The Structural Role of Cholesterol in Biological Membranes

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Cholesterol is a major component of mammalian cell membranes. In erythrocytes and in myelin, it is present in concentrations that approach those of phospholipids.[1−3] Despite numerous investigations involving monolayers and bilayers derived from cholesterol and phospholipids, the structural role that this sterol plays in producing condensed, fluid membranes has remained a mystery. In particular, the mechanism by which cholesterol uncoils phospholipids has yet to be elucidated.[4] Here we report the use of the nearest-neighbor recognition (NNR) method—a chemical technique that takes "molecular-level snapshots" of membrane organization—to clarify this long-standing issue.[5]

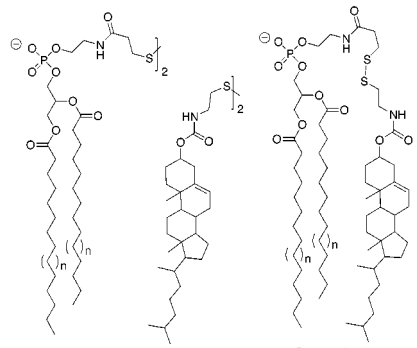
In essence, NNR experiments detect and measure the thermodynamic tendency of two lipids to become nearest-neighbors in the bilayer state.[5] Thus, two lipids of interest are converted into exchangeable dimers, which are allowed to undergo monomer interchange via thiolate−disulfide displacement. The equilibrium dimer distribution that is produced is then analyzed as formal, noncovalent bonds between pairs of adjacent lipids. Specifically, a membrane that is composed of A and B monomers may be treated as an equilibrium mixture of homodimers and heterodimers according to eqs 1 and 2. Here, K is the equilibrium constant for the AA homodimer, BB homodimer, and the AB heterodimer. When phase separation occurs, however, K represents an apparent equilibrium constant. If a membrane is made from an equimolar quantity of AA and BB, and if A and B are randomly distributed after equilibrium is reached, the observed dimer distribution would be statistical. In other words, 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 hetero-associations existed, however, this would be reflected by a value of K that is greater than 4. In contrast, favored homo-associations are indicated by a value of K that is less than 4.

$$\mathrm{AA} + \mathrm{BB} \overset{K}{\rightleftharpoons} 2\mathrm{AB} \tag{1}$$

$$K = [\mathrm{AB}]^2/[\mathrm{AA}][\mathrm{BB}] \tag{2}$$

Two features of the NNR method are noteworthy. First, the NNR method is a highly sensitive technique that can detect differences in nearest-neighbor interactions that are as low as ca. 50 cal/mol. It is well-suited, therefore, for probing sterol−phospholipid interactions, where differences in nearest-neighbor interactions are expected to be small. Second, although this method involves the use of exchangeable dimers, it provides thermodynamic information that relates to nearest-neighbor interactions between *individual* lipid monomers; that is, the disulfide bridge has a negligible influence on K values.[8]

Exchangeable forms of cholesterol and phospholipids that were selected for these NNR studies were **1a**, **1b**, **1c**, **2**, **3a**, **3b**, and **3c**. The design of **2** was based on two considerations. A carbamate moiety was introduced as a headgroup to provide a means for attaching a pendant thiol group while maintaining a hydrogen-bonding element off of the C-3 (β) position. In addition, having the thiol moiety distal from the A-ring was expected to avoid conformational strain within dimers **2**, **3a**, **3b**, and **3c**. With these ideas in mind, cholesteryl chloroformate was condensed with cystamine to give homodimer, **2**. The corresponding heterodimers, **3a**, **3b**, and **3c** were synthesized by condensing cholesteryl chloroformate with 2-amino-1-ethyl-2′-pyridyl disulfide, followed by displacement with the thiol monomer of **1a, 1b**, or **1c**. The syntheses of **1a**, **1b**, and **1c** have previously been described.[6]



To ensure the appropriateness of these lipids in model studies, we compared the monolayer properties of **1a** and **2** with those of 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylglycerol (DMPG) and cholesterol at the air/water interface (Figure 1). It should be noted that both **1a** and DMPG have identical acyl chains, very similar melting behavior, and negatively charged phosphate headgroups.[6] Similar to literature reports, cholesterol yielded a surface pressure−area isotherm having low compressibility and a limiting area of 0.395 $nm^2 \cdot molecule^{-1}$.[7] Examination of **2**, under identical conditions, showed slightly greater compressibility in the region of 0 to 15 $mN \cdot m^{-1}$ and a limiting area of 0.809 $nm^2 \cdot molecule^{-1}$. The greater compressibility of this dimer is a likely consequence of the bridging disulfide unit, which imparts greater flexibility to the surfactant. The fact that the limiting area of **2** is close to twice that of cholesterol indicates a strong similarity in their packing behavior. Additionally, the fact that **1a** has a limiting area (1.58 $nm^2 \cdot molecule^{-1}$), which is twice that of DMPG (0.80 $nm^2 \cdot molecule^{-1}$), also indicates similar packing behavior. Finally, the suitability of **2** as an exchangeable form of cholesterol was confirmed by comparing its condensing effect on **1a** with the condensing effect of cholesterol on DMPG. Thus, a series of surface pressure−area isotherms were recorded using varying mixtures of **1a** plus **2**, and also DMPG plus cholesterol (not shown). Plots of average molecular areas as a function of the mole fraction of phospholipid at 25 $mN \cdot m^{-1}$ are also given in Figure 1. As is evident from this figure, the condensing effect of cholesterol on DMPG is very similar to the condensing effect of **2** on **1a**.

Lipid bilayers, which were used for NNR experiments, were prepared in the form of large unilamellar vesicles via reverse phase

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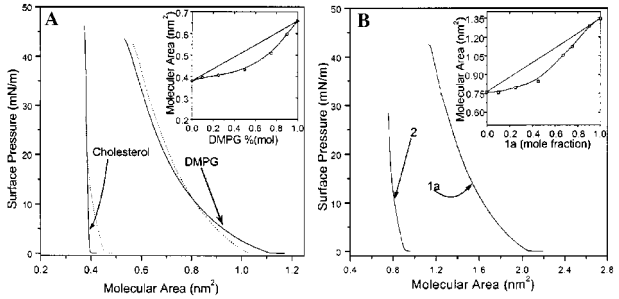

Figure 1. Surface pressure−area isotherms for (A) DMPG and cholesterol, and (B) **1a** and **2** over an aqueous solution that was 0.10 M in NaCl at 25 °C. Isotherms for **1a** and **2** (•••) are also shown in (A) as area/monomer for comparison. The insets show average molecular areas at 25 $mN \cdot m^{-1}$ as a function of mole fraction of phospholipid; isotherms for each mixture are not shown.

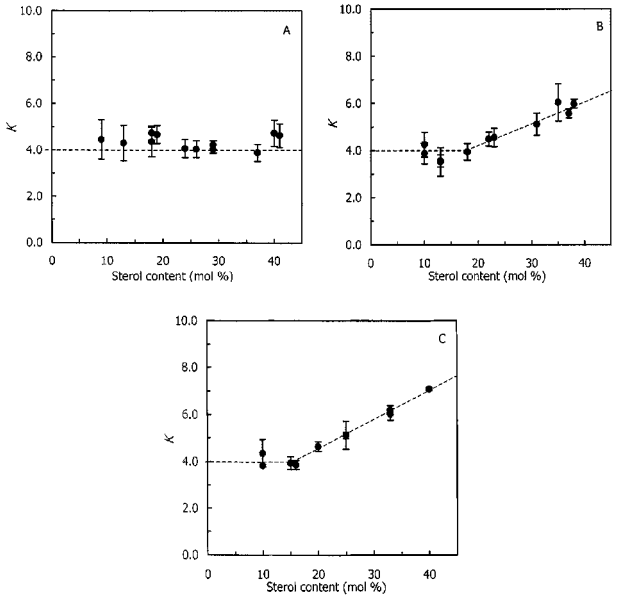

Figure 2. Plot of *K* versus mol % of exchangeable sterol present in bilayers made from (A) **1a**, (B) **1b**, and (C) **1c**; equilibration temperatures were 40, 60, and 68 °C, respectively. All thiolate−disulfide interchange reactions were carried out using vesicles made from varying molar ratios of phospholipid homodimer/heterodimer.

evaporation methods. Varying concentrations of sterol were included in each membrane type, using varying percentages of a given phospholipid homodimer and the corresponding heterodimer. In all cases, dispersions were made using an aqueous solution that was 140 mM in NaCl, 2 mM in NaN_3 and 10 mM in borate buffer (pH 7.4). Prior to monomer exchange, each dispersion was taken to a temperature that was ca. 20 °C above the gel to liquid-crystalline phase-transition temperature of the phospholipid homodimer.[6] Thus, vesicles that were made using **1a**, **1b**, and **1c** were maintained at 40, 60, and 68 °C, respectively. Experimental protocols that were used in forming large unilamellar vesicles, promoting monomer interchange via thiolate−disulfide displacement [after partial reduction (less than 10%) with dithiothreitol], and analyzing dimer distributions by HPLC were similar to those that have been reported.[5,8,9] In all cases, equilibrium was reached within 6 h, as indicated by dimer distributions that became constant with time. Specific values of *K*, the apparent equilibrium constant, which were calculated from observed dimer distributions, were then plotted as a function of the mol % of the sterol monomer present in the membrane (Figure 2).

For bilayers that were made using **1a** and **2**, a random distribution of dimers was found with sterol concentrations that ranged from 9 to 41 mol % at 40 °C; increasing the equilibration temperature to 60 °C gave similar results (not shown). For analogous membranes made from **1b** and **2**, however, a departure from randomness was evident at sterol concentrations that were in excess of ca. 20 mol %. In this case, the sterol and the phospholipid were now favored as nearest-neighbors. A similar trend was observed in the case of membranes made from **1c**, except that the affinity between the sterol and the phospholipid was even greater at the higher sterol concentrations. Despite some scatter in these data, it is clear that *K* increases, significantly, above 4 at high sterol concentrations for membranes derived from **1b** and **1c**. In the presence of 40 mol % of sterol, monomers of **1c** and **2** are favored as nearest-neighbors by a free energy difference of ca. 380 cal/mol.

Additional evidence that sterol−phospholipid affinity increases, as the length of the phospholipid increases, was obtained by carrying out a competition experiment in which the exchangeable sterol produced **3a** and **3c** in the same membrane. Thus, when mixed membranes were prepared using an equimolar quantity of **1a** and **1c** plus an equimolar quantity of **3a** and **3c**, such that the total sterol content was 40 mol %, the equilibrated product mixture showed a molar ratio of **3c**/**3a** of 1.1 at 60 °C; that is, the exchangeable sterol favored association with the longer phospholipid.

The main conclusion that emerges from this study is that significant affinity exists between the palmitoyl (C16)-, and also the stearoyl (C18)-, based phospholipids and the exchangeable form of cholesterol, when the sterol concentration reaches biologically relevant levels. This affinity readily accounts for the long-known, and poorly understood, condensing effect of cholesterol; that is, the uncoiling of the phospholipids is driven by hydrophobic interactions between their acyl chains and the rigid hydrophobic framework of neighboring sterols. At the same time, these findings support the proposal that cholesterol and phospholipids form "complexes" in fluid bilayers.[10−12] Our results indicate, however, that such complexes are likely to form only when those phospholipids are of an appropriate chain length, and only when there is a sufficient sterol concentration present in the membrane.[12] The chain length dependency on phospholipid−sterol affinity that has been observed is best accounted for in terms of attractive hydrophobic interactions between (i) the sterol nucleus and the acyl chains of the phospholipids (the fatty acyl chain is flexible and is able to complement, perfectly, the shape of cholesterol such that the number of hydrophobic contacts is high and the packing is tight) and (ii) the acyl chains of two or more phospholipids that become part of the complex—the longer the acyl chains, the greater the hydrophobic contributions to the stability of the complex. The discontinuity that is apparent in Figure 2, B and C, is a likely consequence of a transition from the liquid-disordered state to a liquid-disordered/liquid-ordered coexistence region, where higher sterol concentrations result in a greater percentage of the membrane being placed in the liquid-ordered phase and where tighter packing leads to a higher value of *K*.

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Supporting Information Available: Synthesis of **2**, **3a**, **3b**, and **3c** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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