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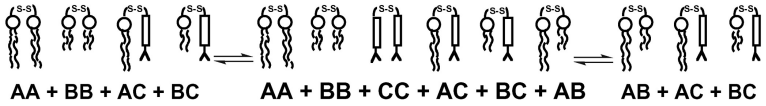

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Selective Association of Cholesterol with Long-Chain Phospholipids in Liquid-Ordered Bilayers: Support for the Existence of Lipid Rafts

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Recent nearest-neighbor recognition (NNR) studies have shown that cholesterol selectively associates with high-melting phospholipids relative to low-melting analogues in fluid bilayers.[1,2] Here, we show that such sterol-phospholipid recognition is limited to the liquid-ordered phase. The relevance of these findings to the notion of lipid raft formation in biological membranes is briefly discussed.

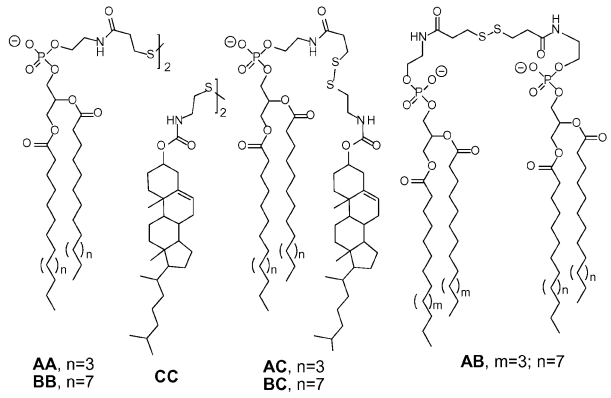
The mixing behavior of cholesterol and phospholipids in the physiologically relevant fluid phase has become the subject of intense interest. Much of this interest stems from the notion that cholesterol and high-melting lipids form "rafts" in biological membranes that are in a liquid-ordered state, which "float in a sea" of liquid-disordered membrane.[3−5] It has also been postulated that lipid rafts play a major role in a variety of cellular processes. In a recent series of monolayer studies, McConnell and co-workers have presented evidence that cholesterol and certain high-melting phospholipids form "condensed complexes".[6−8] Our own work in this area, which has been based on the nearest-neighbor recognition method, has allowed us to detect sterol-phospholipid recognition in fluid bilayers.

The purpose of the investigation reported herein was to clarify the relationship between sterol-phospholipid recognition and the phase state of the bilayer. Specifically, we sought to determine whether such recognition is intimately associated with the liquid-ordered phase.[8,9]

With this aim in mind, we examined the mixing behavior of two exchangeable phospholipids (**A** and **B**) and cholesterol (**C**) by analyzing chemically equilibrated mixtures of **AA**, **BB**, **CC**, **AC**, **BC**, and **AB** (Chart 1). The temperature that was used in these experiments was 30 °C, which lies above the gel to liquid-crystalline phase transition temperature of **AA** (T_m = 22.7 °C) and below that of **BB** (T_m = 55.4 °C).[10] At this intermediate temperature, incremental addition of **C** has a fluidizing effect on the gel portion of the bilayer and a condensing effect on the liquid-disordered portion, the net result being a conversion of both regions of the membrane to a common phase, the liquid-ordered phase.[9,11]

As discussed elsewhere, equilibrium mixtures of exchangeable lipid dimers, generated via thiolate-disulfide interchange reactions, provide "molecular level snapshots" of membrane organization by quantifying the thermodynamic tendency of the monomers to become nearest-neighbors of one another.[1,2] Thus, the mixing properties of **A**, **B**, and **C** are defined by three independent equilibria. In particular, 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 these lipids mix ideally, this is reflected by an equilibrium constant that equals 4.0.[1] When homo-associations are favored, the equilibrium constant is less than 4.0; favored hetero-associations are indicated by a value that is greater than 4.0. 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.[2]

Chart 1



$$\mathbf{AA} + \mathbf{BB} \overset{K_1}{\rightleftharpoons} 2\mathbf{AB} \quad (1)$$

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

$$\mathbf{AA} + \mathbf{CC} \overset{K_2}{\rightleftharpoons} 2\mathbf{AC} \quad (3)$$

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

$$\mathbf{BB} + \mathbf{CC} \overset{K_3}{\rightleftharpoons} 2\mathbf{BC} \quad (5)$$

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

Each of the requisite dimers was prepared using methods previously described.[1,10] Experimental procedures that were used in forming liposomes, carrying out monomer interchange reactions, and analyzing dimer distributions (HPLC) were also similar to those previously described.[1] To ensure that product mixtures were thermodynamically controlled, liposomes were prepared from appropriate combinations of **AA**/**BB**/**AC**/**BC**, and also from 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** plus 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 interchange reactions were carried out at 30 °C. Values reported in Table 1 are averages from both sets of experiments.

$$\mathbf{AA} + \mathbf{BB} + \mathbf{AC} + \mathbf{BC} \rightarrow$$
$$[\underset{\text{equilibrium mixture}}{\mathbf{AA} + \mathbf{BB} + \mathbf{CC} + \mathbf{AC} + \mathbf{BC} + \mathbf{AB}}] \leftarrow \mathbf{AB} + \mathbf{AC} + \mathbf{BC}$$

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Table 1. Equilibrium Dimer Distributions at 30 °C[a]

	equilibrium mole fractions					
C (mol %)[b]	**AA**	**BB**	**CC**	**AC**	**BC**	**AB**
0	0.368 ± 0.006	0.368 ± 0.006				0.264 ± 0.013
15	0.245 ± 0.002	0.279 ± 0.005	0.026 ± 0.000	0.159 ± 0.007	0.092 ± 0.005	
25	0.168 ± 0.006	0.182 ± 0.018	0.055 ± 0.002	0.209 ± 0.008	0.183 ± 0.016	0.203 ± 0.018
33	0.130 ± 0.005	0.123 ± 0.017	0.086 ± 0.003	0.239 ± 0.008	0.251 ± 0.016	0.173 ± 0.016
40	0.101 ± 0.002	0.084 ± 0.003	0.117 ± 0.001	0.262 ± 0.002	0.294 ± 0.003	0.142 ± 0.001

[a] Equilibrium was reached in all cases within 3 h. Values listed are averages (±1 SD) of the data obtained from liposomes prepared from **AA**/**BB**/**AC**/**BC** and **AB**/**AC**/**BC**, where a minimum of three values from each dispersion was used. [b] The mol % reflects the quantity of sterol monomer units that are present in the membrane, where each dimer counts as two lipids.

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

entry	**C** (mol %)	K_1	K_2	K_3
1	0	0.51 ± 0.03		
2	15	0.58 ± 0.01	3.97 ± 0.32	1.17 ± 0.11
3	25	1.35 ± 0.06	4.73 ± 0.02	3.35 ± 0.13
4	33	1.87 ± 0.02	5.11 ± 0.03	5.96 ± 0.27
5	40	2.38 ± 0.10	5.81 ± 0.08	8.79 ± 0.21

[a] Calculated from the data in Table 1.

Because the gel to liquid-crystalline melting properties of **AA** and **BB** are nearly identical to those of DMPC (T_m = 24.0 °C) and DSPC (T_m = 54.1 °C), respectively, the approximate ternary phase diagram that has previously been determined for analogous DMPC/DSPC/cholesterol bilayers can be used to estimate the onset of the liquid-ordered phase for **A**/**B**/**C** bilayers.[12] Specifically, at 30 °C, this onset is expected to occur at ca. 5 mol % **C**. At higher concentrations, the amount of liquid-ordered phase increases at the expense of both the gel and the liquid-disordered phases. Beyond ca. 45 mol % **C**, the membrane is fully in the liquid-ordered phase.

Examination of K_1 values as a function of **C** confirms the fluidizing effect by the sterol (Table 2). Thus, in the absence of **C**, the apparent equilibrium constant (reflecting the overall mixing of **A** and **B** in the solid and the liquid-disordered phases) shows a strong preference for homo-phospholipid association (entry 1). As discussed previously, this preference is driven by the formation of gel phase domains that are rich in **B**.[10] Introduction of **C** decreases the amount of gel phase in the membrane, allowing **A** and **B** to mix more ideally. Note the steady increase in K_1, reflecting a shift toward ideal mixing (entries 2−5). As reported previously, **A** and **B** mix ideally in the fluid bilayers (60 °C), even in the presence of high sterol concentrations.[1]

In sharp contrast, inspection of K_3 as a function of **C** reveals the sterol's fluidizing and condensing effects. Specifically, a crossover occurs in which favored homo-lipid associations change to favored sterol-phospholipid associations (entries 3 and 4). At the higher sterol concentrations, more of the membrane is converted into the liquid-ordered phase, which favors sterol-phospholipid recognition. Because the shorter phospholipid cannot form a gel phase at 30°, **C** can only exert a condensing effect. The increase in K_2 beyond 4.0, therefore, parallels the condensing effect found with K_3. In addition, the larger values of K_3 relative to K_2 at the higher sterol concentrations reflect a stronger association between **B** and **C**, relative to **A** and **C**, similar to what has previously been found at 60 °C.[1]

The steady increase in K_2 and K_3, which we have observed with increasing percentages of **C**, indicates that cholesterol-phospholipid recognition is limited to the liquid-ordered phase. Additional support for this conclusion comes from a plot of K_3 versus mol % **C** at 60 °C, where an onset for sterol-phospholipid recognition is evident at ca. 12 mol % **C** (Figure 1).[1] This value is in reasonable agreement with the onset of the liquid-ordered/liquid-disordered coexistence region for DMPC/DSPC/cholesterol bilayers, which occurs at ca. 18 mol % cholesterol at 60 °C.[12]

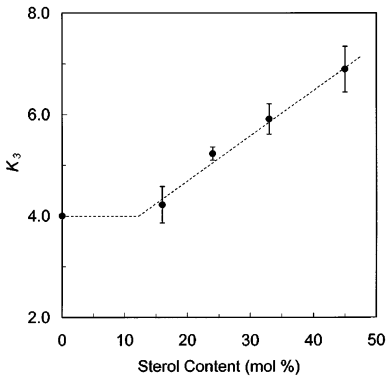

Figure 1. Plot of K_3 versus mol % C at 60 °C.

The notion that cholesterol combines with high melting lipids and lipid-anchored proteins in biological membranes to form "lipid rafts" has generated a large amount of debate. The present findings provide strong support for the existence of lipid rafts by showing that at sufficiently high cholesterol concentrations, where the liquid-ordered phase is produced (most likely as the result of "condensed complexes" being formed), cholesterol will favor high-melting lipids as nearest-neighbors.

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