Solution synchrotron x-ray diffraction reveals structural details of lipid domains in ternary mixtures

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The influence of cholesterol on lipid bilayer structure is significant and the effect of cholesterol on lipid sorting and phase separation in lipid-raft-forming model membrane systems has been well investigated by microscopy methods on giant vesicles. An important consideration however is the influence of fluorescence illumination on the phase state of these lipids and this effect must be carefully minimized. In this paper, we show that synchrotron x-ray scattering on solution lipid mixtures is an effective alternative technique for the identification and characterization of the l_o (liquid ordered) and l_d (liquid disordered) phases. The high intensity of synchrotron x rays allows the observation of up to 5 orders of diffraction from the l_o phase, whereas only two are clearly visible when the l_d phase alone is present. This data can be collected in ~ 1 min/sample, allowing rapid generation of phase data. In this paper, we measure the lamellar spacing in both the liquid-ordered and liquid-disordered phases simultaneously, as a function of cholesterol concentration in two different ternary mixtures. We also observe evidence of a third gel-phaselike population at 10–12 mol % cholesterol and determine the thickness of the bilayer for this phase. Importantly we are able to look at phase coexistence in the membrane independent of photoeffects.

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I. INTRODUCTION

Cellular membranes are composed of a dynamic mixture of lipids, proteins, and carbohydrate molecules. Evidence is accumulating that the lipid composition and membrane architecture play important roles in transport, signal transduction, metabolism, and other characteristics of cell physiology; however many aspects of membrane organization, dynamics, and how membrane structures relate to function remain poorly understood. Historically, a fluid mosaic model for the membrane was accepted, in which lipids and embedded proteins were described as randomly distributed [1]. It is however now known that this is not the case but that biological membranes exhibit a much more complicated structure and that particular lipids and embedded proteins may laterally cluster into submicron domains [2–4]. These lipid domains often referred to as lipid rafts are postulated to take the form of lateral patches in the membrane, of differing composition to the surrounding areas [5]. Lipid rafts may be composed of lipids in a more ordered (l_o) phase, where the alkyl chains are ordered but there exists lateral mobility of the molecules [6,7], and both cholesterol and sphingolipids have been hypothesized to be essential for lipid-raft formation in the cellular membrane [8–11].

Current interest in biological membrane microdomains or lipid rafts is primarily due to their functional role in fundamental processes in cell biology. Rafts were first proposed to mediate sorting in the trans-Golgi network [5]. Recent results suggest that rafts may be important in sorting in the endocytic pathway, serving as docking sites for certain pathogens and toxins [12]. Sorting of proteins into rafts might help to concentrate proteins at different regions of the cell membrane, thus facilitating their intermolecular interactions. The ordered lipid environment of the raft may also influence their function, possibly by altering protein conformation.

Cholesterol is an essential membrane constituent and has been implicated in a wide variety of cell functions, including cell permeability [13,14], membrane fusion [15], and receptor function. The critical importance of cholesterol in the cell may lie in its ability through steric interactions with adjacent lipids to affect the fundamental properties of the lipid bilayer. Biological membranes can contain up to 50% cholesterol [16] and the presence of this molecule in the fluid membrane suggests that structural properties will be modified significantly from a membrane composed of pure lipids. Distribution of cholesterol within membranes is not homogenous, and some evidence shows that cholesterol-rich domains called lipid rafts are present in cell membranes [11]. This phenomenon has been documented by several authors in recent years [17–19], and the phase diagram for various lipid mixtures with cholesterol has been investigated in detail using a variety of techniques including fluorescence microscopy, nuclear magnetic resonance (NMR), and atomic force microscopy (AFM) [20–22].

Although it is well accepted that lipid rafts are mainly composed of sphingolipids and cholesterol, the lateral phase separation is a feature not restricted to membranes containing sphingolipids. Model membrane studies on ternary lipid mixtures composed of cholesterol and two phospholipids with a high-melting temperature (T_m) and a low T_m show that the coexistence l_o phase and l_d phase can occur in the absence of sphingolipids [18,23]. In these mixtures, l_o phase domains enriched in the high T_m lipid and cholesterol separate from surrounding l_d phase regions (enriched in the low T_m lipid). In addition, a recent time-of-flight secondary-ion mass spectrometry (TOF-SIMS) investigation of ternary lipid mixtures revealed that the acyl chain saturation rather than

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the head group difference is the dominant factor in determining phase separation [24]. According to these results, the sphingolipid is not an essential component to form lipid rafts, and any saturated phospholipids with similar acyl chains such as DPPC and DSPC should be capable of fulfilling the same role. However in the cell membrane, functional lipid rafts are always related to the presence of sphingolipids. Therefore, there must be some advantage for the natural cell membrane to prefer sphingolipids but not the saturated phospholipids for raft formation. A detailed comparison of egg sphingomyelin and DPPC might help us to understand the head group contribution to the rafts formation in model membrane systems.

Despite the powerful contributions x-ray techniques can make to this field, relatively limited work has been carried out specifically toward the raft hypothesis [25–27]. Due to the high intensity of synchrotron x-ray diffraction (XRD), its application in these diffraction studies of lipid-water phases results in a substantial reduction in exposure time and accumulation of high-resolution diffraction patterns over typical in-house x-ray sources.

In this paper, we describe small-angle x-ray diffraction measurements of two different model lipid membrane systems, containing varying concentrations of cholesterol. Our results demonstrate how solution x-ray diffraction can provide a reliable global measurement of the phase behavior of the lipid system and we observe a complex dependence on cholesterol concentration, measuring bilayer thickness in different phases simultaneously. Our observations are in fairly good agreement with data published by fluorescence methods, but domains are observed over a narrower cholesterol concentration range. This is in good agreement with the observation by Zhao *et al.* [28] that light-induced domain formation occurred near the phase coexistence boundary and therefore may explain the wider cholesterol range over which domains are observed for fluorescence measurements. In addition, the structural differences between two ternary lipid mixtures with (2S,3R,4E)-2-acylaminooctadec-4-ene-3-hydroxy-1-phosphocholine (eSM) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) have been observed.

II. MATERIALS AND METHODS

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), DPPC, eSM, and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Figure 1 shows the molecular structures of the lipids used in this paper.

Lipids were dissolved in chloroform (HPLC grade) to a concentration of 50 mM and stored under −20 °C. Aqueous multilamellar vesicle (MLV) solutions of DOPC-DPPC-cholesterol or DOPC-eSM-cholesterol mixtures were prepared by first mixing the individual lipid stock solutions thoroughly at the desired molar ratios. All the lipid mixtures were prepared with equal molar ratios of one high T_m lipid (DPPC or eSM) and one low T_m lipid (DOPC) with different amounts of cholesterol up to a maximum of ~50%. Solutions were then dried with nitrogen and placed under vacuum overnight to remove excess solvent. Millipore water

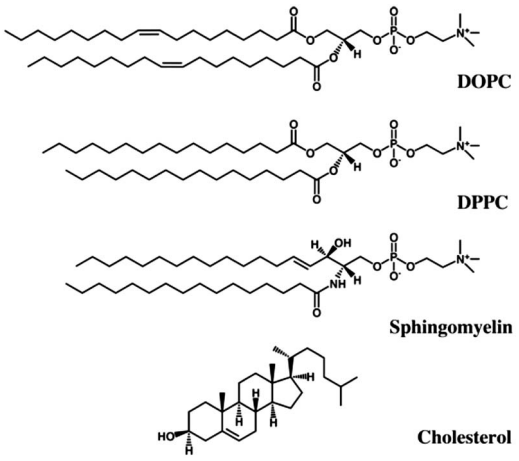

FIG. 1. Molecular structures for the lipids used in this study.

(18.2 MΩ cm^{-1}) was used to rehydrate the lipids to a final concentration of 50 mM. After incubation at 45 °C for at least 24 h, samples were vortexed for several minutes until well mixed, resulting in uniform milky white solutions. Each x-ray sample was prepared by injecting the concentrated solution into a quartz x-ray capillary (Φ=1.5 mm, Charles Supper) and centrifuging to concentrate further, producing a bulk sample of the lamellar phase. Capillaries are sealed with silicone rubber sealant to prevent evaporation.

XRD measurements were carried out at the National Synchrotron Light Source (NSLS), beamline X6B at Brookhaven National Laboratory. The X6B beamline was configured to have a fairly small beam-spot size at the sample (0.2×0.3 mm^2) and a wavelength of 1.033 Å (12 keV). Quartz capillaries containing lipid mixtures were mounted in a transmission configuration. Unoriented (powder) diffraction patterns were recorded for each solution using a CCD detector at room temperature (22 °C). All data were corrected for variations in incident-beam intensity and background air scatter. To obtain a graph of scattering intensity as a function of scattering vector $\boldsymbol{q}$, images were analyzed using FIT2D software (freeware developed at the ESRF). Area detector images are azimuthally averaged to produce a one-dimensional(1D) plot of scattered intensity vs $\boldsymbol{q}$. The technique provides an ensemble measurement over the entire sample volume exposed to the beam. For lipid samples, typical data provide measurements of the lamellar repeat distance (d) for a fully hydrated solution sample, which is composed of the complete 1D unit cell (lipid bilayer and water layer). Calibration of the XRD patterns was performed using silver behenate (d_{001}=58.38 Å) [29] and the measurement of d is sensitive to a fraction of 1 Å.

III. EXPERIMENTAL RESULTS

A. Observation of l_o domains in both ternary systems

Figure 2 shows solution x-ray diffraction data for both ternary lipid mixtures investigated. They each have equal molar ratios of DOPC-eSM or DOPC-DPPC with varying molar percentages of cholesterol. Two reflections corresponding to $\boldsymbol{q}=2\pi/d$ and $4\pi/d$ can be clearly observed for

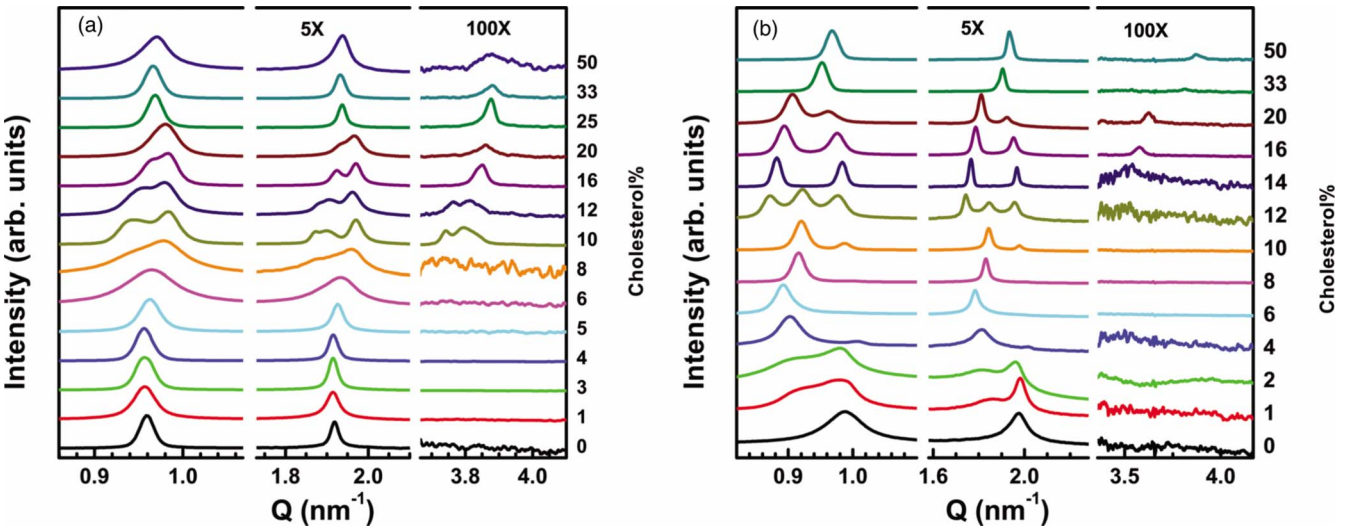

FIG. 2. (Color online) Synchrotron x-ray diffraction data for 1:1 mixtures of DOPC with (a) eSM and (b) DPPC containing varying concentrations of cholesterol (mol %). Only the (001), (002), and (004) Bragg peaks are shown. This data indicates the presence of the l_o phase where the 004 peak is present.

all the samples characteristic of the bilayer d spacing. Notice that above a certain threshold of cholesterol (10% for DOPC-eSM-cholesterol and 16% for DOPC-DPPC-cholesterol), a fourth-order reflection ($q=8\pi/d$) is clearly present, while at cholesterol ratios lower than this, only two reflections are observed. The thresholds observed here correspond fairly well with the previously reported cholesterol fractions [20] required to induce phase separation into the liquid ordered (l_o) and liquid disordered (l_d) phases with similar mixtures, although they are a little higher in our case.

It is well known that as the lamellar structure of a phase becomes more defined, a greater number of Bragg peaks are observed. The limits of this being (a) a bulk sample having a sinusoidal electron-density distribution from layer to layer, in which a *single Bragg peak* would be observed at $q=2\pi/d$ or (b) sharply defined lamellae, yielding a diffraction pattern with many orders of diffraction present at $q=2\pi/d$, $4\pi/d$, $6\pi/d$, etc. Therefore, the observation of the Bragg (004) peak can be ascribed to the appearance of a new phase in the bulk sample, i.e., the liquid-ordered phase (l_o). Increased chain ordering in randomly distributed domains of the l_o phase will have the effect of—on average—stiffening the layer, thus suppressing bilayer fluctuations in the lamellar stack. Such an effect will be most obvious if l_o domains have a tendency to stack on top of each other. Another factor which may influence this behavior is the increased structural order in these domains.

Lateral coexistence of the l_o and l_d phases in membranes formed from ternary lipid mixtures containing cholesterol has been well documented by fluorescence microscopy [18–21] and AFM in the literature [30,31], although there are limitations to these methods. AFM and fluorescence microscopy are local techniques probing single bilayers at length scales on the order of 10–1000 nm and ~1–100 μm, respectively. By using x-ray scattering, we are able to average the diffraction from a bulk sample over a large volume of ~0.01 mm^3 to give a global measurement of the system. We assume that as the l_o and l_d phases have differing lipid compositions—therefore different chain arrangements and ordering—they should exhibit differences in bilayer spacing. Since the alkyl chains in the l_o phase are more highly ordered, we expect this to produce a bilayer of increased thickness. This is indeed the case and this effect can be observed in the data presented in Fig. 2(a) shown most effectively in the mixture with 16% cholesterol. The second-order Bragg peak (002) in this case is clearly split into two components at $q=1.924$ and 1.970 nm^{-1}. If both components were to persist to the fourth order, we would expect to see two peaks at $q=3.848$ and 3.940 nm^{-1}. However, only the first of these (corresponding to the larger d spacing) is present. Therefore, we can deduce that the split (002) reflection in this mixture originates from two different populations of bilayer: one is the l_o phase, which has multiple orders of diffraction and larger lamellar spacing ($d=6.53$ nm), and another one is the l_d phase, which is less ordered with a smaller lamellar spacing of 6.38 nm.

B. Lipid bilayer thickness as a function of cholesterol fraction

Based on this analysis, we have plotted the lamellar spacing of different phases as a function of cholesterol fraction for each of our ternary mixtures, as shown in Fig. 3. In the case of the DOPC-eSM-cholesterol mixtures [Fig. 3(a)], we found no peak splitting below 8% of cholesterol and the average d *spacing* is about 6.55 ± 0.02 nm. Although the coexistence of the liquid-crystalline (L_α) phase and the gel phase in 1:1 DOPC-eSM mixture has been proposed by several authors using AFM and fluorescence microscopy, we did not observe double peaks for this mixture, and this is repeatable even in a 100 mM NaCl solution. This observation is in good agreement with Lindblom and co-workers' [32] NMR result, where monoexponetial diffusion was observed for the same 1:1 DOPC-eSM lipid mixture. Therefore, our x-ray result suggests that certain amount of cholesterol is required to generate two distinct liquid phases in this system.

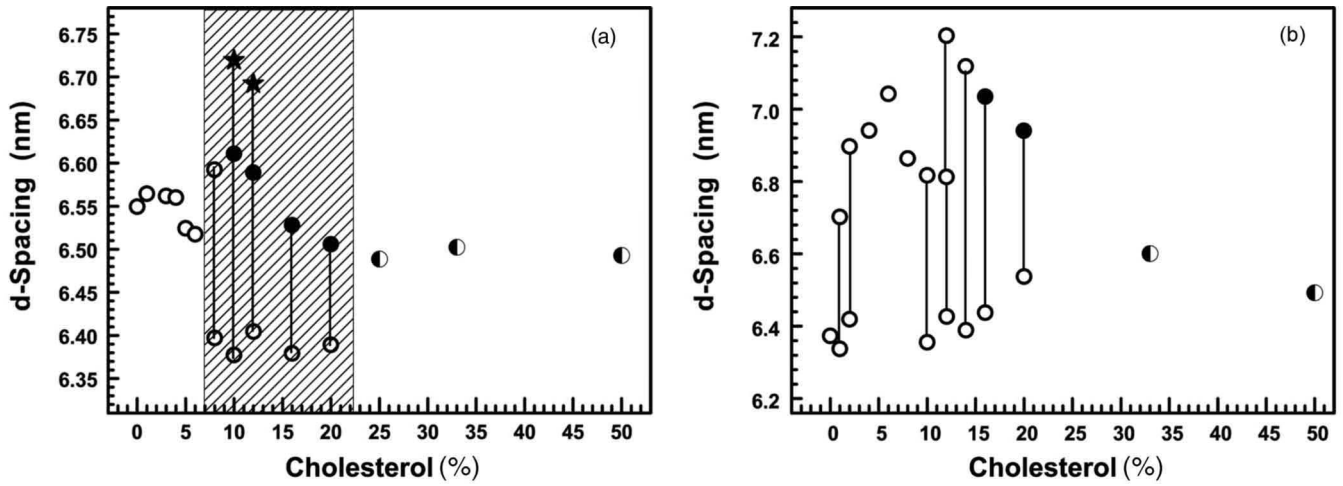

FIG. 3. Lamellar spacings (d) for the l_o phase (solid symbols) and the l_d phase (open symbols) as a function of cholesterol percentage at a constant 1:1 molar ratio of (a) DOPC-eSM and (b) DOPC-DPPC.

Based on the presence of the (004) peak, the onset of l_o phase formation is observed at 10% cholesterol. In Fig. 3, solid and open circles represent d spacings of l_o and l_d phases, respectively. The shaded region covers the lipid mixtures with peak splitting, therefore roughly corresponding to the region of l_o/l_d coexistence. There is one exception however. For lipid mixtures with 8% cholesterol, peak splitting was observed, but no (004) peak is present. This could possibly indicate an intermediate state, in which two fluidlike phases start to phase separate, thus have different d spacings but neither are ordered. The average d spacing of l_d phase is 6.39 ± 0.01 nm very close to the reported DOPC lamellar d spacing (6.32 nm) [33]. This observation fits well with the basic idea that DOPC enriches the l_d phase in a raft-containing membrane. It should be noted that while the d spacing in this coexistence region remains almost constant for the l_d phase, it decreases from 6.61 to 6.50 nm for the l_o phase as the cholesterol fraction is increased. Decreasing of the l_o phase d-spacing might be ascribed to the smaller molecular size of cholesterol, as the eSM side chains adopt a less extended conformation to minimize the void volume created by cholesterol. Solid stars in this region represent a very interesting observation, and this will be discussed in detail in Sec. III C.

Above 25 mol % cholesterol in this DOPC-eSM-cholesterol ternary mixture, no peak splitting is observed but an (004) peak is still present, as indicated by the half-filled symbols in Fig. 3(a). This is consistent with the previous fluorescence and AFM results and may suggest the formation of a continuous l_o phase above certain cholesterol amount. The average bilayer thickness (6.49 ± 0.01 nm) is very close to the decreased thickness of the l_o phase (at 20 mol% cholesterol) and is about 0.1 nm larger than that of the l_d phase.

Bilayer thickness data for DOPC-DPPC-cholesterol mixtures [Fig. 3(b)] show similar behavior with some important differences. First, the range over which we observe the (004) peak (l_o phase) is much larger and for the mixture with 12% cholesterol three defined peaks are observed but no (004) peak is present. Moreover, while DOPC-eSM-cholesterol mixtures show an almost constant d spacing before the threshold at 8% cholesterol, a clear peak shift is observed for this DOPC-DPPC-cholesterol mixture. Possible explanations for these differences will be thoroughly discussed in Sec. III D.

C. Three-phase coexistence in DOPC-eSM-cholesterol mixtures

One of the more striking results observed in this experiment is the clear presence of a three-phase region in the DOPC-eSM-cholesterol mixtures at 10% and 12% cholesterol. In Fig. 3(a) we have marked an additional *d spacing* on the graph with a solid star, as a third lamellar spacing was observed. The d spacing of this third phase (most likely the gel phase) is about 0.1 nm larger than that of the l_o phase for the same lipid composition, suggesting a more extended and ordered packing of molecules in this new phase. Wide angle scattering data for these samples could potentially confirm this observation as the in-plane ordering for the gel phase is well defined [27]. However, owing to the likely very small proportion of gel phase present in this case, such a peak was not observed in these samples.

Figure 4 shows in detail each of the Bragg peaks observed for the mixture with 10% cholesterol; a mixture in which this third bilayer thickness is observed. As can be seen in Fig. 4(a), the first component of the split (001) peak is fairly broad, indicating the possible presence of more than two individual peaks. On viewing the (002) region [Fig. 4(b)], three distinct peaks are clearly observed, two of which persist to a fourth order and even a fifth-order reflection. In addition, we noticed that the first component associated with the gel phase has a particularly narrow peak width, when compared with the l_o phase (Table I). This also indicates a more highly ordered molecular packing and arrangement than the standard l_o phase.

Coexistence of three phases in a similar ternary system (POPC (1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine)-pSM (2S,3R,4E)-2-acylaminooctadec-4-ene-3-hydroxy-1-Phosphocholine)-cholesterol) was previously observed by Prieto and co-workers [22] using the fluorescence

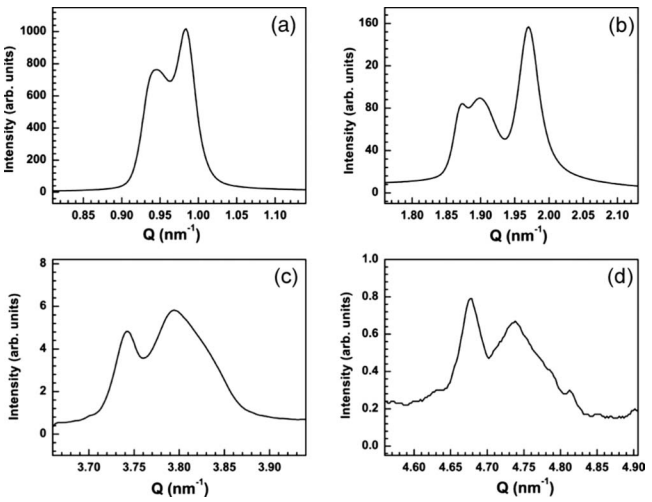

FIG. 4. A More detailed view of scattering from the DOPC-eSM-cholesterol (10%) mixture, highlighting scattering at (a) $q=2\pi/d$, (b) $4\pi/d$, (c) $8\pi/d$, and (d) $10\pi/d$.

resonance energy transfer (FRET), and according to their results, we should observe a much wider three-phase region (4∼25% cholesterol for POPC-PSM cholesterol). Similar studies by Zhao *et al.* [34] also predicted a significant three-phase region. However in our case, the coexistence of three phases was only found for the samples with 10∼12% cholesterol. Although the difference due to lipidlike (DOPC vs POPC and eSM vs PSM) might contribute to the inconsistency somehow, the 4% quencher they introduced into the lipid mixtures also needs to be taken into account. This is a considerable concentration of impurity and could alter the phase behavior remarkably. Veatch and Keller [20] also discussed the possible coexistence of three phases in giant unilamellar vesicles (GUVs) with ternary lipid mixtures; however current fluorescence microscopy techniques are not able to easily distinguish the gel phase from the l_o phase in such a system if the region of gel phase is small (<500 nm). In another model membrane system [supported lipid bilayer (SLB)], phase separation in such a ternary lipid system has been well investigated with AFM [30,31], but so far no clear evidence exists for the three-phase coexistence. This problem can be now explained by our x-ray results because the difference of lamellar thicknesses between the gel phase and l_o phase was observed to be as small as 0.1 nm. Such a small height difference would be very difficult to detect by AFM, especially if this difference appears in the domain boundaries between l_o and l_d phases.

TABLE I. Q values and peak widths of reflections from individual phases in the DOPC-eSM-cholesterol (45:45:10) sample. Peak width is determined by fitting a Gaussian curve to the peak.

	Q (nm^{-1})			Peak width		
Peak	Gel	l_o	l_d	Gel	l_o	l_d
(002)	1.870	1.901	1.971	0.0160	0.0416	0.0303
(004)	3.740	3.800		0.0208	0.0660	
(005)	4.676	4.738		0.0244	0.0554	

D. Difference between two ternary lipid systems and corresponding models

In the case of the DOPC-DPPC-cholesterol mixtures, a more defined triple peak is clearly observed at 12% cholesterol, as shown in Fig. 2(b). Both the (001) and (002) peaks are split into three distinct peaks, respectively; however none of these peaks have a corresponding (004) reflection present. Observation of triple peaks sounds like possible evidence for a three-phase region, but if this is the case all three phases are more l_d phaselike. This indicates that we might need to comprehend this phase behavior in a different way.

First, we should consider the structural differences between DPPC and eSM, which are likely to play essential roles in regulating phase behavior in the ternary lipid mixtures. As depicted in Fig. 1, they both have the same polar head group (phosphocholine) and two saturated alkyl chains, with the only difference being the interfacial region that bridges the nonpolar alkyl chains to the polar head group. In eSM, both hydrogen bonding donor (hydroxyl and amino) and acceptor (carbonyl) are simultaneously present at two sides of this molecule, offering the possibility to form an abundance of intermolecular hydrogen bonds [35–37]. This remarkably contrasts to the two carbonyl groups (acceptor only) in DPPC in the same interfacial region and may be important for the phase behavior difference between these two systems.

1. DOPC-eSM-cholesterol

In this ternary system, due to the intermolecular hydrogen bonding interaction, eSM molecules may laterally cluster into rigid membrane domains (gel or L_β phase), in which their alkyl chains adopt an all-trans conformation and the long axis of eSM molecules is approximately normal to the membrane surface [38,39]. We found that before adding cholesterol, no peak splitting is present and the observed bilayer d spacing (6.55 nm) is right in the middle of that of the gel phase and the l_d phase (6.7 nm and 6.4 nm, respectively) in the "three-phase coexistence" mixture with 10 mol % cholesterol. Considering the high in-plane diffusion of lipid molecules, this suggests well-mixed bilayers depicted as the first structure in Fig. 5(a). Such asymmetric stacking is very similar to the reported mixed bilayers with one monolayer of disordered phase and the other of ordered phase [40,41], therefore could stay in thermodynamic equilibrium [42]. Such a structure is in contradiction to the observations from fluorescence microscopy by Korlach *et al.* [43] that the l_o phaselike domains are superposed in opposing monolayers. However, this observation may only apply to macroscopic phase separation and is not necessarily the case for nanoscale domains which we have the potential to observe.

The addition of ∼10 mol % cholesterol produces a dramatic change in the phase behavior manifested as the appearance of three-phase coexistence. As we already know, small amounts of cholesterol preferentially localize along the boundary of lipid-raft domains [44,45], in an effort to minimize the unfavorable energetic effects created along the interface. Also in a sphingolipid-cholesterol binary system, the bilayers d spacing was reported to decrease linearly with increasing cholesterol content [46] indicating a possible

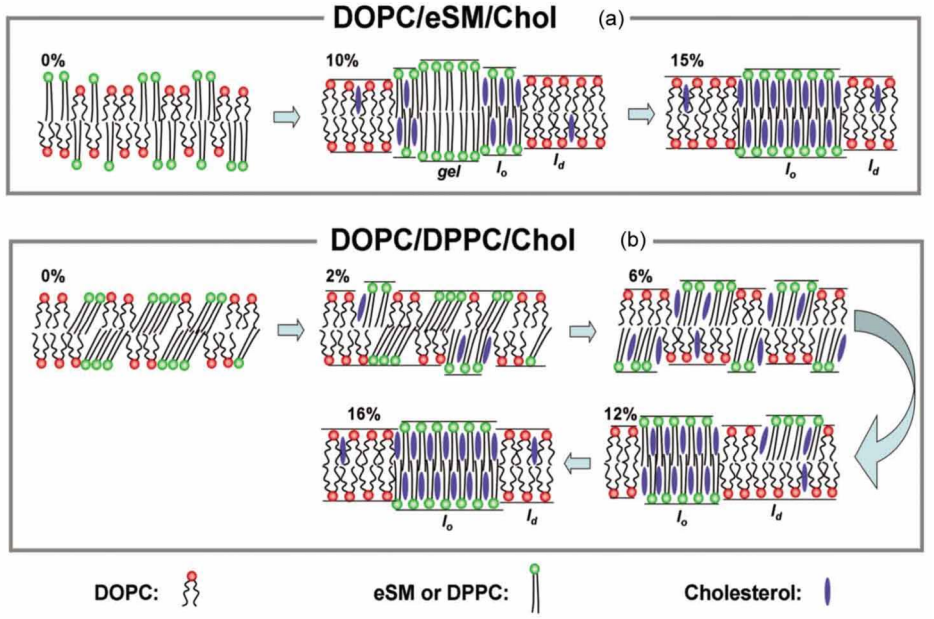

FIG. 5. (Color online) Schematic showing the membrane structure of (a) 1:1 DOPC/eSM and (b) 1:1 DOPC/DPPC with increasing cholesterol.

membrane structure of transbilayer alkyl chain interdigitation in the l_o phase bilayers [7,47]. In our x-ray measurements, comparing with d spacings of lipid mixtures containing $0\sim6\%$ cholesterol, the stars in Fig. 3(a) can be attributed to a bilayer domain composed of the gel phase in both leaflets. The corresponding open circles represent a symmetrical l_d phase. Solid circles here represent a symmetrical l_o phase with a reduced d spacing due to transbilayer alkyl chain interdigitation. Therefore, a possible model to describe the three-phase-coexistence is a symmetric membrane distribution with lipids laterally sorting into highly ordered eSM domains (gel phase), eSM-cholesterol enriched boundaries (l_o phase), and DOPC enriched fluid surroundings (l_d phase). Further increases in cholesterol concentration up to 20 mol % result in the disappearance of the gel phase, i.e., coexistence of l_o and l_d phases.

2. DOPC-DPPC-cholesterol

For DPPC mixtures, the alkyl chains in the gel phase ($L_{\beta'}$) are tilted at an angle of $\sim30°$ with respect to the bilayer normal [48–50]. Therefore, although the fully extended DPPC has a longer molecular length than DOPC, the d spacing of the DPPC gel phase (6.34 nm) [51,52] is very close to that of the DOPC liquid-crystalline phase (6.32 nm) [33]. In such a case, even if the mixture separates into two phases, they cannot be distinguished by d-spacing measurements alone. Also if we consider the membrane as a well-mixed DOPC-DPPC bilayer, the lamellar thickness should also be approximately 6.33 nm. This is in good agreement with our x-ray results as we do not observe peak splitting at 0% cholesterol and the measured d spacing is equal to 6.4 nm.

The presence of small amounts of cholesterol (<4 mol %) produces a broad peak with a larger d spacing and represents an intermediate state as more cholesterol is incorporated into the membrane and packs next to the saturated lipids [Fig. 5(b)]. This phenomenon was not observed in the DOPC-eSM-cholesterol mixtures. This peak is broad and not a direct superposition of the 0% and 4% peaks because we must also consider the effects on DPPC tilt angle upon incorporation with cholesterol [50]. Once the tilt angle is reduced, the effective molecular length should increase. Due to the possible nonuniform distribution of cholesterol into different DPPC domains, a relatively wide range of tilt angles may be observed. This effect can be clearly seen in Fig. 2(b). If we look at the progression of the (001) peak, the lower **q** component (with larger d spacing) is very broad, thus reflecting a wide distribution of bilayer thicknesses.

For mixtures containing $4\sim8$ mol % of cholesterol, again a single peak is observed. Lamellar spacings for these mixtures are almost in the middle of the maximum and minimum d spacings observed for the mixture with the triple peak present (12 mol % cholesterol). This is very similar to the DOPC-eSM-cholesterol system before the threshold for l_o phase formation is reached. Therefore, we can surmise that in this region DPPC molecules adopt a favorable tilt angle to incorporate with cholesterol, and since the DPPC alkyl chains are still tilted, there is little coupling between two leaflets. A possible model of asymmetrical distribution of bilayer is depicted as the third one in Fig. 5(b).

The most interesting result for this mixture is the triple peak observed in the mixture at 12 mol % cholesterol. Different from the DOPC-eSM-cholesterol mixtures with similar triple peaks, here the d spacing of the second component (6.8 nm) is right in the middle of that of the first and third components (7.2 nm and 6.4 nm, respectively), and none of them have a (004) or higher-order reflections. Comparing with the l_o phase d spacing at higher cholesterol concentrations, we consider the first component (with the largest d spacing) as a precursor to the l_o phase, although no (004) peak is present. Formation of the l_o phase can be understood as a further extension of the DPPC molecules as they further incorporate with the increased numbers in cholesterol molecules. This eventually results in a perpendicular arrangement of DPPC/cholesterol molecules and thus transbilayer alkyl chain interdigitation. The d spacing of the second peak

component is only slightly smaller than that of the mixture with 8% cholesterol, indicating a similar asymmetric lipid distribution in the bilayer. The third component with a d spacing of 6.4 nm is clearly a symmetric l_d phase and this is consistent with the DOPC-eSM-cholesterol result. A possible model to describe the triple peak in this mixture is a coexistence of DPPC-cholesterol-enriched l_o phase regions, DOPC enriched l_d phase regions, and an asymmetric distribution of bilayers with tilted DPPC cholesterol in one leaflet and fluid DOPC in another [fourth model in Fig. 5(b)].

It should be emphasized that the x-ray data do not prove this model to be correct but that the results are consistent with such a hypothesis. Chen *et al.* [26,53] recently reported the same observation of triple peaks in DOPC-DPPC-cholesterol mixtures using x-ray diffraction. According to their result, triple peaks can be observed in mixtures containing $9\sim15$ mol % cholesterol. While in our case, such a phenomenon only exists in the sample with 12 mol % cholesterol and was not observed for either 10% or 14% cholesterol. This discrepancy might be ascribed to the buffer solutions they used to prepare samples, as we know that counterions can potentially screen electrostatic interactions between lipid head groups, resulting in an alteration of phase boundaries. The d spacings they observe have the same feature as that in our case, i.e., the d spacing of the second component is in the middle of that of the first and third components [26,53]. In that paper, authors assign the first peak with the largest d spacing to gel phase, without further explanation or experimental evidence such as wide angle x-ray scattering (WAXS). We propose that the origin of the third peak observed by this group is not the gel phase but instead a result of the asymmetric distribution of domains in the bilayer.

In Fig. 3(b), it can be clearly seen that further increasing cholesterol up to 20% results in the coexistence of l_o and l_d phases. This indicates a complete coupling of DPPC-cholesterol domains between leaflets, thus a symmetrical distribution of bilayers. Similar to the DOPC-eSM-cholesterol system, reduction in the l_o phase d spacing can be ascribed to the smaller molecular size of cholesterol compared with the hydrophobic region of DPPC molecules because a few terminal carbons in DPPC side chains would be forced to adopt a less extended conformation to minimize the void volume created by cholesterol. We also noticed a slight increase of 0.1 nm in l_d phase d spacings, which is contrary to the constant l_d phase d spacing for the DOPC-eSM-cholesterol mixtures in the same cholesterol region. This difference might suggest that cholesterol has a preference for sphingolipids over phosphocholine. In the DOPC-eSM-cholesterol system, cholesterol prefers eSM domains through a hydrogen bond between the 3β-OH group of cholesterol and the amide group of sphingomyelin [7,47,54]; therefore at less than 25 mol % cholesterol it has little influence on the l_d phase d spacing (which contains little eSM). In the DOPC-DPPC-cholesterol system, cholesterol has a similar affinity for DOPC and DPPC, although the cholesterol molecule slightly prefers the more ordered DPPC domains. We observe that the packing of the DOPC rich l_d phase and the DPPC rich l_o phase were simultaneously altered by the addition of cholesterol either increasing or decreasing their d spacings.

Above 20 mol % cholesterol (up to ~50 mol %), both sets of mixtures behave in the same way. A single Bragg peak is observed, with higher orders of diffraction present. This directly demonstrates the presence of the l_o phase and we can assume either a possible continuous fluid l_o phase [55] or coexistence with the l_d phase based on previously published work by several authors [20,21].

IV. DISCUSSION AND CONCLUSIONS

While solution scattering appears to characterize the lamellar phase behavior in these systems very well, there is an important caveat to our analysis, which must be discussed. Solution x-ray diffraction is a global measurement of a bulk sample and indicates different d-spacing populations in the sample. This data represents the lamellar phase for our mixtures and not an isolated membrane, as used in the diffraction from small unilamellar vesicles (SUVs). If there is domain formation, we are always scattering from a mixture of the phases present.

The lateral domain size and arrangement will affect the data somewhat. At one extreme, the bilayers could completely partition into a stack of l_o lamellae and a stack of l_d lamellae. This would give the most well-defined data but is entropically unlikely. In the other extreme, a domain of one phase will prefer not to stack over a domain of the same phase, resulting in a checkerboardlike appearance. This is also highly unlikely to occur and would result in a broad (001) peak with no visible splitting. The most likely configuration for the sample is therefore a mosaic of small domains of varying sizes consisting of lamellar stacks of the same phase as it is favorable for bilayers of the same phase to stack together up to a certain stack size. Packing defects could accommodate the stacking of such a structure with two different lamellar spacings. It is not possible to determine the relative quantities of each phase (although we can assume to some extent that they are relatively equally present if the two Bragg peaks form l_o and l_d phases which are of similar heights).

There are advantages and disadvantages to the different methods used to study domain formation. GUVs are more representative of the geometry of the living cell; however they reveal less quantitative information about the nature of the phase compared to the bulk methods described here, leaving aside the fact that large scale phase separation can be promoted by the photoperoxidation of unsaturated lipids originating in the fluorescent probes [56,57]. Bilayer thickness measurements cannot be made using GUVs; whereas the solution XRD allows an analysis of the bilayer spacing and ordering within the two different phases as a function of changing composition. This bilayer thickness (d) does include the water layer, however for the complete unit cell and any swelling effects due to salt must be taken into account. It is more appropriate to use the results as an indicator of more than one bilayer population or to observe variations in spacing as a function of another parameter (e.g., cholesterol content) than to obtain an absolute measurement of bilayer thickness. More detailed measurements of d can be made by scattering from SUVs to obtain the electron-density profile

of the bilayer. In that case however, the small vesicle size rules out lipid domain studies.

In this paper, we demonstrate that solution x-ray scattering from the lamellar phase of ternary lipid mixtures is an invaluable tool in the characterization of l_o and l_d phase coexistence. The technique can potentially be used to generate the phase diagram for such mixtures in a more global measurement than used previously by examining the details of the Bragg peaks. This is an average measurement of a macroscopic sample, therefore not subject to local anomalous variations in mixing. In addition, the technique is free from the photoinduced effects of the fluorescence microscope. No evidence of x-ray beam damage on the samples is observed and also no probe molecule is required which can potentially affect the phase structure.

We have measured the lamellar spacings of the l_o and l_d phases simultaneously in two different ternary lipid mixtures as a function of cholesterol fraction, and interestingly our data show that a possible three-phase region occurs in the DOPC-eSM-cholesterol mixtures with the presence of the gel phase.

It is clear that the l_o and l_d phases in a bulk sample can be distinguished by this method and on comparison with previously published data using the GUV identification method; the results appear to coincide fairly well with ours, although the phase coexistence region observed in this paper is notably narrower. It is possible that the addition of probe molecules (either through steric interactions or as a photooxidation initiator) may widen the coexistence region. Using our analysis, we can observe a distinction between the initial onset of domain formation (i.e., the point where we start to observe peak splitting) and the true onset of the l_o phase formation [as defined by the appearance of the (004) peak]. It appears that at lower cholesterol fractions domains of different lamellar spacings may form but they do not have the well-defined layers characteristic of l_o. This observation accounts for the slightly lower cholesterol concentrations reported previously for the onset of phase separation in GUVs [20]. Using this method, data acquisition can be carried out rapidly at a synchrotron x-ray source and a further exploration of the complete phase diagram including differing ratios of high T_m and low T_m lipids and temperature dependence will be extremely interesting.

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