Cholesterol Interactions with Fluid-Phase Phospholipids: Effect on the Lateral Organization of the Bilayer

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ABSTRACT The lateral organization of lipids and proteins in cell membranes is recognized as an important factor in several cellular processes. Cholesterol is thought to function as a modulator of the lateral segregation of lipids into cholesterol-poor and cholesterol-rich domains. We investigated how the affinity of cholesterol for different phospholipids, as seen in cholesterol partitioning between methyl-β-cyclodextrin and large unilamellar vesicles, was reflected in the lateral organization of lipids in complex bilayers. We especially wanted to determine how the low-T_m lipid affected the lateral structure. Partition experiments showed that cholesterol had a higher affinity for *N*-oleoyl-sphingomyelin (OSM) than for palmitoyl-oleoyl-phosphatidylcholine (POPC) bilayers, but the highest preference was for *N*-palmitoyl-sphingomyelin (PSM)-containing bilayers. Partial phase diagrams of POPC/PSM/cholesterol and OSM/PSM/cholesterol bilayers at 23°C and 37°C were used to gain insight into the lateral organization of lipids in bilayers. Analysis of phase diagrams revealed that the phospholipid composition of cholesterol-poor and cholesterol-rich domains reflected the affinity that cholesterol exhibited toward bilayers composed of different lipids. Therefore, the determined affinity of cholesterol for different phospholipid bilayers was useful in predicting the cholesterol-induced lateral segregation of lipids in complex bilayers.

INTRODUCTION

The heterogeneous lateral organization of lipids and proteins in membranes is recognized as an essential feature of cell membranes in processes such as cell signaling, protein and lipid sorting, and interactions between cells and various pathogens, as reviewed recently (1,2). In mammalian cell membranes, cholesterol was suggested to function as a major modulator of lipid lateral segregation and packing density. Because of its flat and rigid ring structure and small polar headgroup, cholesterol is well-suited for close contact with phospholipids that have saturated acyl chains, and is thus able to influence the packing of surrounding lipids. In lipid-model bilayers, close contact between the sterol and adjacent phospholipids results in the formation of the so-called liquid-ordered (l_o) phase (3). This phase state was also suggested to be relevant for the lipid portion of cholesterol-containing cellular membranes.

A number of studies have been focused on the effects of lipid properties and composition on lateral heterogeneity in lipid bilayer membranes. Ternary bilayers containing a low-T_m lipid, a high-T_m lipid, and cholesterol are commonly used as simple models of cell membranes. Studies based on fluorescence and atomic-force microscopy, NMR, calorimetry, and fluorescence measurements, with their specific resolution, have provided useful knowledge on domain formation and phase coexistence in bilayers containing biologically relevant lipids. Previous studies have demonstrated that macroscopic liquid-liquid phase separation occurs in cholesterol-containing bilayers in which the low-T_m lipid and the high-T_m lipid are represented by two phosphatidylcholines, or one phosphatidylcholine (PC) and one sphingomyelin (SM), which differ significantly in the number of *cis*-unsaturations, chain lengths, or branching, as reviewed recently (4,5). In the plasma-membrane outer leaflet, phosphatidylcholines commonly have a saturated *sn-1* chain and an unsaturated *sn-2* chain, whereas the amide-linked acyl chain of sphingomyelins is generally saturated. Ternary mixtures of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), *N*-palmitoyl-D-*erythro*-sphingosylphosphorylcholine (PSM), and cholesterol thus belong to the biologically more relevant simple models of the plasma-membrane outer leaflet. Based on fluorescence and NMR measurements, mixtures of POPC, PSM, and cholesterol were demonstrated to separate laterally, and to form segregated domain sizes in the nanometer range (6–8). Such bilayers apparently contain domains rich in PSM and cholesterol, and domains enriched in POPC, respectively (7).

Domain size and demixing temperatures appear to be connected to the relative properties of the low-T_m and the high-T_m lipids, as illustrated by a recent fluorescence and atomic force microscopy study in which the length of phosphatidylcholine monounsaturated acyl chains in ternary PC/*N*-stearoyl SM/cholesterol bilayers was varied (9). The dependence of domain formation on low-T_m lipid acyl-chain unsaturation is indicated by the finding that micrometer-scale domains can be detected for a much larger compositional and thermal range when 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) replaces POPC as the low-T_m lipid in cholesterol-containing ternary bilayers (10). Phospholipids with polyunsaturated acyl chains were proposed to support the lateral segregation of membrane lipids in multicomponent mixtures as a conse-

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quence of their low miscibility with saturated phospholipid, and of their inability to create close interactions with cholesterol (11–13). This is in line with pulsed-field gradient NMR experiments on the role of lipid acyl-chain unsaturation in lipid lateral diffusion (14), where PC/egg-SM/cholesterol bilayers showed a clear separation into two diffusion rate components, with *sn-2*-polyunsaturated PC indicating liquid-liquid phase separation, whereas with mono- or diunsaturated PC, only one diffusion rate component could be discerned. A recent fluorescence quenching study showed the effect of many unsaturated glycerophospholipids with different degrees of unsaturation and different headgroups on domain formation by cholesterol and DPPC (15). It was concluded that there are different mechanisms by which low-T_m lipids can stabilize ordered domains formed by high-T_m lipids and cholesterol, depending on both the headgroup and the hydrophobic portion of the low-T_m lipid molecule in a nonadditive way. Based on the above studies, it can be concluded that the lipid in liquid crystalline (l_d) phase is of critical importance for lateral domain formation and for cholesterol partitioning between domains in ternary mixed-lipid bilayers.

This study investigated how the affinity of cholesterol for different phospholipids, as seen in cholesterol partitioning between methyl-β-cyclodextrin (mβCD) and large unilamellar vesicles (LUVs), is reflected in the lateral organization of lipid bilayers. In the partition experiments, cholesterol had a high affinity for *N*-oleoyl-D-*erythro*-sphingosylphosphorylcholine (OSM) bilayers compared with POPC bilayers, but the highest preference was evident in PSM-containing bilayers. An analysis of binary and ternary phase diagrams, determined with different fluorescence methods, showed that the lateral organization in bilayers reflected the results of the partition study.

EXPERIMENTAL PROCEDURES

Materials

The PSM was purified from egg-yolk sphingomyelin (Avanti Polar Lipids, Alabaster, AL) by reverse-phase high-performance liquid chromatography (dimensions of 250 × 21.2 mm, 5-μm particle size, Supelco (Bellefonte, PA) Discovery C18 column), using methanol/water (95:5, volume ratio) as the eluent. The POPC and OSM were obtained from Avanti Polar Lipids. Cholesterol was purchased from Sigma Chemicals (St. Louis, MO), and the ^{3}H-labeled cholesterol from Perkin-Elmer (Waltham, MA). The 1,6-diphenyl-1,3,5-hexatriene (DPH) was obtained from Molecular Probes (Eugene, OR). We produced *trans*-parinaric acid (tPA) through an iodine-catalyzed isomerization reaction of *cis*-parinaric acid (Cayman Chemical, Ann Arbor, MI). The procedure was based on the method by Sklar et al. (16). The product was purified by reverse-phase high-performance liquid chromatography (same column as above), using methanol/water/acetic acid (900:50:3, volume ratio) as the eluent. The concentrations of fluorophore stock solutions were determined according to previously reported extinction coefficients (DPH, 88,000 $cm^{-1}M^{-1}$ at 350 nm in methanol, as reported by the vendor; tPA, 92,000 $cm^{-1}M^{-1}$ at 299 nm in methanol (17)). Phospholipid and cholesterol stock solutions were prepared in hexane/2-propanol (3:2, volume ratio). Solutions were stored at −20°C, and warmed to ambient temperature before use. The water used in all experiments was purified by reverse osmosis, followed by passage through a Millipore UF Plus water-purification system (Millipore, Billerica, MA), to yield a product with a resistivity of 18.2 MΩcm.

Partitioning of ^{3}H-labeled cholesterol between methyl-β-cyclodextrin and phospholipid vesicles

The distribution of cholesterol between methyl-β-cyclodextrin (mβCD; Fluka, Buchs, Switzerland) and phospholipid vesicles was determined according to the procedure of Niu and Litman (18). Briefly, vesicles were prepared by filtering a lipid suspension 10 times through a filter with a pore size of 200 nm (Whatman International, Maidstone, UK). Complexes of cholesterol (including a small portion of ^{3}H-labeled cholesterol) and mβCD were formed at a molar ratio of 1:50. Phospholipid vesicles and mβCD/cholesterol were incubated on a shaker for 2 h at 37°C. The phospholipid, mβCD, and cholesterol concentrations in the mix were 1 mmol/L, 10 mmol/L, and 0.2 mmol/L, respectively. Vesicles were separated from mβCD by filtration through Microcon YM-30 filters (Millipore) under centrifugation at 12,000 × g and 37°C. The equilibrium distribution of cholesterol between phospholipid vesicles and mβCD was determined by measuring the relative radioactivity of ^{3}H-labeled cholesterol in the material retained on the filter (in vesicles) and in the filtrate (in complex with mβCD). Partitioning experiments were performed using Tris buffer (10 mmol/L Tris, 140 mmol/L NaCl, pH 7.4). The mole fraction partition coefficient (K_x) was calculated according to Tsamaloukas et al. (19), assuming a 1:2 cholesterol/cyclodextrin stoichiometry in the complexes:

$$K_x = \frac{C_{CHOL}^{LUV}(C_{CD})^2}{(C_L + C_{CHOL}^{LUV})C_{CHOL}^{CD}} \quad (1)$$

where C_L is the phospholipid concentration, C_{CD} is the cyclodextrin concentration, C_{CHOL}^{LUV} is the cholesterol concentration in lipid bilayers, and C_{CHOL}^{CD} is the concentration of cholesterol in complex with mβCD. The partitioning of cholesterol between vesicles composed of different phospholipids was calculated as:

$$K_A^B = \frac{K_x^B}{K_x^A} \quad (2)$$

where K_x^A and K_x^B are the partitioning coefficients between cyclodextrin and vesicles of type A and B, respectively (18).

Fluorescence experiments

The appropriate amounts of lipids dissolved in organic solvent were mixed, and the solvent was evaporated and the dry samples were kept in high vacuum for at least 2 h. The lipids were hydrated in water at a temperature above the gel-to-liquid-crystalline phase-transition temperature of the lipid component with the highest transition temperature. When tPA was included, argon-saturated water was used to minimize oxidation of the probe. Multilamellar vesicles were prepared by vortexing the samples at a temperature above T_m. The vesicle suspensions analyzed using fluorimetry were further bath-sonicatated (Branson 2510, Branson Ultrasonics, Danbury, CT) at ~70°C for a short time.

The DPH anisotropy measurements were performed on a PTI Quanta-Master-2 spectrofluorimeter (Photon Technology International, Lawrenceville, NJ), operating in the T format. Samples were scanned within the indicated temperature ranges at a temperature rate of 1°C/min. The excitation and emission wavelengths were 358 and 430 nm, respectively, and all slits were set to a width of 5 nm. The G value was determined before each measurement. The DPH/lipid ratio was 1:200, and the total lipid concentration was 100 μmol/L.

The fluorescence decay, steady-state anisotropy, and intensity of tPA in phospholipid-containing and cholesterol-containing bilayers were measured to determine the boundaries between gel phase (s_o)-containing and gel phase-free areas in the ternary-phase diagrams. Lifetime-based quantum yield was

calculated from the time-resolved data according to Lakowicz (20). Time-resolved fluorescence data were collected using a PTI TimeMaster fluorimeter (Photon Technology International, Lawrenceville, NJ), with a nitrogen laser as the light source. Rhodamine 6G (Invitrogen, Carlsbad, CA) was used as the laser dye, and the laser output was frequency-doubled. The steady-state fluorescence anisotropy and intensity were analyzed using a PTI QuantaMaster-2 spectrofluorimeter, operating in the T format. In time-resolved experiments, samples were excited at 295 nm and emission was measured at 405 nm, and in steady-state experiments, the excitation and emission wavelengths were set to 305 nm and 430 nm, respectively. The total lipid concentration was 200 μmol/L, and the tPA/lipid ratio was 1:200. Samples with a constant X_{CHOL} of 0.1–0.2 and X_{PSM} between 0.05–0.80 were prepared, and measurements were performed at 23°C and 37°C.

Preparation of phase diagrams

The data obtained in fluorescence experiments were used to prepare phase diagrams. Boundaries between regions with different properties in the composition space were determined by measuring composition series that were expected to cross these boundaries. By plotting the resulting fluorescence data as a function of composition, the boundaries could be determined from clear changes in the fluorescence signal. This procedure is described in more detail in Results. The distinct regions in the resulting phase diagram are pure phases (l_d, l_o, and s_o) or regions where two phases coexist (l_d-s_o and s_{o1}-s_{o2}), or else the regions between the l_d and l_o phases are heterogeneous, containing various amounts of l_o-like domains of different sizes (7,8).

RESULTS

Cholesterol-partitioning between mβCD and phospholipid bilayers

To obtain information about interactions between cholesterol and sphingomyelins or phosphatidylcholines in fluid bilayers, the partitioning of ^{3}H-cholesterol between mβCD and LUVs was studied, using the approach of Niu and Litman (18). Cholesterol-partitioning between mβCD and pure OSM or POPC bilayers, and binary mixtures (2:1, molar ratio) of an unsaturated lipid (OSM or POPC) and a saturated phospholipid (PSM or DPPC), was studied at 37°C. The obtained partition coefficients (K_x) for cholesterol equilibration between mβCD and the different phospholipid bilayers are shown in Fig. 1. Based on the results, it is clear that cholesterol had a higher affinity for OSM than for POPC bilayers. When Eq. 2 was used to calculate the partition coefficient for cholesterol equilibration between OSM and POPC LUVs, a K_{POPC}^{OSM} of 1.86 was obtained, and the free energy (ΔG) for transfer of cholesterol from POPC to OSM LUVs was −0.38 kcal/mol, indicating that cholesterol had a clear preference for OSM over POPC. The addition of 33 mol % saturated phospholipid to the fluid LUVs increased cholesterol's affinity for lipid bilayers, as expected according to previous observations (18,19,21). However, cholesterol still had a higher affinity for pure OSM than for the POPC/DPPC bilayers ($K_{PC}^{OSM} = 1.36$), and the addition of 33 mol % PSM had a much greater effect on cholesterol's affinity for LUVs than did the addition of the same amount of DPPC. The partition coefficient ($K_{POPC/DPPC}^{POPC/PSM}$) for the distribution of cholesterol between POPC/PSM and POPC/DPPC vesicles was calculated at 1.96. Hence, it is clear that the association of cholesterol with a bilayer was significantly stabilized by SM compared with PC, irrespective of whether the lipids had saturated or unsaturated acyl chains. These results are in agreement with previous studies (18,22–24).

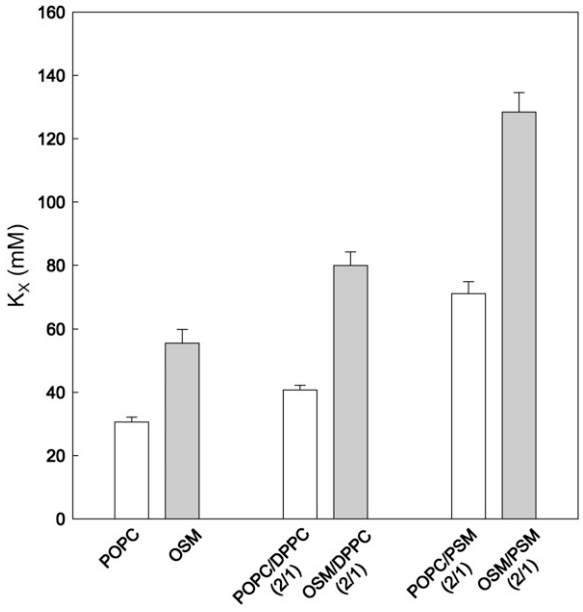

FIGURE 1 Partitioning of cholesterol between mβCD and phospholipid vesicles. The equilibrium distribution of ^{3}H-labeled cholesterol between large unilamellar vesicles and mβCD at 37°C is described using partition coefficients.

Lateral heterogeneity in bilayers of cholesterol and OSM or POPC

The effect of cholesterol on the lateral organization of OSM and POPC bilayers was studied by measuring DPH anisotropy in POPC and OSM bilayers as a function of cholesterol concentration, as described by de Almeida et al. (7). The steady-state anisotropy of DPH (0.5 mol %) in samples composed of OSM or POPC with 0–57 mol % cholesterol was measured as a function of temperature between 10–50°C. Representative data are shown in Fig. 2. When anisotropy was plotted versus mol fraction cholesterol, the obtained curve consisted of two plateau regions at low and high cholesterol concentrations, separated by a region where anisotropy increased as a linear function of cholesterol concentration. The two plateau regions at low and high cholesterol concentrations are interpreted as more or less homogenous bilayers, whereas the intermediate linearly rising region is assumed to be the result of a heterogeneous organization (possibly ordered and disordered domains) of lipids in bilayers (7). Therefore, the starting and end points of this central region of the curves withhold information about interactions between cholesterol and phospholipids. These points were resolved by performing a linear fit to the three regions, and determining

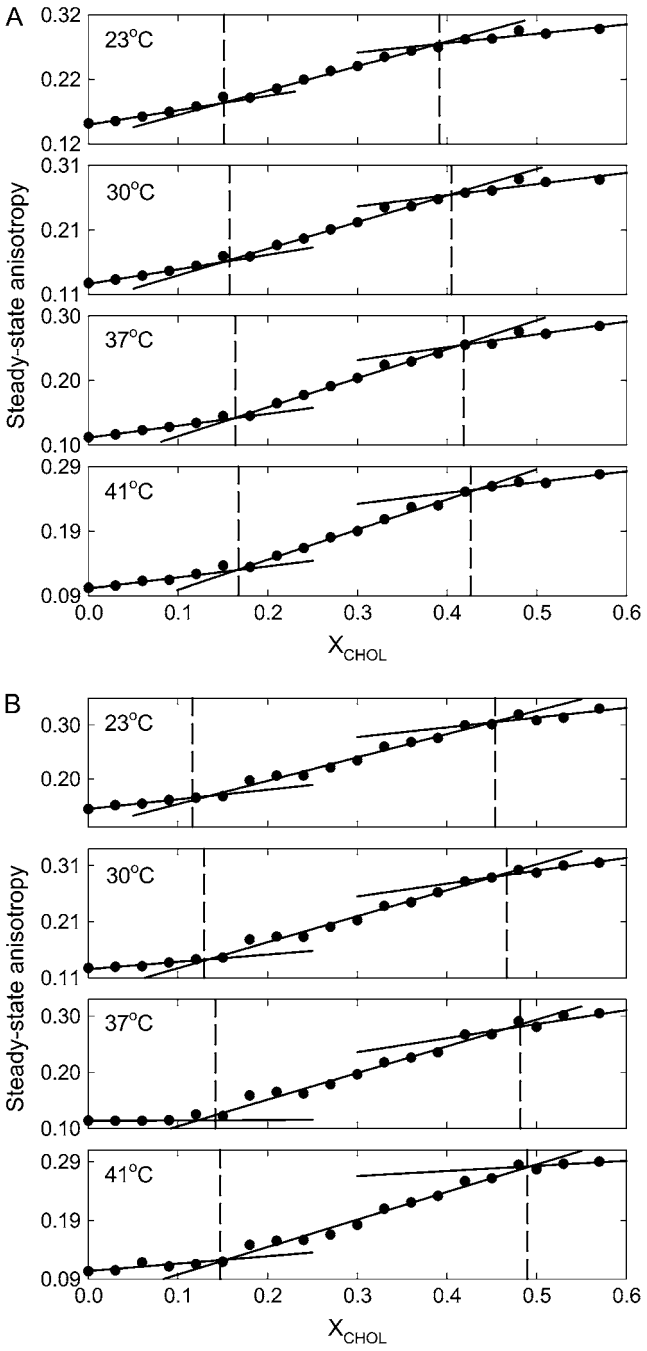

FIGURE 2 Representative DPH anisotropy data used for determination of phase boundaries in binary OSM/cholesterol and POPC/cholesterol bilayers. Boundaries were determined at temperatures between 15–41°C for OSM/cholesterol (*A*) or POPC/cholesterol (*B*) bilayers. Linear fitting of DPH anisotropy data revealed three regimes, corresponding to an l_d-phase region, a heterogeneous region containing cholesterol-poor and cholesterol-rich domains, and an l_o-phase region at low, intermediate, and high cholesterol concentrations, respectively. Dashed lines represent phase boundaries from the fitted line in Fig. 3.

the crossing points of the lines. The resulting points are shown in Fig. 3. In both OSM/cholesterol and POPC/cholesterol bilayers, the boundaries of the intermediate slope region showed a fairly good linear dependence on temperature

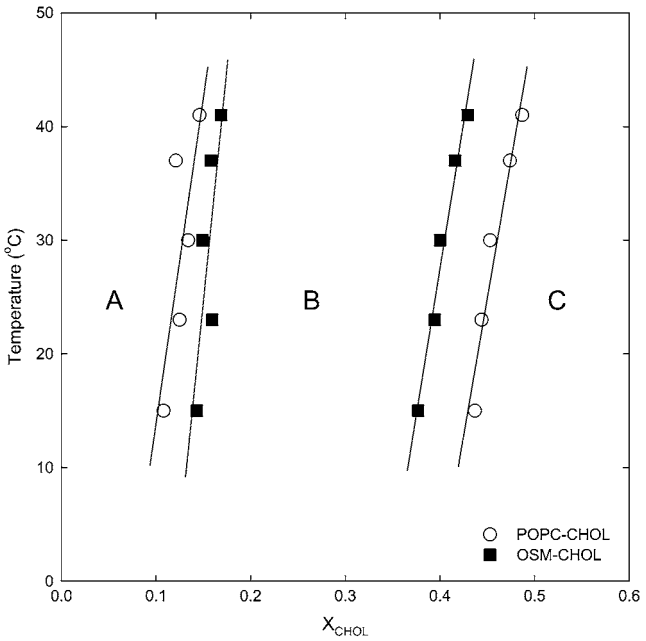

FIGURE 3 Binary OSM/cholesterol and POPC/cholesterol phase diagrams. The boundaries between regions *A*, *B*, and *C* were based on the dependence of DPH anisotropy on bilayer composition. Linear regressions for each data set are shown as solid lines. In region *A*, bilayers are in l_d phase. In region *B*, l_d-phase bilayers contain an increasing number of cholesterol-rich domains. In region *C*, bilayers are in l_o phase. CHOL, cholesterol.

within the studied temperature range, suggesting that more cholesterol is needed to create ordered domains in the bilayer and to form a homogenous high-order bilayer at higher temperatures.

The boundary at high X_{CHOL} predicts the formation of a homogenous, ordered liquid bilayer that should be analogous with the l_o phase. Based on the DPH anisotropy functions, it therefore seems that more cholesterol is needed to form a uniform l_o phase in POPC bilayers than in OSM bilayers. The lower boundary, at low cholesterol concentrations, is interpreted as the boundary where the bilayer starts to become heterogeneous, and where small, dynamic ordered domains enriched in cholesterol start to form. In POPC bilayers, the low X_{CHOL} boundary appears at a lower cholesterol concentration than in OSM bilayers, suggesting that the OSM bilayer remains homogenous up to a higher cholesterol concentration than do POPC bilayers.

Phase behavior of PSM/OSM and PSM/POPC bilayers

In multicomponent membranes, lateral heterogeneity and phase behavior are expected to be a result of the interplay between all lipids present. To gain better insight into how OSM and POPC interact with PSM, the phase behavior of binary mixtures of PSM and either OSM or POPC was examined, using the DPH anisotropy approach. In addition, a

few samples were analyzed using differential scanning calorimetry, to verify the results of fluorescence anisotropy experiments (the agreement was good; results are not shown). Representative results for pure phospholipid bilayers are shown in Fig. 4. In pure PSM bilayers, the anisotropy of DPH was ~0.37 up to ~40–42°C, at which temperature the gel-to-liquid-crystalline phase transition was clearly visible as a dramatic drop in anisotropy. In pure POPC bilayers, a similar transition was observed at ~−2°C, in agreement with previous results (25). In pure OSM bilayers, no phase transition was observed between −5°C and 60°C using DPH anisotropy. The DPH anisotropy showed a temperature dependence in the pure OSM bilayers similar to that in the POPC bilayers in the liquid-crystalline state. In POPC/PSM bilayers, a single phase transition was observed in all samples, except in the samples containing 20–50 mol % PSM (two transitions detected), indicating gel-phase immiscibility of POPC and PSM. For the OSM/PSM bilayers, only one phase transition was observed within the studied temperature range. Because we do not have the T_m of OSM melting, the OSM/PSM phase diagram is incomplete at lower temperatures. A closer examination of phase boundaries at 23°C and 37°C in Fig. 5 reveals that the two-phase region is shifted to a lower X_{PSM} in OSM/PSM bilayers compared with POPC/PSM bilayers, suggesting that PSM has a lower solubility in an OSM-enriched l_d phase than in a POPC-enriched l_d phase. On the other hand, OSM seems to exhibit higher solubility in the PSM-rich s_o phase than does POPC, because a complete gel phase is formed at lower PSM concentrations with OSM. Both of these observations can be explained by the higher degree of order in the OSM bilayers than in the POPC bilayers, which favors the formation of the s_o phase.

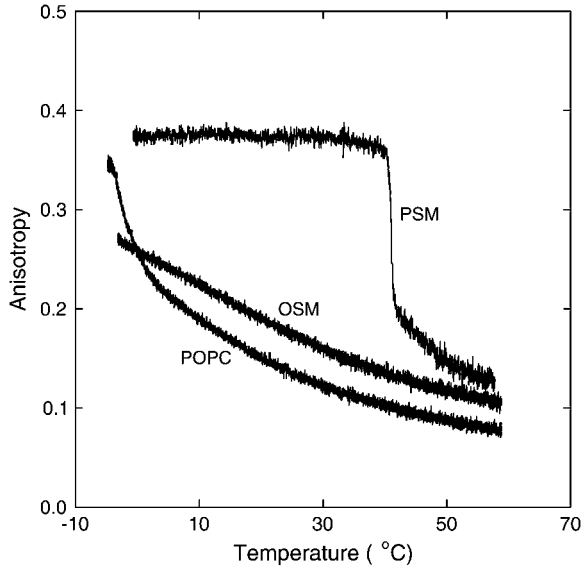

FIGURE 4 Steady-state anisotropy of DPH as a function of temperature in pure phospholipid bilayers.

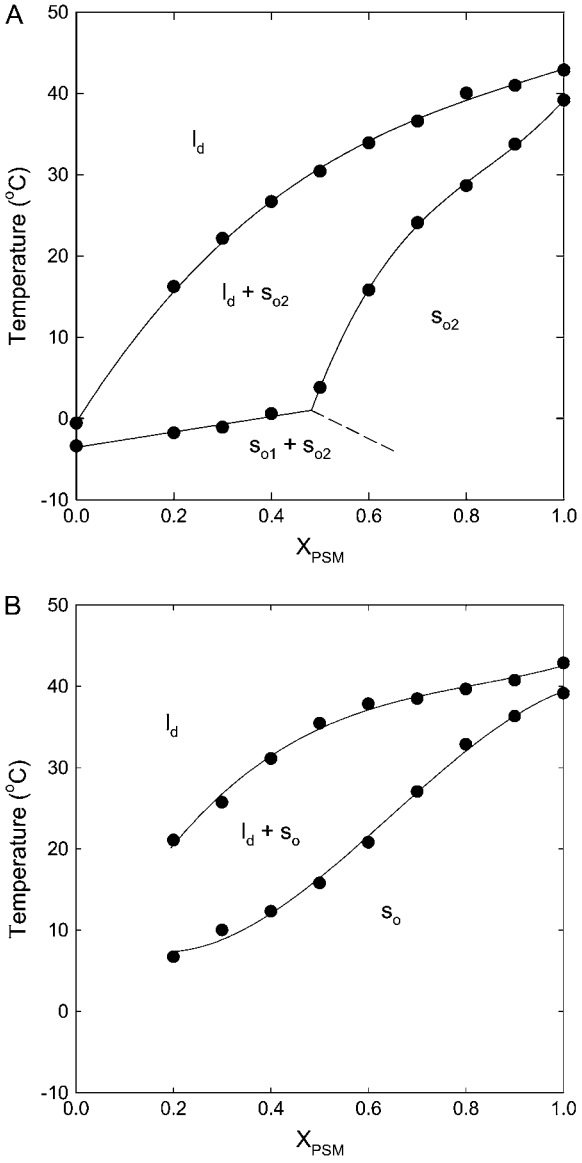

FIGURE 5 Binary-phase diagrams. Phase boundaries were based on the dependence of DPH anisotropy on POPC/PSM (*A*) and OSM/PSM (*B*) bilayers.

Determination of the onset of gel-phase formation in ternary systems

The focus of this project was on fluid phospholipid bilayers. However, we needed information about where in the composition space the gel phase starts to form: first, to be able to focus on the all fluid lipid compositions, but also because the onset of s_o phase formation gives information about the interactions of cholesterol with the phospholipids. For these determinations, two different approaches were used. At low X_{CHOL} ($\leq$0.05), where the bilayer was expected to be in a homogenous fluid phase before gel-phase formation, we applied the DPH anisotropy approach, which was also used for l_d-s_o phase boundary determination in binary bilayers. The

DPH anisotropy was measured as a function of temperature in samples with ≤ 0.05 X_{CHOL} and varying fractions of PSM and POPC or OSM (results not shown), after which the phase boundaries were determined as described above (shown in Fig. 8).

At higher X_{CHOL}, in samples expected to form a heterogeneous fluid phase, the onset of gel-phase formation was determined using tPA. The fluorescence properties of this probe are known to be sensitive to the lipid environment (16,26,27), and tPA was shown to have a high gel-phase affinity, whereas it partitions rather equally between cholesterol-rich and cholesterol-poor domains (27,28). Representative results from these experiments are shown in Fig. 6. The lifetime-based quantum yield, fluorescence intensity, and anisotropy increased slightly as a linear function of PSM concentration up to a point, after which the increase was more strongly dependent on X_{PSM}. This marked change in fluorescence properties originated in the formation of the gel phase, and the boundary was determined as the point of intercept between the two linear functions obtained. The intercepts determined at 23°C and 37°C at X_{CHOL} from 0.1–0.2 are plotted in Fig. 7. As shown in Fig. 7, the effect of cholesterol on the gel-phase boundary was dependent on temperature. At 23°C, onset of the gel phase was clearly shifted toward higher X_{PSM} with increasing X_{CHOL}, whereas at 37°C, the gel-phase onset boundary was only slightly affected by an increased X_{CHOL}. This temperature effect could have occurred because the difference between PSM and the low-T_m lipid (POPC or OSM) becomes smaller as the experimental temperature approaches the main phase transition of PSM at ~41°C. At both temperatures, gel-phase formation was induced at lower PSM concentrations in OSM-containing bilayers than in bilayers with POPC.

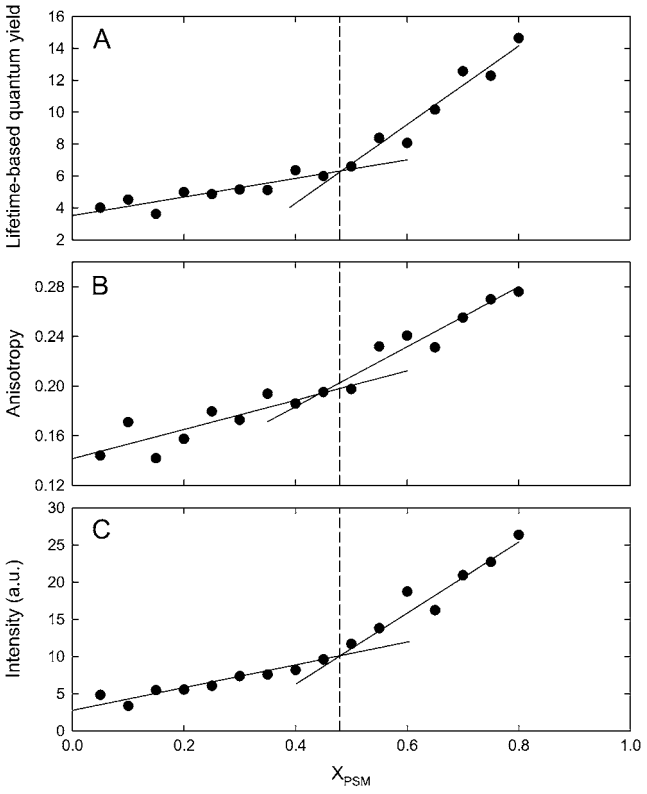

FIGURE 6 Representative tPA fluorescence data used for the determination of gel-phase boundary in ternary systems. Lifetime-based quantum yield (*A*), steady-state anisotropy (*B*), and intensity for tPA (*C*) were determined in OSM/PSM/cholesterol with $X_{CHOL} = 0.12$ at 37°C. Dashed line represents gel-phase boundary.

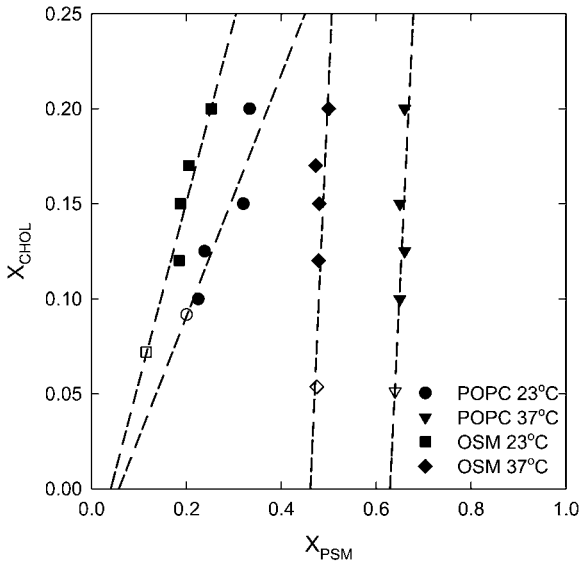

FIGURE 7 Gel-phase boundaries in ternary systems. Boundaries in OSM/PSM/cholesterol and POPC/PSM/cholesterol at 23°C and 37°C were determined using tPA fluorescence. Linear regressions for each data set are shown as dashed lines. Intercept points between gel-phase and l_o-phase boundaries are included as open symbols.

Lateral heterogeneity in ternary lipid bilayers

To acquire insights into how cholesterol induces the formation of ordered domains in ternary bilayers, the POPC/PSM/cholesterol and OSM/PSM/cholesterol bilayers were studied by determining DPH anisotropies at 23°C and 37°C as a function of cholesterol concentration. When plotted against X_{CHOL}, the anisotropy function was similar to that in the binary phospholipid-cholesterol bilayers, with two linear plateau regions flanking a linear intermediate region (data not shown). As in the binary systems, boundaries were determined as the point at which the intermediate linear function crosses the plateau regions. The resulting boundaries are shown as solid circles in Fig. 8.

The general shapes of regions in the OSM/PSM/cholesterol and POPC/PSM/cholesterol phase diagrams where ordered domains are formed were very similar but highly temperature-dependent. A detailed comparison of boundaries revealed that bilayers containing OSM formed a uniform l_o phase at lower X_{CHOL} than bilayers containing POPC (true

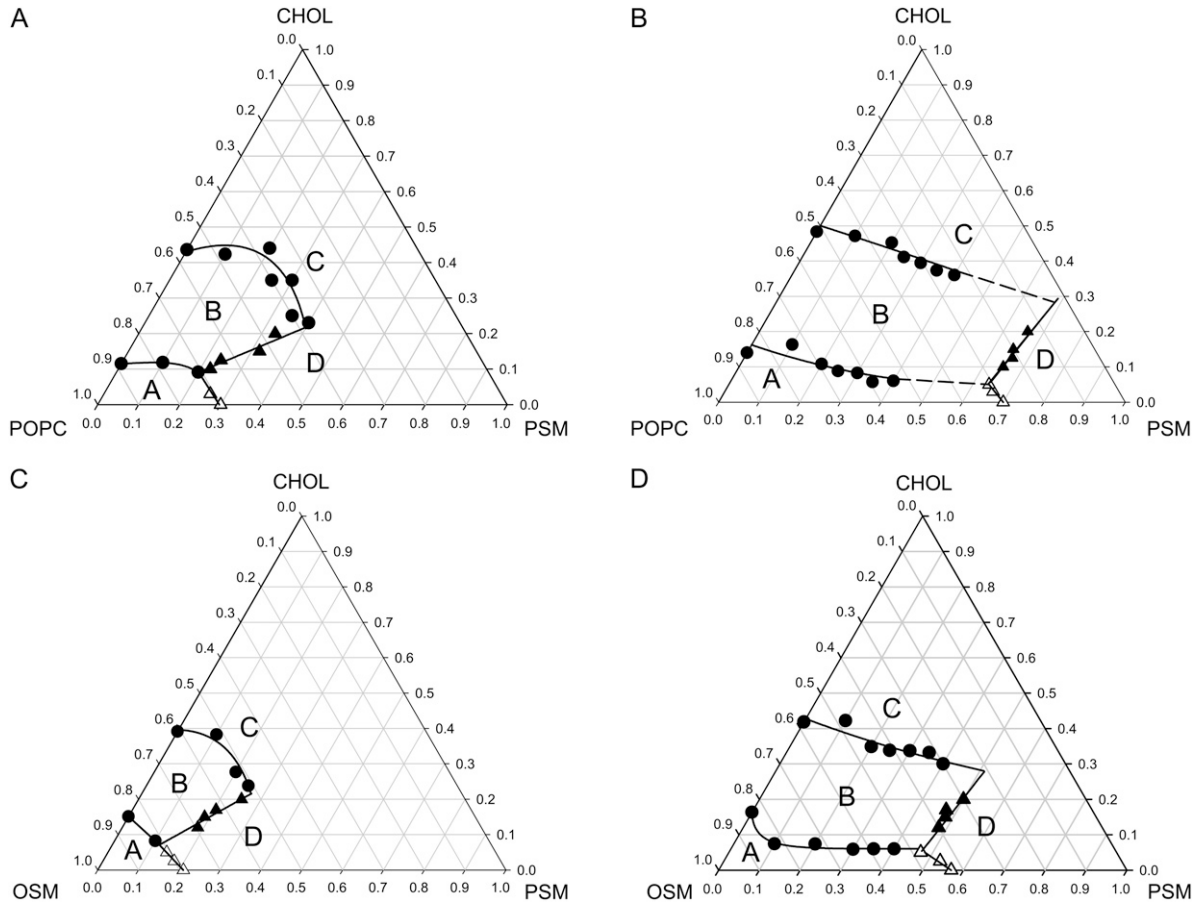

FIGURE 8 Partial ternary-phase diagrams of OSM/PSM/cholesterol and POPC/PSM/cholesterol bilayers. Phase boundaries were determined at 23°C (*A* and *C*) and 37°C (*B* and *D*). Solid circles represent l_d-l_o phase boundaries, determined using DPH anisotropy. Solid triangles represent gel-phase onset boundaries, determined using tPA fluorescence. Open triangles represent gel-phase onset boundaries determined using DPH anisotropy. In region *A*, bilayers are in l_d phase. In region *B*, l_d-phase bilayers contain an increasing number of cholesterol-rich domains. In region *C*, bilayers are in l_o phase. Region *D* was not studied, but the s_o phase was also present. CHOL, cholesterol.

for 23°C and 37°C). This finding indicates that OSM is more soluble in the l_o phase than is POPC. The low X_{CHOL} boundary shows a similar trend in both OSM-containing and POPC-containing bilayers, i.e., the addition of PSM decreases the cholesterol concentrations needed to induce ordered domain formation in the bilayers. In OSM-containing bilayers, the inclusion of low PSM concentrations had clearer effects on the low X_{CHOL} boundary than in POPC-based bilayers. This may be attributable to the observed lower solubility of PSM in OSM bilayers, compared with POPC bilayers, in the fluid disordered phase (Fig. 5).

DISCUSSION

Affinity of cholesterol for different phospholipid bilayers

The approach of using cyclodextrins to measure the affinity of cholesterol for phospholipid membranes has proved valuable (18,21,24,29). Studies of the desorption of cholesterol to β-cyclodextrin from phospholipid-cholesterol monolayers showed that cholesterol removal from SM monolayers is less efficient than removal from acyl chain-matched PC monolayers (24). Studies in which the affinity of cholesterol for OSM and POPC monolayers was compared suggested a higher affinity for unsaturated SM than for unsaturated PC monolayers (23). This was supported by the observation that the efflux rates of dehydroergosterol and cholestatrienol from OSM vesicles were slower than from POPC vesicles (22). These observations were verified in this study, where ^{3}H-cholesterol was shown to have a higher affinity for OSM than for POPC bilayers (Fig. 1). In addition, including phospholipids with saturated acyl chains (33 mol % DPPC or PSM) in the fluid bilayer led to increased cholesterol affinity, in agreement with previous observations (18,21,29). It was also clear that cholesterol had a higher affinity for SM-containing bilayers in general, because the addition of DPPC to the fluid bilayer had a small effect on the affinity compared with the addition of PSM.

When the obtained K_x values are compared with the anisotropy of DPH in different phospholipid bilayers, it becomes clear that the affinity of cholesterol for bilayers with a

certain phospholipid composition corresponds well with the order in the particular phospholipid bilayer before the addition of cholesterol (Fig. 9). According to the condensed complex model of Radhakrishnan and McConnell (30), phospholipid-cholesterol complexes become more stable as the lateral packing increases, leading to a lower chemical activity of cholesterol (31). Insofar as a lowered chemical activity would decrease cholesterol efflux from the bilayer, this model is in agreement with our observations. Based on the order in pure OSM bilayers, a higher K_x than was actually obtained could have been expected. It is tempting to speculate that the lower-than-expected K_x was observed because unsaturated phospholipids are less efficient complex-formers than are saturated phospholipids (31). This is in line with the less efficient cholesterol-induced ordering of unsaturated acyl chains, compared with fluid-saturated acyl chains of phospholipids (6). In our study, this hypothesis was supported by the observation that a higher cholesterol concentration was needed to induce ordered cholesterol-enriched domains in OSM bilayers compared with OSM/PSM (2/1) bilayers (~16 mol % in OSM and 6 mol % in OSM/PSM at 37°C).

Another explanation for the generally higher affinity of cholesterol for SM than for PC bilayers may involve the more constrained headgroup conformation in SM than in PC (32,33). A less flexible headgroup conformation, possibly facilitated by intramolecular hydrogen bonds, could partly explain why SM molecules pack tightly with cholesterol. This interpretation is in accordance with the umbrella model (34), which predicts that the degree to which cholesterol favors interactions with different phospholipids is related to how well cholesterol is shielded from the surrounding water by the phospholipid headgroup. Recent molecular dynamics simulation data suggest that charge-pairing between the SM headgroup and cholesterol would further stabilize SM-cholesterol interactions (35). These observations regarding the headgroups of SM and PC molecules are in agreement with previous observations that cholesterol removal from PC monolayers is largely influenced by the length of the acyl chains, whereas in SM monolayers, the acyl chain length is of little importance (24).

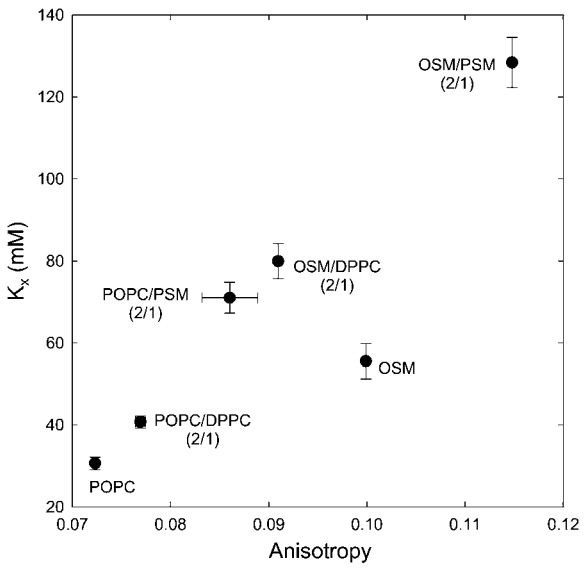

FIGURE 9 Affinity of cholesterol for phospholipid bilayers correlates with degree of order. Steady-state anisotropy of DPH measured in phospholipid bilayers without cholesterol at 37°C is plotted against the measured K_x.

Lateral heterogeneity and the formation of cholesterol-rich domains

Macroscopic liquid-liquid phase separation can occur in cholesterol-containing bilayers with a low-T_m phospholipid and a high-T_m phospholipid (36–38). If and to what degree phase separation takes place in such ternary lipid bilayers will depend on the structure of the phospholipid components, how they interact with each other, and how they interact with cholesterol. For example, a higher degree of *cis*-unsaturation, or the introduction of branches in the acyl chains of the low-T_m lipid, led to more pronounced phase separation (14,36,38). In ternary systems, like those studied here, in which the low-T_m lipid is a monounsaturated lipid such as POPC, no macroscopic phase separation seems to occur, but nanoscopic domains are formed (8). The same holds true for binary phospholipid-cholesterol bilayers, from which it follows that in our study, cholesterol inclusion may at most lead to the formation of nanoscopic domains. Although such domains may be very dynamic and perhaps short-lived, they are considered to be of biological significance, and information about their formation is crucial in understanding the structure-function relation of biological membranes.

How and to what degree a specific phospholipid forms cholesterol-rich domains must be dependent on molecular structure and interactions with cholesterol. Hence, the formation of domains in binary OSM-cholesterol and POPC-cholesterol bilayers reflects how these particular lipids interact with cholesterol. The phase behavior of POPC-cholesterol bilayers has been thoroughly studied by a number of different methods (7,39–41), and the phase diagram determined in this study is similar to those previously reported. However, as recently indicated, there is most likely no macroscopic phase separation in POPC-cholesterol bilayers, but instead, nanoscopic domains are likely formed, at least at lower temperatures (39).This suggests that the low X_{CHOL} boundaries in the binary-phase diagrams in Fig. 3 are boundaries at which domain formation begins.

The lower boundary in the phase diagrams, i.e., the cholesterol concentration at which ordered domains start to form, shows that more cholesterol is needed to induce domains in OSM than in corresponding POPC bilayers. The formation of ordered domains may be linked to the well-documented ordering effect of cholesterol on neighboring phospholipids (23). The initial step in domain formation is probably taken when cholesterol orders the acyl chains of neighboring

phospholipids. The larger the ordering effect, the more favorable it should be to the formation of ordered domains. Thus, a larger ordering effect should reduce the cholesterol concentration needed to initialize domain formation, suggesting that cholesterol has a smaller ordering effect on neighboring OSM than on POPC molecules. This was shown in a molecular dynamics simulation comparing the effects of cholesterol on POPC and OSM (42), in agreement with observations in the anisotropy measurements in this study.

The addition of a high-T_m phospholipid (e.g., PSM) to the bilayers should increase the tendency for heterogeneity in the bilayers, and lead to the formation of larger domains. Hence, domain formation should be directly influenced by the structural differences and miscibility of the two phospholipids. Accordingly, the addition of PSM lowered the cholesterol concentration needed to form ordered cholesterol-rich domains below that needed in pure OSM or POPC bilayers as well as in pure fluid PSM, as reported (7). In OSM bilayers, the effect of low concentrations of PSM was greater than in POPC bilayers. This may be a result of the observed lower solubility of PSM in the OSM-rich l_d phase compared with the POPC-rich l_d phase (Fig. 5).

Formation of a homogenous l_o phase

The upper boundary in the binary and ternary phase diagrams shows the composition of the l_o phase formed by cholesterol together with the different phospholipids (Figs. 3 and 8). In the binary lipid bilayers, the composition of the l_o phase formed by OSM is compared with the POPC l_o phase. According to the phase diagram, less cholesterol is needed to form a uniform l_o phase in OSM than in POPC bilayers at all temperatures. The same trend was also observed in ternary systems. This could be attributable to the higher initial order of pure OSM bilayers. As PSM is added to the bilayers, the phospholipid/cholesterol ratio increases in the l_o phase in both OSM-containing and POPC-containing bilayers, in agreement with observations that the ordering effect of cholesterol on fluid saturated phospholipids is greater than on fluid phospholipids with unsaturated acyl chains (6).

Information about how different lipids partition between cholesterol-rich and cholesterol-poor domains would be available from tie lines passing through a two-phase region. Because the systems discussed here do not exhibit true phase separation, rather nanoscopic domains are formed, it is uncertain how well the behavior of these domains follows phase rules. However, assuming that the lever rule applies, we can insert theoretical tie lines, which basically would be placed like a fan between the end tie lines (the outer left axis and the gel-phase boundary) (7). At 37°C, such tie lines would more or less follow the z axis. In fact, such tie lines agree well with the observation that DPH anisotropy increased approximately linearly with X_{PSM} at 37°C. According to these tie lines, PSM would partition almost equally between cholesterol-rich and cholesterol-poor domains both in OSM-containing and POPC-containing bilayers at 37°C, whereas POPC and OSM would partition predominantly into cholesterol-poor bilayer regions. At 23°C, the boundary of gel-phase onset is tilted more toward the x axis. This indicates that PSM partitions preferentially into cholesterol-rich domains at this temperature. This observation is in agreement with the results of a deuterium NMR study of POPC/PSM/cholesterol bilayers (6). It also fits well with the fact that at 23°C, the inclusion of PSM has a more dramatic effect on phospholipid-cholesterol stoichiometry in the l_o phase than at 37°C.

Predicting lateral bilayer organization based on mβCD-LUV partitioning data

One goal of this project was to establish to what degree the cholesterol affinity measured in mβCD-LUV partitioning experiments can be used to predict the lateral organization and domain formation in bilayers composed of phospholipids and cholesterol. Based on the high preference of cholesterol for PSM bilayers, it might be predicted that PSM and cholesterol would separate from POPC or OSM in ternary systems. According to theoretical tie lines, PSM partitions equally between cholesterol-rich and cholesterol-poor domains, whereas POPC and OSM preferentially partition into cholesterol-poor domains at 37°C. Hence, cholesterol seems to prefer PSM over the monounsaturated phospholipids used in this study. The fact that complete separation does not occur can be explained by the entropic cost of phospholipid demixing, which works against cholesterol-driven phase segregation (29). At 23°C, this barrier is more easily overcome because of the less favorable mixing of PSM and unsaturated lipids at this temperature. This results in an increased enrichment of PSM in the cholesterol-rich domains. Hence, it seems that predictions based on the partitioning data are valid.

Based on cholesterol's lower affinity for POPC/DPPC bilayers compared with POPC/PSM, and on a previous comparison of cholesterol desorption from DPPC and PSM monolayers (24), it may be predicted that because of the lower affinity for DPPC, domain formation in this system should be less efficient than in POPC/PSM bilayers upon the addition of cholesterol. This was observed in microscopy experiments with giant unilamellar vesicles (38), supporting the usefulness of partition studies in the prediction of the lateral organization in lipid bilayers.

Our major focus, however, was on clarifying how the increased affinity of cholesterol for OSM bilayers, compared with POPC bilayers, influences the lateral organization in lipid bilayers. Based on the different affinities that cholesterol showed toward the two low-T_m lipids, it seemed possible that cholesterol would be distributed differently between the disordered and ordered domains in ternary lipid bilayers, so that the OSM-rich (cholesterol-poor) domains would be slightly more enriched in cholesterol than the POPC-rich domains. Indeed, the high X_{CHOL} boundaries in Figs. 3 and 8 indicate

that less cholesterol is needed to form a uniform l_o phase in OSM-containing bilayers than in similar POPC-based bilayers. This is in line with predictions that could be made based on the partition data. Overall, it seems that the results of the partitioning study are reflected in the lateral organization of the ternary lipid bilayers.

CONCLUSIONS

In this study, it was observed that the affinity of cholesterol for different phospholipid bilayers correlates well with the initial chain order in the bilayer, which may explain cholesterol's preference for sphingomyelin over chain-matched phosphatidylcholines. Further, the results indicate that the measured partition coefficients for cholesterol between mβCD and different phospholipid bilayers can be used to make predictions of how cholesterol will influence the lateral organization of multicomponent phospholipid bilayers. However, the success rate of such predictions will be increased if all possible phospholipid-phospholipid interactions are taken into consideration.

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