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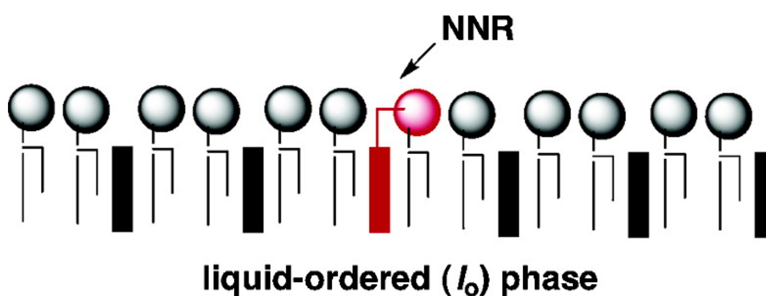

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A Chemical Sensor for the Liquid-Ordered Phase

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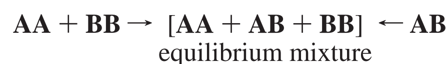
Abstract: The mixing properties of exchangeable phospholipids, derived from 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine, with an exchangeable form of cholesterol have been used to monitor the transition from the liquid-disordered to the liquid-ordered phase in cholesterol-containing bilayers, made from 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine and 1,2-distearoyl-*sn*-glycero-3-phosphocholine, respectively.

Introduction

A modern view of the structure of cellular membranes is one in which the lateral distribution of the lipids is nonrandom.[1] In particular, it is generally presumed that cholesterol associates with high-melting lipids (sphingolipids) to form a "liquid-ordered" (l_o) phase, and that this phase is separated from liquid-disordered (l_d) regions, which are rich in glycerophospholipids.[2−10] It has further been proposed that this liquid-ordered phase is divided into discrete regions ("lipid rafts"), which house signaling proteins.[8,9] Despite the acceptance of the lipid raft hypothesis by a significant segment of the scientific community, the lack of direct evidence has led others to question its validity. One of the early pioneers of the liquid-ordered phase has even quipped, "the latest hype in membrane biology focusing on membrane structure and function in terms of so-called 'rafts' has renewed the interest in coming to grips with one of the most difficult problems in membrane biology concerning the lateral molecular organization in the plane of the membrane and the importance of this organization; e.g., signaling, growth and ligand−receptor interactions".[10]

Our own efforts in this area have focused, sharply, on the use of the nearest-neighbor recognition (NNR) method as a tool for elucidating the two-dimensional structure of fluid bilayers.[11] In essence, NNR measurements provide molecular-level snapshots of bilayer organization by detecting and quantifying the thermodynamic tendency of exchangeable monomers to become nearest-neighbors of one another.[11] Typically, two lipids of interest (**A** and **B**) are converted into exchangeable dimers (**AA**, **AB**, and **BB**), which are then allowed to undergo monomer interchange via thiolate−disulfide interchange. These equilibria are then defined by a constant, *K*, which governs the monomer interchange among **AA**, **BB**, and **AB** (eqs 1 and 2). When monomers **A** and **B** mix ideally, this is reflected by an equilibrium constant that equals 4.0. 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.



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

In one previous NNR investigation, we found that an exchangeable phospholipid (**1**) and an exchangeable form of cholesterol (**2**) mix nearly ideally in fluid bilayers at 60 °C when the sterol concentration is less than ca. 15 mol %.[12] At higher sterol concentrations, however, **1** and **2** became favored nearest-neighbors. Because this change in mixing closely matched the onset of the liquid-ordered phase in bilayers made from 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and cholesterol, and since the melting behavior of **1** and DPPC, and the condensing properties of **2** and cholesterol on fluid bilayers, are nearly identical, we interpreted these results in terms of an analogous phase transition taking place.[12−14] Similar results were

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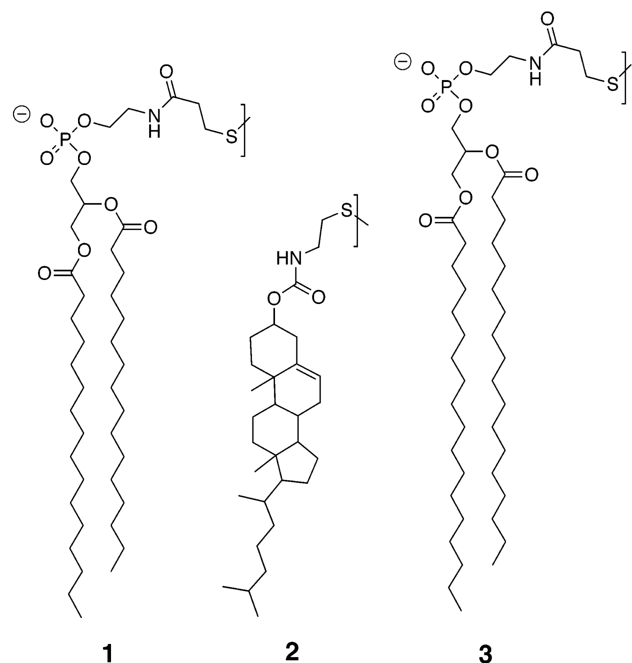
Chart 1



also obtained for the mixing of **2** with a longer homologue, **3**.[12] This apparent connection between nearest-neighbor recognition and a transition from the liquid-disordered to the liquid-ordered phase suggested to us that the NNR method could be used as a *chemical sensor* for detecting and quantifying phase changes taking place in bilayers composed of nonexchangeable phospholipids. In addition, given the high-sensitivity of the nearest-neighbor recognition method, we reasoned that NNR measurements could provide fundamental insight into the liquid-ordered phase by defining the energy involved in its formation. Moreover, such chemical sensing could provide unique insight into the action of a variety of membrane-perturbing agents on the liquid-ordered phase that are of biomedical importance, for example, general anesthetics.[15−18]

With these ideas in mind, we sought to test the feasibility of using the NNR method as a chemical sensor of the liquid-ordered phase. For this purpose, we examined a classic system whose phase behavior has been studied in considerable detail by a variety of techniques, that is, bilayers prepared from DPPC plus varying mole percentages of cholesterol.[13] In addition, we also examined bilayers made from 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) plus cholesterol.[19]

Experimental Section

***N*-[1-(Carboxyethyldithio)-2-ethyl]cholesteryl Carbamate.** To a solution that was made from 3-(2-aminoethyldithio)propanoic acid (300 mg, 1.34 mmol), triethylamine (577 μL, 4.14 mmol), and 15 mL of chloroform was added of cholesteryl chloroformate (743 mg, 1.66 mmol) (Aldrich). After stirring for 12 h, removal of solvent under reduced pressure, and purification by column chromatography [silica gel, starting with chloroform/ethyl acetate (5/1, v/v), followed by chloroform/methanol (10/1, v/v/)] afforded 705 mg (86%) of *N*-[1-(carboxyethyldithio)-2-ethyl]cholesteryl carbamate having R_f = 0.38 [silica gel, chloroform/methanol (10/1, v/v)] and ^{1}H NMR ($CDCl_3$, 500 MHz, rt): δ 5.35 (s, 1H), 5.02 (s, 1H, OCONH), 4.47 (br, 1H), 3.47 (m, 2H), 2.92 (t, 2H), 2.78 (m, 4H), 2.00−2.32 (m, 2H), 1.80−1.97 (m, 5H), 0.83−1.52 (m, 33H), 0.65 (s, 3H).

{1,2}. To a solution that was made by dissolving *N*-[1-(carboxyethyldithio)-2-ethyl]cholesteryl carbamate (42 mg, 0.07 mmol) in 2 mL of chloroform were added *N*-hydroxysuccinimide (NHS) (9 mg, 0.078 mmol) and DCC (16 mg, 0.078 mmol). After stirring for 5 h at room temperature, triethylamine (29 μL, 0.21 mmol) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (50 mg, 0.07 mmol) were added. After stirring for 12 h at room temperature, the solution was diluted with 30 mL of chloroform and washed, sequentially, with 50 mL of aqueous HCl (pH ∼ 2−3) and 30 mL of brine. The organic layer was then separated and concentrated under reduced pressure. Purification by preparative thin-layer chromatography [silica, chloroform/methanol/water (40/10/1, v/v/v)] afforded 65 mg (70%) of {**1**,**2**} having an R_f (0.52) and a ^{1}H NMR spectrum that were identical to those of an authentic sample. ^{1}H NMR ($CDCl_3$, 500 MHz, rt), ^{1}H ($CDCl_3$, 500 MHz, 50 °C): δ 0.67 (s, 3H), 0.85−1.58 (m, 81H), 1.81−1.98 (m, 5H), 2.25−2.32 (m, 6H), 2.61 (t, 2H), 2.81 (t, 2H), 2.96 (t, 2H), 3.46 (br, 4H), 3.92 (br, 4H), 4.15 (m, 1H), 4.38 (m, 1H), 4.46 (m, 1H), 5.20 (s, 1H), 5.35 (br, 2H, =CH + OCONH), 7.40 (br, 1H, CONH).

{1,1}. A solution was prepared from dithiobis(succinimidyl propionate) [Pierce] (20.0 mg, 0.05 mmol), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine [Avanti Polar Lipids] (76.0 mg, 0.11 mmol), triethylamine (15.2 μL), and 5 mL of anhydrous dichloromethane and stirred for 12 h at room temperature. Removal of solvent under reduced pressure followed by purification by preparative thin-layer chromatography [silica, chloroform/methanol/water/30% NH_4OH (65/25/4/1.9, v/v/v/v)] afforded 49.0 mg (64%) of the desired homodimer, {**1**,**1**}, having a ^{1}H NMR spectrum that was identical to that of an authentic sample. ^{1}H NMR ($CDCl_3$, 500 MHz, rt): δ 7.87 (s, 2H), 5.18 (br, 2H), 4.34 (q, 2H), 4.12 (q, 2H), 3.91 (br, 8H), 3.42 (d, 4H), 2.95 (t, 4H), 2.60 (t, 4H), 2.26 (q, 8H), 1.54 (br, 8H), 1.31−1.22 (m, 96H), 0.84 (t, 12H).

Nearest-Neighbor Recognition Measurements. In a typical liposome preparation, thin films of DPPC, cholesterol, and {**1**,**2**} heterodimer or DPPC, cholesterol, {**1**,**1**}, and {**2**,**2**} homodimers were prepared using 16.2 μmol of DPPC, 6.6 μmol of cholesterol, and 0.6 μmol {**1**,**2**} heterodimer or 16.2 μmol of DPPC, 6.6 μmol of cholesterol, 0.3 μmol of {**1**,**1**}, and 0.3 μmol of {**2**,**2**} in chloroform. After drying overnight under reduced pressure, 2.0 mL of 10 mM Tris−HCl buffer (10 mM tris−HCl, 150 mM NaCl, 2 mM NaN_3, and 1 mM EDTA, pH = 7.4) was added to the dried film. The mixture was then vortex-mixed for 1 min and incubated for 5 min at 60 °C, and followed by another 1 min of vortex and another 20 min incubation. The dispersion was then subjected to a freeze/thaw cycle for five times in liquid nitrogen and 60 °C water, followed by sequential extrusion through 400 and 200 nm membranes (10 times for each membrane). After extrusion, the dispersion was incubated for about 30 min and then started the thiolate disulfide exchange reaction.

Thiolate−disulfide interchange reactions were initiated after the dispersions were equilibrated at 60 °C by injecting 25.5 μL of a Tris buffer solution of 37.65 mM *threo*-dithiothreitol (0.96 μmol) and 24 μL of a Tris buffer solution that was 8.4 μM in monensin (0.204 μmol), with brief vortex mixing, and finally increasing the pH to 8.5 via addition of ca. 10 μL of 1.0 M NaOH. All dispersions were maintained under an argon atmosphere throughout the course of the interchange reactions. Aliquots (0.30 mL) were withdrawn as a function of time and quenched with 85 μL of 30 mM HCl (final pH 5.0). After removal

(14) Previous high-sensitivity differential scanning calorimetry measurements have shown that the melting properties of homodimers of **1** closely match those of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine and 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylglyerol: Krisovitch, S. M.; Regen, S. L. *J. Am. Chem. Soc.* **1992**, *114*, 9828−9835.

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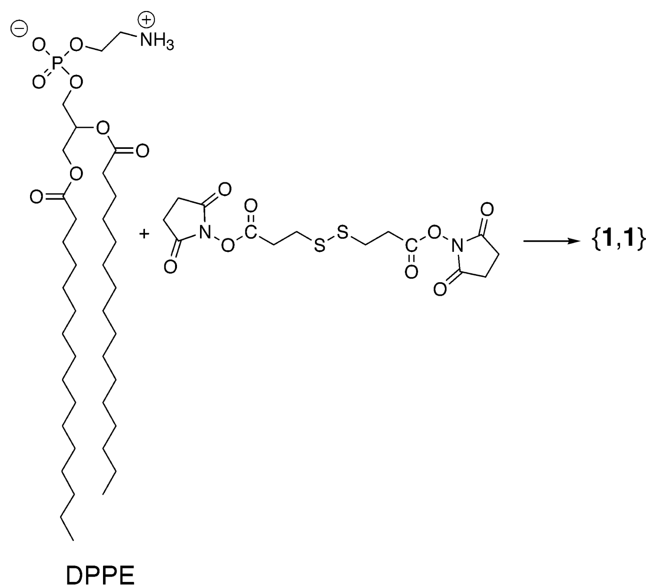
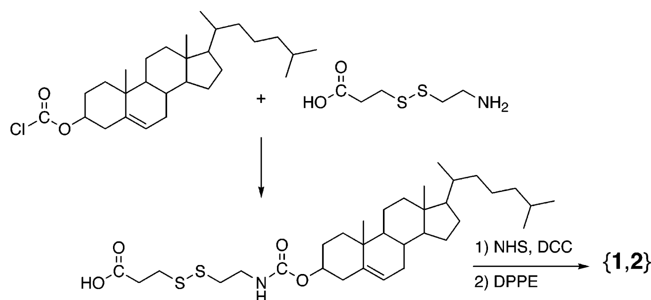
Scheme 1



Scheme 2



of water under reduced pressure, the residue was dissolved in 2 mL of chloroform and centrifuged for 20 min. The chloroform layer was then removed under reduced pressure to yield a clear film, which was, subsequently, dissolved in 10 μL of chloroform and 90 μL of the mobile phase that was used for HPLC analysis. Product mixtures were analyzed by C18 reverse phase HPLC using a mobile phase that was composed of 10 mM tetrabutylammonium acetate in denatured ethanol/water/hexane (76/13/10, v/v/v) with flow rate of 0.9 mL/min. The column was maintained at 31 °C, and the components were monitored at 205 nm by a Waters 996-photodiode-array UV detector.

Results and Discussion

Chemical Sensing of the Liquid-Ordered Phase. To test whether the mixing of **1** and **2** can sense changes in the phase of a host membrane derived from natural lipids, we investigated bilayers composed of 2.5 mol % of **1**, 2.5 mol % of **2**, and 95 mol % of varying molar ratios of DPPC/cholesterol. Thus, in contrast to our previous studies, where both the molar ratio of **1**/**2** and the phase of the membrane were varied at the same time, the present experiments allow us to keep the exchangeable lipid composition constant and to vary only the composition and phase of the host membrane.

The homodimer of **1** (i.e., {**1**,**1**}) that was used in this work was prepared by an improved method of synthesis. Specifically, direct acylation of two molecules of 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) with dithiobis[succinimidyl propionate] (DSP) afforded the desired homodimer in a single step (Scheme 1).[20] The corresponding heterodimer {**1**,**2**} was also prepared by an improved procedure in which cholesteryl chloroformate was first condensed with 3-(aminoethyldithio)-propanoic acid and the product then condensed with DPPE (Scheme 2).[21] The requisite sterol homodimer,{**2**,**2**}, was obtained using methods similar to those previously described.[12]

Protocols that were used for carrying out NNR exchange reactions in vesicles prepared via extrusion, and for analyzing dimer compositions, are described in the Experimental Section. Thus, in a typical NNR experiment in which the total sterol content was 30 mol %, vesicles were prepared from 16.2 μmol of DPPC, 6.6 μmol of cholesterol, and 0.6 μmol of {**1**,**2**}. Thiolate−disulfide interchange was then performed at 60 °C and the dimer composition monitored as a function of time. To ensure that equilibrium was reached in every case, similar experiments were carried out using the same DPPC and cholesterol content, but now the heterodimer was replaced with 0.3 μmol of {**1**,**1**} and 0.3 μmol of {**2**,**2**}. Equilibrium constants were then calculated using average dimer compositions that were determined from *both* sets of data. Typically, six different time intervals (i.e., 12 sets of data) were used to obtain each value of *K* and one standard deviation.

A plot of *K* versus the mol % of total sterol present is shown in Figure 1. In this plot, solid lines have been included to help guide the eye; the dashed lines represent compositions in which abrupt changes in the dependency of *K* on the mol % of sterol have taken place. As is readily apparent, the value of *K* sharply increases as the sterol concentration is raised beyond ca. 14 mol %. In addition, *K* levels off at ca. 5.5 when the sterol concentration reaches ca. 30 mol %. This behavior parallels, exactly, the onset of the liquid-ordered phase (ca. 14 mol % of cholesterol) and the transition from the liquid-disordered/liquid-ordered coexistence region to the liquid-ordered region (ca. 30 mol % of cholesterol) for DPPC/cholesterol.[13] Thus, the results that are presented in Figure 1, together with the well-established phase diagram for DPPC/cholesterol, show that the mixing of **1** and **2** is directly linked to the phase of its host membrane, and that association between **1** and **2** is favored in the liquid-ordered phase. These results also indicate that there is significant mixing between the exchangeable and nonexchangeable lipids.

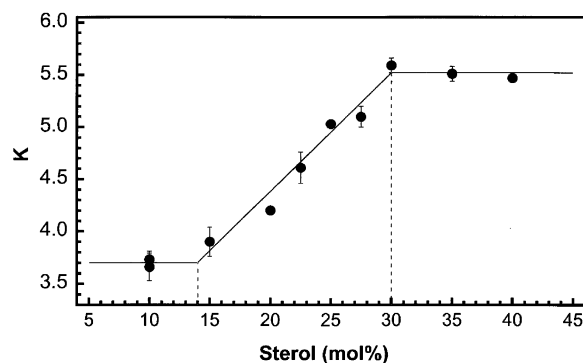
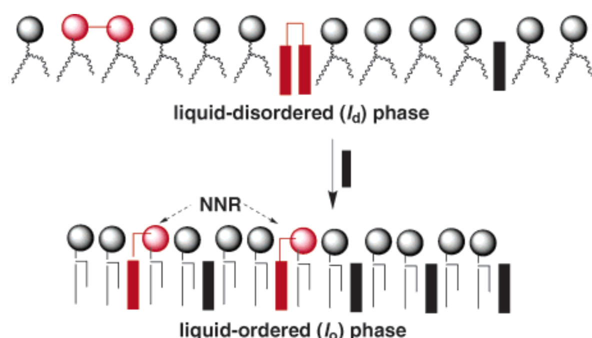


Figure 1. Plot of *K* (60 °C) versus the mol % of total sterol present in bilayers made from 95 mol % of nonexchangeable lipids (i.e., DPPC plus varying percentages of cholesterol) and 5 mol % of exchangeable lipids (2.5 mol % of **1** and 2.5 mol % of **2**). In all cases, equilibrium was reached within 2 h.

(20) An improved synthesis of related phospholipid heterodimers has recently been reported elsewhere: Zhang, J.; Jing, B.; Tokutake, N.; Regen, S. L. *Biochemistry* **2005**, *44*, 3598−3603.
(21) 3-(Aminoethyldithio)propanoic acid was synthesized using established methods: Marchand-Brynaert, J.; Bouehet, M.; Touillauxc, R.; Beauve, C.; Fastrez, J. *Tetrahedron* **1996**, *52*, 5591−5606.

Scheme 3



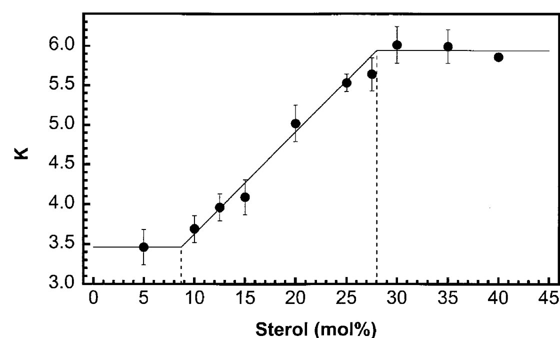


Figure 2. Plot of K (60 °C) versus the mol % of total sterol present in bilayers made from 95 mol % of nonexchangeable lipids (i.e., DSPC plus varying percentages of cholesterol) and 5 mol % of exchangeable lipids (2.5 mol % of **2** and 2.5 mol % of **3**). In all cases, equilibrium was reached within 2 h.

On the basis of the difference in K that is observed, on going from the liquid-disordered to the liquid-ordered region, the energy involved in forming the liquid-ordered phase can be estimated to be ca. $\Delta G = -135$ cal/mol of dimer at 60 °C. A stylized illustration of the effect of cholesterol in promoting the formation of {**1**,**2**} and the liquid-ordered phase is shown in Scheme 3. Here, the solid (red) rectangles represent the sterol nucleus of **2**, and the solid (red) circles represent the polar headgroup of **1**; the black rectangles and black circles are those of the corresponding natural lipids.

Analogous experiments that were carried out for the mixing of **2** and **3** in bilayers made from cholesterol and DSPC gave similar results (Figure 2). In this case, however, the transition from the liquid-disordered to the liquid-disordered/liquid-ordered coexistence region and also the transition from the liquid-disordered/liquid-ordered coexistence region to the liquid-ordered phase appear to take place at slightly lower sterol concentrations, which is consistent with the phase diagram that has been reported for cholesterol/DSPC membranes based on ESR measurements.[19]

Implications Regarding the Formation of Lipid Aggregates in the Liquid-Ordered Phase and Cholesterol Dimers in the Liquid-Disordered Phase. Although these NNR measurements, by themselves, do not define the average size of the lipid aggregates that are formed, they do suggest a *minimum* value in the liquid-ordered phase. In particular, the fact that {**1**,**2**} and {**2**,**3**} are more stable as the bilayer becomes richer in cholesterol, implies that a microdomain that contains these heterodimers also contains at least one cholesterol molecule. Since the liquid-ordered phase is fully formed at ca. 30 mol % of cholesterol for both DPPC and DSPC, this further suggests that there is a preference for phospholipid/cholesterol clustering in a molar ratio of 2/1. Taken together, these results suggest, therefore, that the minimum size of a microdomain for both systems consists of two sterols plus four phospholipids, for a total of *six* lipids.[13,19]

Finally, the slight preference that we have observed for homodimer formation at low sterol concentrations in the liquid-disordered phase is noteworthy. This finding is fully consistent with previous differential scanning calorimetry results for DPPC/cholesterol membranes, which supported the existence of sterol dimers.[22] Our results, however, indicate that cholesterol dimers are favored *within the same leaflet* and not across the bilayer, as was previously proposed.[22] The crossover from favored sterol−sterol association to favored sterol−phospholipid association with increasing the sterol concentrations further implies that the preference for sterols and phospholipids to become nearest-neighbors and the stability of the liquid-ordered phase are due, in large part, to long-range interactions; that is, associative interactions that extend beyond two nearest-neighbors.

Conclusions

Nearest-neighbor recognition measurements provide a sensitive means for monitoring the transition from the liquid-disordered to the liquid-ordered phase in cholesterol-containing bilayers derived from DPPC, and also from DSPC. On the basis of a growing body of evidence, which supports the hypothesis that signaling proteins are housed in liquid-ordered regions of biological membranes, the question of how membrane-active drugs influence sterol−phospholipid association becomes especially relevant.[6−11] In this regard, NNR measurements of the type reported herein offer a unique opportunity for addressing this question in a direct and quantitative way.

Acknowledgment. We are grateful to the National Institutes of Health (PHS GM56149) for support of this research.

Supporting Information Available: Tables of equilibrium dimer compositions used to calculate K values (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA0513988

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