipid dimer **3c** was prepared by related methods.[9] Specific procedures that were used in forming liposomes, carrying out monomer interchange reactions, and analyzing dimer distributions (HPLC) were similar to those previously described.[7] To ensure that product mixtures were thermodynamically controlled, liposomes were prepared from appropriate combinations of **AA**/**BB**/**AC**/**BC**, and also from corresponding combinations of **AB**/**AC**/**BC** *having the same mole percentages of* **A**, **B**, and **C**. Thus, for each sterol concentration investigated, liposomes 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** (Chart 2). All interchange reactions were carried out at 60 °C to maintain the fluid phase.[10] Convergence of both data sets in all cases was excellent. Values reported in Table 1 are averages from both sets of experiments.

Chart 2

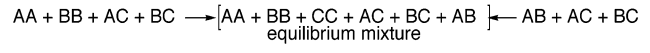


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Table 1. Equilibrium Dimer Distributions at 60 °C[a]

		equilibrium mole fractions					
C (mol %)[b]	**AA/BB**	**AA**	**BB**	**CC**	**AC**	**BC**	**AB**
0	**1a**/**1b**[c]	0.250 ± 0.004	0.250 ± 0.004				0.500 ± 0.009
24		0.137 ± 0.004	0.141 ± 0.006	0.052 ± 0.003	0.193 ± 0.006	0.184 ± 0.002	0.293 ± 0.016
40		0.079 ± 0.004	0.089 ± 0.005	0.136 ± 0.004	0.272 ± 0.002	0.247 ± 0.006	0.177 ± 0.001
0	**1a**/**1c**	0.275 ± 0.002	0.275 ± 0.002				0.450 ± 0.005
29		0.128 ± 0.009	0.149 ± 0.010	0.082 ± 0.006	0.220 ± 0.010	0.176 ± 0.012	0.244 ± 0.021
40		0.179 ± 0.009	0.158 ± 0.010	0.290 ± 0.018	0.076 ± 0.008	0.117 ± 0.010	0.179 ± 0.012

[a] **A**, **B**, and **C** refer to an exchangeable long phospholipid, a short phospholipid, and sterol, respectively; equilibrium was reached in all cases within 3 h. Values listed are averages (±1 SD) of the data obtained from liposomes prepared from appropriate ratios of **AA**/**BB**/**AC**/**BC** and **AB**/**AC**/**BC**, where a minimum of three values from each dispersion was used. [b] mol % reflects the quantity of sterol monomer units that are present in the membrane, where each dimer counts as two lipids. [c] All data for **1a**/**1b** are taken from ref 7.

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

entry	**C** (mol %)	**AA/BB**	K_1	K_2	K_3
1	0	**1a**/**1b**	4.00 ± 0.02		
2	24		4.44 ± 0.17	5.23 ± 0.13	4.62 ± 0.36
3	40		4.46 ± 0.43	6.89 ± 0.45	5.04 ± 0.19
4	0	**1a**/**1c**	2.68 ± 0.02		
5	29		3.12 ± 0.11	4.61 ± 0.24	2.54 ± 0.18
6	40		1.13 ± 0.02	0.11 ± 0.01	0.30 ± 0.01

[a] Calculated from the data in Table 1.[11]

A comparison of the equilibrium constants for membranes derived from **1a**, **1b**, and **2** with ones made from **1a**, **1c**, and **2**, is presented in Table 2. For convenience, monomers of **1a** are designated as **A** in both Tables. Similarly, monomers of each of the shorter phospholipids (**1b** and **1c**) appear as **B**; monomers of **2** are represented by **C**, and dimers **3** and **4** are given by **AC**, **BC**, and **AB**. In the absence of sterol, **A** and **B** derived from **1a** and **1b** mix ideally (entry 1). Introduction of a high concentration of **C** (40 mol %) in these bilayers leads to a strong preference for **A** and **C** to become nearest neighbors, but only a modest preference for **B** and **C** to cluster; the effects of **C** on the mixing of **A** and **B** are almost negligible (entries 2 and 3). In contrast, when a greater mismatch between the phospholipids exists (i.e., when **1b** is replaced by **1c**), favored homophospholipid associations are observed, even in the absence of **C** (entry 4). Addition of a moderate concentration of **C** (29 mol %) results in **A** and **C** becoming favored nearest neighbors, while **A** and **B**, as well as **B** and **C**, favor homoassociations (entry 5). The most striking results are those in which a high concentration of **C** (40 mol %) has been included in the bilayer. In this case, very strong homoassociations are favored for all three pairs of lipids (entry 6).

Why the crossover to favored homolipid associations on going from **1b** to **1c**? At present, we believe that this is due to the extreme shortness of the lauroyl chains in **1c**, and the substantial mismatch in chain length between **1a** and **1c**. Our working hypothesis is that a favored *transbilayer* arrangement is one in which the short phospholipids in each monolayer lie opposite to the long phospholipids in the adjoining monolayer. Such an arrangement would minimize exposure of the hydrocarbon chains to the aqueous phase and maximize hydrophobic interactions. Due to the extreme shortness of the lauroyl chains of **1c**, we also hypothesize that the stearoyl chains of **1a** have a reduced ability to "wrap around" neighboring sterols to form a condensed complex. Specifically, we suggest that their full length is needed to compensate for the very short lauroyl chains to be able to maintain a minimum thickness and a stable bilayer.[10,12] The strong influence that the sterol has in inducing homoassociations, especially at high sterol concentrations, can then be attributed to a reduction in the conformational freedom of the phospholipids. Thus, by sterols serving as a rigid hydrocarbon "wall" in the bilayer, the conformational freedom of the acyl chains of neighboring phospholipids is significantly restricted, forcing them to interact more strongly with other neighboring phospholipids. Homophospholipid associations are then favored due to a perfect matching of the acyl chains and an optimization of van der Waals attractions within the bilayer.

In principle, this unusual lipid-mixing behavior that we have discovered could have biological relevance. One can imagine, for example, that significant thinning of a biomembrane, induced by a hydrophobic segment of a protein, could promote homolipid associations. One can also imagine that for those biological processes that require major lipid reorganization (e.g., membrane fusion), homoclustering may well play a significant role.

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Supporting Information Available: Synthesis of **3c**, spectral data for **1c** and **4b** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(11) Error values (Δ) were calculated from the mole fractions of the dimers, their standard deviations (SD), and the corresponding *K* value, according to: $\Delta = [2K/\mathrm{AB}][\mathrm{SD_{AB}}] - [K/\mathrm{AA}][\mathrm{SD_{AA}}] - [K/\mathrm{BB}][\mathrm{SD_{BB}}]$. Rade, L.; Westergren, B. *Mathematics Handbook for Science and Engineering*; Birkhauser: Cambridge, MA 1995; Chapter 16.
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