

Closed-Loop Miscibility Gap and Quantitative Tie-Lines in Ternary Membranes Containing Diphytanoyl PC

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ABSTRACT Vesicles containing ternary mixtures of diphytanoylphosphatidylcholine, dipalmitoylphosphatidylcholine (DPPC), and cholesterol produce coexisting liquid phases over an unusually large range of temperature and composition. Liquid domains persist well above the DPPC chain melting temperature (41°C), resulting in a closed-loop miscibility gap bounded by two critical points at fixed temperature. Quantitative tie-lines are determined directly from ²H NMR spectra using a novel analysis, and are found to connect a liquid-disordered phase rich in diphytanoyl PC with a liquid-ordered phase rich in DPPC. The direction of the tie-lines implies that binary DPPC/cholesterol mixtures are in one uniform phase above 41°C. All ²H NMR results for tie-lines are verified by independent fluorescence microscopy results.

INTRODUCTION

Over the past five years, our group and others have established that coexisting liquid-ordered (L_o) and liquid-crystalline (L_α) phases form in vesicles composed of a wide variety of ternary lipid mixtures (1–6). These mixtures contain a lipid with high melting temperature (T_m), a lipid with low T_m , and a sterol such as cholesterol. A major question remains: why are three components, rather than only two, necessary to form micron-scale liquid domains in membranes? Furthermore, opinions vary on whether or not large-scale domains in ternary systems arise from small-scale domains in binary systems of lipid and cholesterol (7). Recent work has explored both the experimental (8–11) and theoretical aspects of these questions (12–16). We have found that the ternary system of 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (diPhyPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and cholesterol (Chol) is particularly suited to addressing these fundamental questions experimentally.

The diPhyPC/DPPC/Chol mixture exhibits surprisingly high miscibility transition temperatures, and coexisting liquid phases are observed over a wider range of lipid compositions than we have reported for any other lipid mixture. As a result, a closed-loop miscibility gap is present at high temperatures in the phase diagram, isolated from interference by the lower-temperature gel phase of DPPC at 41°C (17). As an added benefit, both phospholipids are saturated, so our vesicles are less prone to photooxidation during fluorescence imaging. This stability allows for direct observation of critical fluctuations by fluorescence microscopy over the course of several minutes. Moreover, diPhyPC is interesting in itself. It has a very low melting temperature of $<-120^\circ\text{C}$ due

to its branched acyl chains (18). Polyunsaturated lipids also have very low chain melting temperatures, and our observations may apply to membranes containing these biologically important lipids.

Here we map phase diagrams of diPhyPC/DPPC/Chol by fluorescence microscopy and independently determine the composition of coexisting liquid phases by ²H NMR. We use a novel analysis to calculate tie-lines through the composition point 35/35/30 diPhyPC/DPPC/Chol both above and below the DPPC chain melting temperature. Lastly, we present implications of our ternary tie-lines on the phase diagram of binary DPPC/Chol mixtures.

MATERIALS AND METHODS

Commercial reagents

DiPhyPC, DPPC, Chol, and 1,2-dipalmitoyl-d62-*sn*-glycero-3-phosphocholine (DPPCd62) were obtained from Avanti Polar Lipids (Alabaster, AL). All lipids were used without further purification and were stored in chloroform at -20°C until use. Texas Red 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (Molecular Probes, Eugene, OR) was used as a dye for contrast between phases in fluorescence experiments.

Fluorescence microscopy

Giant unilamellar vesicles (GUVs) were prepared using electroformation (19) and viewed using fluorescence microscopy, as described extensively elsewhere (3,20). Vesicles were prepared in $>18\text{ M}\Omega/\text{cm}$ water, grown for 1 h at $60 \pm 3^\circ\text{C}$, and stored at the growth temperature for at most 2 h before observation. Miscibility transition temperatures were measured by monitoring the distribution of a fluorescent probe in vesicles. Specifically, GUVs were viewed after ramping a temperature-controlled stage near the transition ($\sim 0.2^\circ\text{C/s}$) and then waiting $\sim 60\text{ s}$ for the stage and sample to equilibrate.

Although the miscibility transition temperature is well defined in a single vesicle, we find a distribution of transition temperatures among vesicles in a single GUV preparation. We attribute this to slight variations in lipid composition between vesicles, estimated at $<2\text{ mol } \%$. Errors reported in temperatures represent the full range of measured transition temperatures in a single vesicle sample. The midpoint of this range roughly corresponds to the temperature at which half of the vesicles contain coexisting liquid phases. This reproducible midpoint is the transition temperature we report.

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Deuterium NMR

Multilamellar vesicles (MLVs) of 1:1 diPhyPC/DPPCd62 + 30% Chol, 1:2 diPhyPC/DPPCd62 + 40% Chol, and 1:4 diPhyPC/DPPCd62 + 40% Chol were prepared for ^2H NMR experiments. Lipid mixtures containing 10 mg deuterated lipid (DPPCd62) were mixed in chloroform and then dried under nitrogen while rotating to form a thin film. Samples were exposed to vacuum ($<10\ \mu\text{m Hg}$) overnight to remove any remaining solvent. Lipids were hydrated to 50 wt % with deuterium-depleted H_2O , homogenized at 60°C , spun into ground-glass stoppered sample tubes, and sealed with parafilm. Samples were either used immediately after preparation or stored at -80°C until use. Samples were not hydrated at room temperature ($\sim 20^\circ\text{C}$) because the resulting MLVs produced low-resolution spectra at temperatures well above the miscibility transition (Fig. 1). Resolution was restored after vesicles were annealed at high temperature (90°C) for ~ 1 h. We conclude that samples that are mixed and hydrated at room temperature are inhomogeneous.

^2H NMR powder spectra were acquired on a 500 MHz Bruker spectrometer (Billerica, MA) at a resonance frequency of 76.8 MHz using a stationary dual-resonance probe with a 4-mm solenoidal sample coil. Sample temperature was controlled to 0.1°C by a Bruker variable temperature control unit. Absolute sample temperature was calibrated to within 0.5°C . A quadrupolar echo sequence (21) was used with two 1.8-s 90° pulses and an interpulse delay of $50\ \mu\text{s}$. For all experiments, the carrier frequency of the instrument was placed at the center of the spectrum. Typically 1,024 scans were acquired with a spectral width of 200 kHz and a delay time of 0.2 s. Before Fourier transformation, the resonances, detected in quadrature detection mode, were phase-corrected to minimize intensity in the imaginary channel. The echo maximum in the real channel was determined with a resolution of 1/10 of a dwell-time unit. A time-base-corrected free induction decay was calculated by spline interpolation between data points to begin

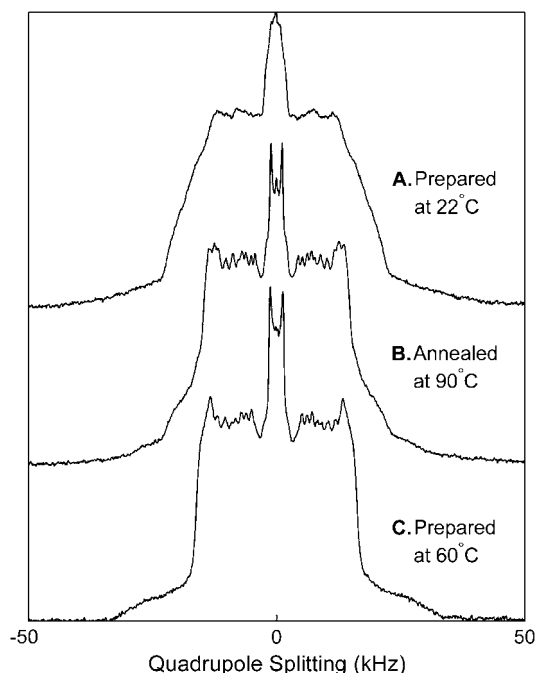


FIGURE 1 Quadrupole spectra of vesicles of 1:1 diPhyPC/DPPCd62 + 30% cholesterol at 60°C under different sample preparations. (A) MLVs hydrated at room temperature ($\sim 20^\circ\text{C}$) produce low resolution spectra, suggesting that lipids are not uniformly distributed between vesicles. (B) Spectral resolution improves after vesicles from A are annealed for 1 h at 80 – 90°C . (C) MLVs hydrated at 60°C give well resolved L_α spectra.

exactly at the echo maximum. An exponential line broadening of 100 Hz was applied to the free induction decay before performing the Fourier transformation.

The first moments, M_1 , of ^2H NMR spectra were evaluated as $M_1 = (1/M_0) \int |\omega S(\omega)|$, where $M_0 = \int S(\omega)$ is the total area under the spectrum, and $S(\omega)$ is the intensity of the ^2H NMR spectrum as a function of frequency ω . The origin of the frequency axis was defined as the center of symmetry of each spectrum. The limits of integration were symmetric around the origin and completely enclose the observed spectra. The magnitude and spectral width of the methyl splittings were determined from the dePaked spectra by fitting with a Lorentzian lineshape.

Tie-line evaluation

Tie-lines were determined through spectral subtraction as previously described in binary systems (22,23). Here we generalize the method for use in ternary systems. First, we show how to calculate the location of the tie-line endpoints when the tie-line slope is known. We then describe a novel method for evaluating tie-line slope using ^2H NMR spectra from three distinct lipid compositions.

Determination of tie-line endpoints with known tie-line slope

Two normalized experimental spectra, S_A and S_B , have compositions that fall along the same tie-line if they can be represented as a weighted superposition of the same two endpoint spectra:

$$S_A = f_A S_\alpha + (1 - f_A) S_o;$$

$$S_B = f_B S_\alpha + (1 - f_B) S_o,$$

where S_α is the L_α -phase endpoint spectrum, S_o is the L_o -phase endpoint spectra, and f_A (f_B) represents the fraction of S_A (S_B) arising from lipids in the L_α phase. Experimentally, the endpoint spectra are initially unknown, and we instead perform a weighted subtraction of the two spectra until only a pure L_α or L_o endpoint spectrum is found:

$$S_\alpha = (S_A - F_A S_B) / (1 - F_A);$$

$$S_o = (S_B - F_B S_A) / (1 - F_B).$$

The terms f_A , f_B , F_A , and F_B are related by simple algebra:

$$f_A = (1 - F_A) / (1 - F_A F_B);$$

$$f_B = (F_B - F_A F_B) / (1 - F_A F_B).$$

In a two-phase region, the fraction of lipids that are in the L_α or L_o phase is given by the lever rule:

$$0 = M_A (\vec{\phi}_A - \vec{\phi}_\alpha) + (1 - M_A) (\vec{\phi}_\alpha - \vec{\phi}_o)$$

$$0 = M_B (\vec{\phi}_B - \vec{\phi}_\alpha) + (1 - M_B) (\vec{\phi}_B - \vec{\phi}_o),$$

where M_A and M_B are the mole fractions of total lipid represented in the spectra S_A and S_B that are in the L_α phase. The vector $\vec{\phi}_i = [\phi_i^{\text{DPPCd62}}, \phi_i^{\text{Chol}}, \dots]$ describes the lipid composition that produces spectrum S_i , and the vectors $(\vec{\phi}_i - \vec{\phi}_\alpha)$ and $(\vec{\phi}_i - \vec{\phi}_o)$ connect the composition of spectrum S_i with the L_α and L_o endpoints, respectively. In our experiments, we measure the partitioning of the DPPCd62 lipid to the L_α phase (recorded in f_A and f_B). The measured values for f_A and $(1 - f_A)$ must be normalized by the concentration of DPPC lipids in each phase ($\phi_\alpha^{\text{DPPCd62}}$ and ϕ_o^{DPPCd62}) to give the partitioning of total lipid. The lever rule becomes:

$$0 = [f_A / \phi_\alpha^{\text{DPPCd62}}] (\vec{\phi}_A - \vec{\phi}_\alpha) + [(1 - f_A) / \phi_o^{\text{DPPCd62}}] (\vec{\phi}_\alpha - \vec{\phi}_o)$$

$$0 = [f_B / \phi_\alpha^{\text{DPPCd62}}] (\vec{\phi}_B - \vec{\phi}_\alpha) + [(1 - f_B) / \phi_o^{\text{DPPCd62}}] (\vec{\phi}_B - \vec{\phi}_o).$$

This system of equations is solved to determine the compositions of the two endpoint spectra ($\vec{\phi}_\alpha$ and $\vec{\phi}_\beta$). In a three-component system, compositions are described using two independent variables (e.g., ϕ^{DPPCd62} and ϕ^{Chol}) and thus four equations must be solved. The tie-line slope is constrained by the choice of compositions $\vec{\phi}_A$ and $\vec{\phi}_B$, whereas the parameters f_A and f_B determine the location of the tie-line endpoints.

Determination of tie-line slope

In our analysis, we do not assume a tie-line slope and instead use three separate ^2H NMR spectra to determine tie-line slope and endpoints through two minimization steps. Our goal is to determine the tie-line that passes through the point $\vec{\phi}_A = 35\hat{\phi}^{\text{DPPCd62}} + 30\hat{\phi}^{\text{Chol}}$ (1:1 diPhyPC/DPPC + 30% Chol). A second composition along the tie-line (ϕ_B) is determined by interpolating between any two additional spectra (here, they are 1:2 diPhyPC/DPPC + 40% Chol and 1:4 diPhyPC/DPPC + 40% Chol) so that the resulting endpoint spectrum S_α (determined by spectral subtraction) is best fit to the reference L_α spectrum (Fig. 2, *a* and *b*). This minimization step determines the tie-line slope as well as the parameter F_A . Spectral subtraction is then conducted by fitting S_β against the reference L_β spectrum to determine the second subtraction parameter F_B (Fig. 2 *c*).

Fitting procedure

Spectral subtraction is accomplished by minimizing the mean-squared difference between the calculated S_α (S_β) spectrum and a known reference L_α (L_β) spectrum using the `fminsearch` routine in Matlab6 (Fig. 2). The reference L_α spectrum is acquired from membranes of 4:1 1,2-di-oleoyl-*sn*-glycero-3-phosphocholine (DOPC)/DPPCd62 + 15% cholesterol, and the reference L_β spectrum is from membranes of either DPPCd62 + 40% cholesterol or DPPCd62 + 50% cholesterol. Reference spectra are scaled along the frequency axis to correct for temperature- and composition-dependent changes in order parameters. This correction assumes that order-parameter profiles of hydrocarbon chains are related by a simple scaling factor. Previous work (e.g., Barry and Gawrisch (24)) and our own analysis in Fig. 2 demonstrate that this assumption is accurate for modest variations of temperature and composition.

Errors in the fitting parameters F_A , F_B , and tie-line slope are determined directly from the covariance matrix and are verified through a visual inspection of the fits. A five-point smoothing filter is applied to the residual before squaring and integrating to reduce errors due to noise. Errors in the fitting parameters are propagated through the tie-line calculation, taking into consideration that fitting parameters do not vary independently. Correlation coefficients between tie-line slope and F_A , which are both determined in the first fitting step, range between -0.51 and -0.59 . The fitting parameter F_B , which is determined in the second fitting step, and tie-line slope are likely also correlated, but it is assumed that this contributes minimally to the reported error bounds. Using this method, tie-line errors are dependent on the reference spectra used. Propagated error limits (see Fig. 7) are deemed to be sufficiently small. Errors could be further reduced by investigating samples with compositions corresponding to the tie-line endpoints.

RESULTS AND DISCUSSION

Phase boundary by fluorescence microscopy

GUVs prepared from ternary mixtures of diPhyPC, DPPC, and cholesterol produce coexisting liquid phases as temperature is lowered through the miscibility transition temperature (Fig. 3). A distinct experimental advantage of the diPhyPC system is that vesicles are generally larger and more stable than for similar domain-forming mixtures. In time, liquid phases ripen completely through a process of domain collision and coalescence. Occasionally, domains remain stable in a dispersed pattern.

The diPhyPC/DPPC/Chol system is remarkable in the extent of its miscibility phase boundary, both in temperature and in composition (Fig. 4). Liquid domains are present in vesicles at temperatures as high as 50°C , well above the DPPC chain melting temperature of $T_m = 41^\circ\text{C}$ (17). As a

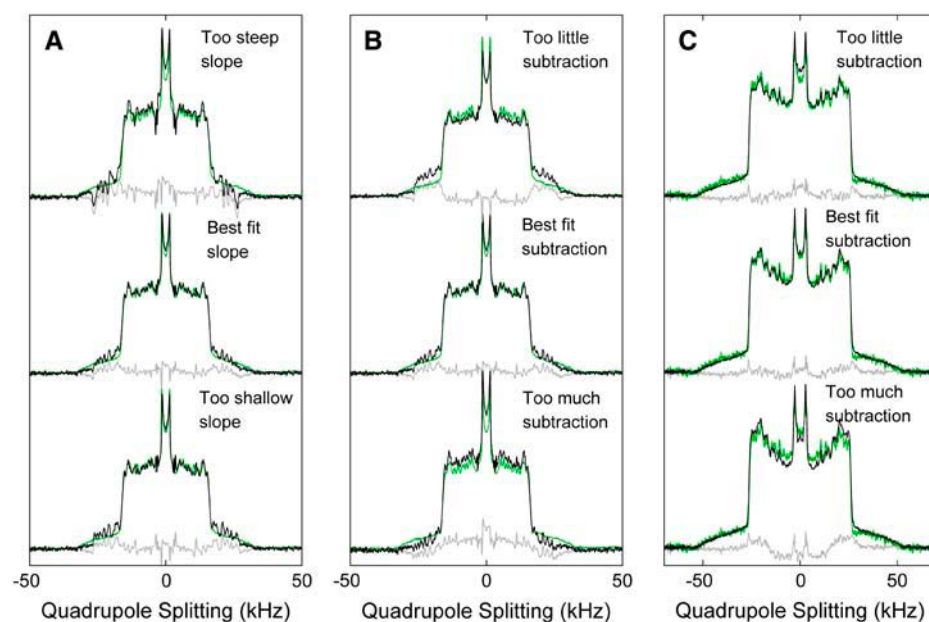
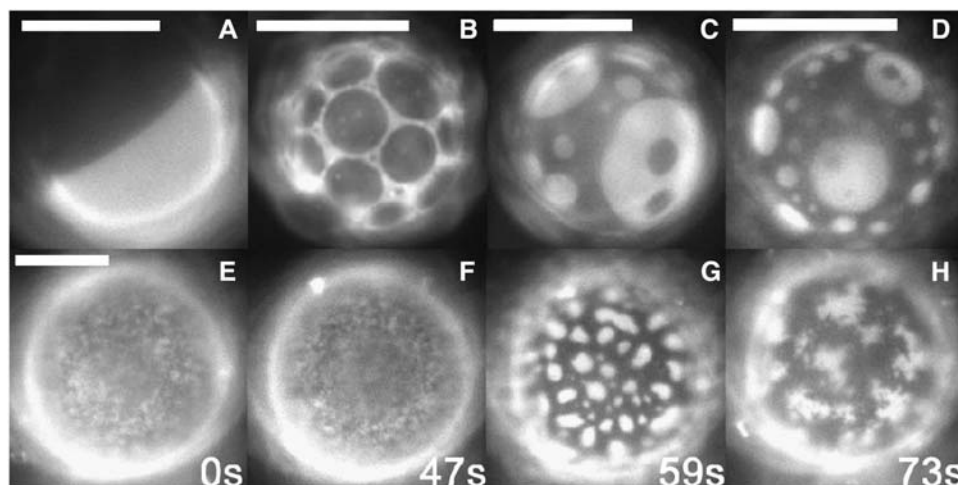


FIGURE 2 Demonstration of spectral subtraction method at 35°C to determine the best fit for tie-lines passing through the point 1:1 diPhyPC/DPPC + 30% cholesterol. Measured reference spectra (green) are subtracted from calculated endpoint spectra (black), yielding residuals in gray. All reference L_α spectra in *A* and *B* are identical, and all reference L_β spectra in *C* are identical. (*A*) Calculated L_α endpoint spectrum with a tie-line slope that is too steep (*top*), best fit (*middle*), and too shallow (*bottom*). The goodness of fit can be assessed by eye by examining the intensity at wide splittings. (*B*) Calculated L_α endpoint spectrum when the subtraction is too little (*top*), best fit (*middle*), and too large (*bottom*). (*C*) Calculated L_β endpoint spectrum when the subtraction is too small (*top*), best fit (*middle*), and too large (*bottom*). In this example, the calculated L_β endpoint is best evaluated by examining narrow splittings and is less sensitive to changes in fitting parameters than the L_α endpoint. For clarity of demonstration, the parameter ranges shown here are larger than those described by the errors obtained directly from the fitting procedure.



panels E–H and was imaged at the times shown. Membrane compositions are (A) 1:2 diPhyPC/DPPC + 40% Chol at 16°C; (B) 1:4 diPhyPC/DPPC + 20% Chol at 23°C; (C) 1:4 diPhyPC/SOPC + 40% Chol at ~5°C; (D) 1:4 diPhyPC/POPC + 40% Chol at ~5°C; and (E–H) 1:1 diPhyPC/DPPC + 50% Chol at ~33°C. Scale bars, 20 μm .

comparison, the related system of DOPC/DPPC/Chol produces liquid domains only below 41°C, although the qualitative shape of the phase boundary is similar in both systems (3). The diPhyPC system also surpasses the DOPC system in the range of compositions that produce domains.

By comparing systems containing diPhyPC to ones containing other lipids with low melting temperatures, it appears that lower chain melting temperatures produce higher miscibility transition temperatures. For example, liquid domains are observed at higher temperatures in vesicles of diPhyPC/DPPC/Chol (diPhyPC $T_m < -120^\circ\text{C}$) than in DOPC/DPPC/Chol (DOPC $T_m = -20^\circ\text{C}$) (17), just as miscibility transition temperatures are higher in DOPC/D-erythro-N-palmitoyl-sphingomyelin (PSM)/Chol than in 1-palmitoyl-2-oleoyl-*sn*-

glycero-3-phosphocholine (POPC)/PSM/Chol (POPC $T_m < -2^\circ\text{C}$) (4). In other words, we expect similar results for membranes in which the high T_m lipid component is a sphingomyelin rather than DPPC.

Observations of high miscibility transition temperatures are biologically significant because an important class of lipids, polyunsaturated lipids, also have very low chain melting temperatures (25). It has been proposed that polyunsaturated lipids can enhance interactions between saturated chains and cholesterol (26,27). Given our results for membranes containing diPhyPC, we predict that membranes with polyunsaturated lipids should sustain coexisting liquid domains at high temperatures. Indeed, there is evidence for domains rich in DPPC and cholesterol at high temperatures (50°C) in rhodopsin-containing membranes of di(22:6)PC/DPPC/Chol

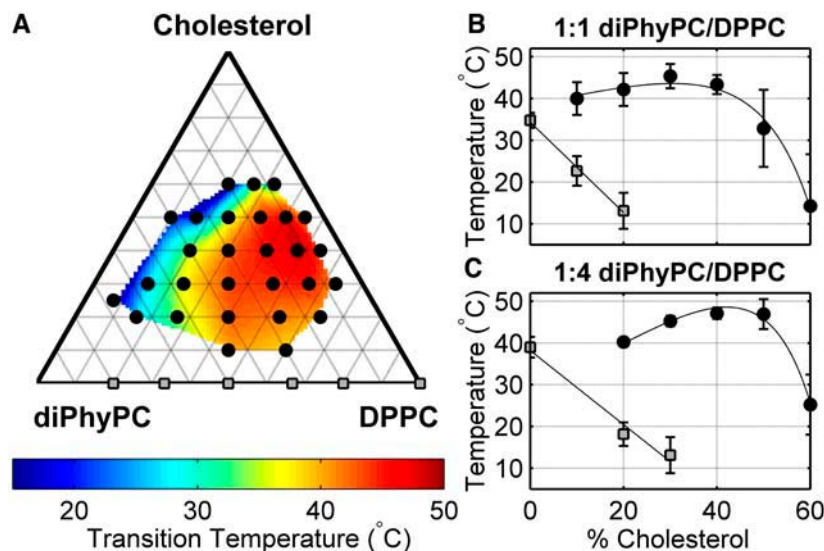


FIGURE 4 (A) Phase behavior determined by fluorescence microscopy for vesicles composed of mixtures of diPhyPC, DPPC, and cholesterol. Black circles denote compositions that form coexisting liquid phases as temperature is lowered. Miscibility transition temperatures follow the color scale, where high temperatures are generally at high DPPC/Chol fractions. Gray squares denote gel-liquid coexistence, which is observed in membranes without cholesterol. (B and C) Cuts through the phase boundary at constant diPhyPC:DPPC ratio. Black circles denote the miscibility transition temperature and gray squares indicate the temperature at which a gel phase is first observed as the temperature is lowered.

(28). Additionally, membranes with polyunsaturated lipids may form domains even in the absence of more traditional “raft-associated” lipids. For example, we observe liquid immiscibility in membranes in which the monounsaturated lipids POPC or 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC) function as the high T_m component (Fig. 3, *C* and *D*), a surprising result.

Fig. 5 shows phase diagrams of diPhyPC/DPPC/Chol vesicles at two different temperatures, 43°C and 16°C. By examining the surface fraction of bright and dark phases as a function of lipid composition at both temperatures, we conclude that the bright phase is strongly enriched in diPhyPC, whereas the dark phase is strongly enriched in DPPC and moderately enriched in cholesterol (3). Above the chain melting temperature of 41°C, we observe a closed-loop miscibility gap, such that the miscibility phase boundary does not intersect any of the three limiting binary mixtures, or any region containing a solid (gel) phase. A striking consequence of this type of miscibility gap is that the diPhyPC/DPPC/

Chol system has two miscibility critical points at any fixed temperature between the DPPC chain melting temperature and the upper consolute critical point. By examining surface fractions of bright and dark phases in Fig. 5, *top*, we infer the presence of two critical points at high and low cholesterol compositions.

In contrast, below the DPPC chain melting temperature, the phase diagram appears similar to those we have observed previously (4) in that the miscibility phase boundary does intersect a gel phase in a three-phase region. We have direct ^2H NMR evidence for coexistence of two liquids plus one gel phase in membranes of DOPC/DPPC62/Chol (S. L. Veatch, S. L. Keller, and K. Gawrisch, unpublished data). We can also infer the presence of a three-phase region in membranes with <30% cholesterol by fluorescence microscopy because the lipid mobility in dark regions and the ripening of dark domains is significantly slower at low temperature (Fig. 5, *bottom*), as we have reported for vesicles of DOPC/PSM/Chol and POPC/PSM/Chol (4).

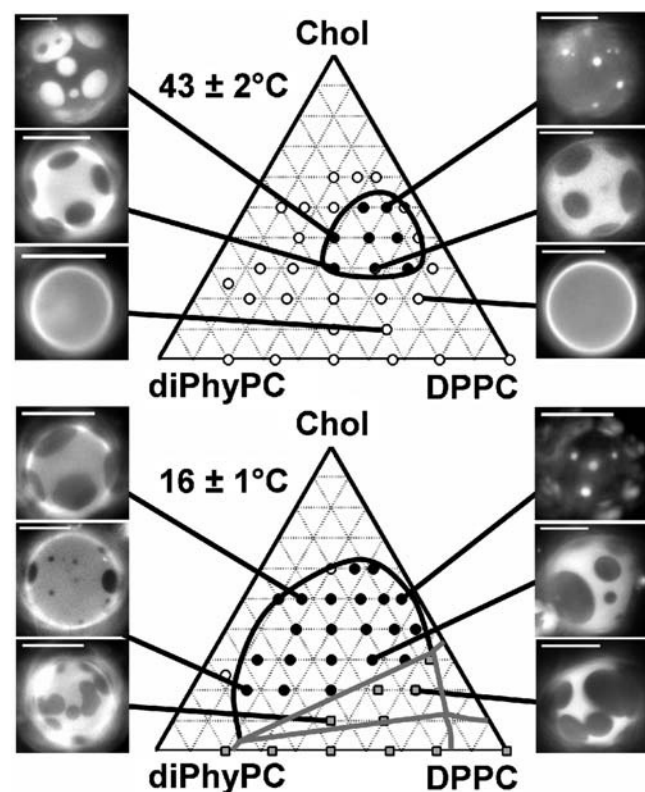


FIGURE 5 Phase diagrams determined by fluorescence microscopy and corresponding fluorescence micrographs for GUVs of ternary mixtures of diPhyPC, DPPC, and cholesterol at constant temperature. Open circles denote one liquid phase. (*Top*) Above the DPPC chain melting temperature of 41°C, the miscibility region denoted by black circles lies within a closed loop and no solid phase is present. (*Bottom*) At a lower temperature, gray squares indicate the presence of solid phase in vesicles. The liquid-liquid coexistence region connects to the gel-liquid coexistence region through a three-phase triangle at low cholesterol. Vesicle compositions are as indicated in the Gibbs triangle. Scale bars, 20 microns.

Liquid immiscibility by ^2H NMR

^2H NMR is a particularly powerful tool for observing coexisting liquid phases, because spectra show a superposition of liquid endpoint spectra (Fig. 6). A significant step forward in our current work is that we have collected detailed ^2H NMR spectra for three separate compositions over a wide range of temperatures of the ternary diPhyPC/DPPC/Chol vesicle system. Our choice of compositions allows us to quantitatively determine the lipid compositions of the two liquid phases along tie-lines (Fig. 7) without relying on phase boundaries determined by fluorescence as in previous work (20). As a result, our current tie-lines benefit from significantly smaller error bounds.

We find that ^2H NMR tie-lines connect an L_o phase rich in both DPPC62 and cholesterol with an L_α phase rich in diPhyPC. A series of tie-lines passing through the point 35:35:30 diPhyPC/DPPC/Chol are shown in Fig. 7. At lower temperatures, the tie-line extends to higher diPhyPC fraction (the L_α endpoint lies closer to the diPhyPC vertex in the Gibbs triangle), and the cholesterol compositions of the two liquid phases become more similar (the tie-line has a shallower slope).

Our results above are useful for critically evaluating the validity of fluorescence microscopy phase boundaries. First, we notice that the tie-lines in Fig. 7 are similar to the tie-lines we previously estimated for the ternary system of DOPC/DPPC/Chol by using a combination of ^2H NMR and fluorescence microscopy results (20). Additional evidence for good correlation between ^2H NMR and fluorescence is clear by directly evaluating phase boundaries both in temperature and in composition. We find good agreement between miscibility transition temperatures measured by ^2H NMR and by fluorescence microscopy for the mixtures probed in Table 1. Moreover, tie-line endpoints derived from ^2H NMR

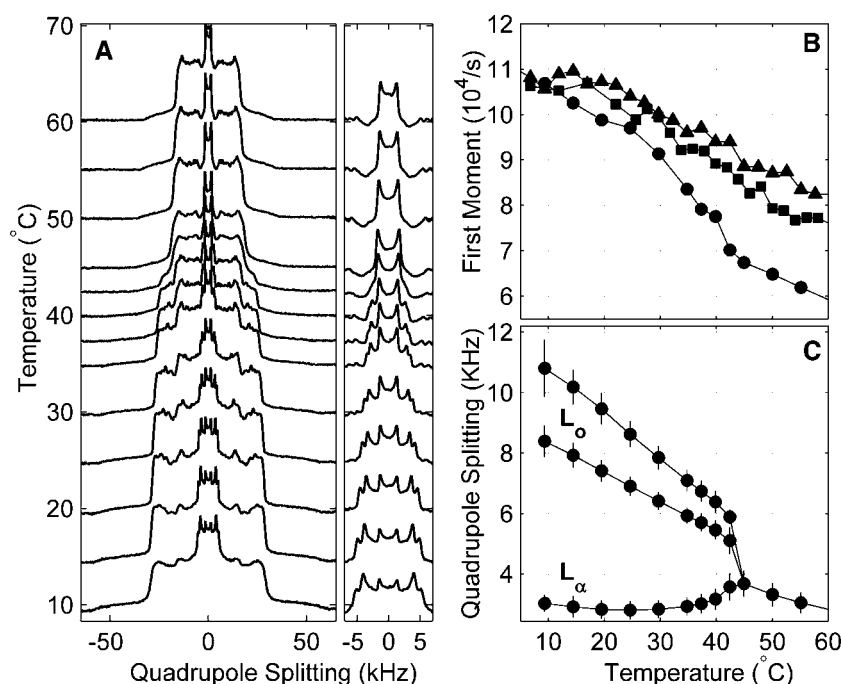


FIGURE 6 ^2H NMR results for multilamellar vesicles containing ternary mixtures of diPhyPC, DPPCd62, and cholesterol. (A) DPPCd62 ^2H NMR spectra at various temperatures for 1:1 diPhyPC/DPPCd62 + 30% cholesterol. (B) DPPCd62 first moments for 1:1 diPhyPC/DPPCd62 + 30% cholesterol (circles), 1:2 diPhyPC/DPPCd62 + 40% cholesterol (squares), and 1:4 diPhyPC/DPPCd62 + 40% cholesterol (triangles). (C) DPPCd62 methyl quadrupole splittings and peak widths (error bars) as a function of temperature for 1:1 DOPC/DPPCd62 + 30% cholesterol. Below the miscibility transition at 42.5°C, three separate methyl splittings are found, indicating coexistence of L_o and L_α phases (20). The L_o phase produces two wide (ordered) splittings, whereas the L_α phase produces a single narrow (disordered) splitting.

are close to fluorescence-microscopy phase boundaries, as can be seen by eye in Fig. 7.

Critical fluctuations

Using fluorescence microscopy, we directly observe composition fluctuations in membranes of diPhyPC/DPPC/Chol with compositions near a miscibility critical point (Fig. 3, *E–H*). In vesicles of diPhyPC/DPPC/Chol, we find that critical fluctuations can persist for long times (>10 min) without ripening into larger liquid domains (Fig. 3, *E–F*). In the same vesicle, fluctuations ripen into distinct domains when temperature is slightly lowered, although fluctuations of the domain boundaries are still visible (Fig. 3 *G*). When temperature is raised again, domains break apart and critical fluctuations resume, indicating that our observations are reversible (Fig. 3 *H*).

A distinct experimental advantage of the diPhyPC/DPPC/Chol ternary system is that it is possible to view critical fluctuations under the microscope for extended time periods. Previously, we were unable to hold vesicles containing unsaturated phospholipids near a miscibility phase boundary using fluorescence microscopy because photooxidation of unsaturated lipids shifts the phase boundary to higher temperature over time (3,7).

In this study, we did not probe lipid compositions near a critical point by ^2H NMR. Critical fluctuations are observed by ^2H NMR studies as broadening of spectral peaks (unpublished data for the system of DOPC/DPPCd62/Chol). We do not observe significant broadening of spectra near the transition temperature for the three mixtures probed in this study.

Implications for the DPPC/Chol binary phase diagram

The direction of our calculated tie-lines in the ternary diPhyPC/DPPC/Chol mixture has direct implications for the phase behavior of binary mixtures of DPPC and cholesterol. Numerous reports have proposed coexistence of L_o and L_α phases in DPPC (or DMPC) and cholesterol above the DPPC chain melting temperature (22,29,30). We (7) and others (12, 13,31) have recently questioned this conclusion, because most evidence for this particular phase separation is indirect, and lipid organization, if present, occurs on a small length-scale. In past work, we documented that large-scale phase separation is not directly observed in binary DPPC/Chol mixtures by fluorescence microscopy (3), yet it was unclear if limited spatial resolution or equal probe partitioning between phases obscured our ability to observe two liquid phases. Our results in the ternary diPhyPC/DPPC/Chol mixture offer much stronger evidence to suggest that binary membranes of DPPC and cholesterol are in one uniform phase above the DPPC chain melting temperature.

Consider the two potential phase diagrams in Fig. 8 for vesicles of diPhyPC/DPPC/Chol at temperatures above the DPPC chain melting temperature. If phase separation is present in the binary DPPC/Chol mixture (Fig. 8 *A*), we expect to find tie-lines roughly parallel to the binary DPPC/Chol edge, at least for mixtures with small amounts of diPhyPC. This is because the tie-line in the binary DPPC/Chol mixture is constrained to run along the binary edge, and thermodynamic rules stipulate that tie-line slopes must vary continuously. On the other hand, if phase separation is not present in the binary mixture, then there is no initial constraint on

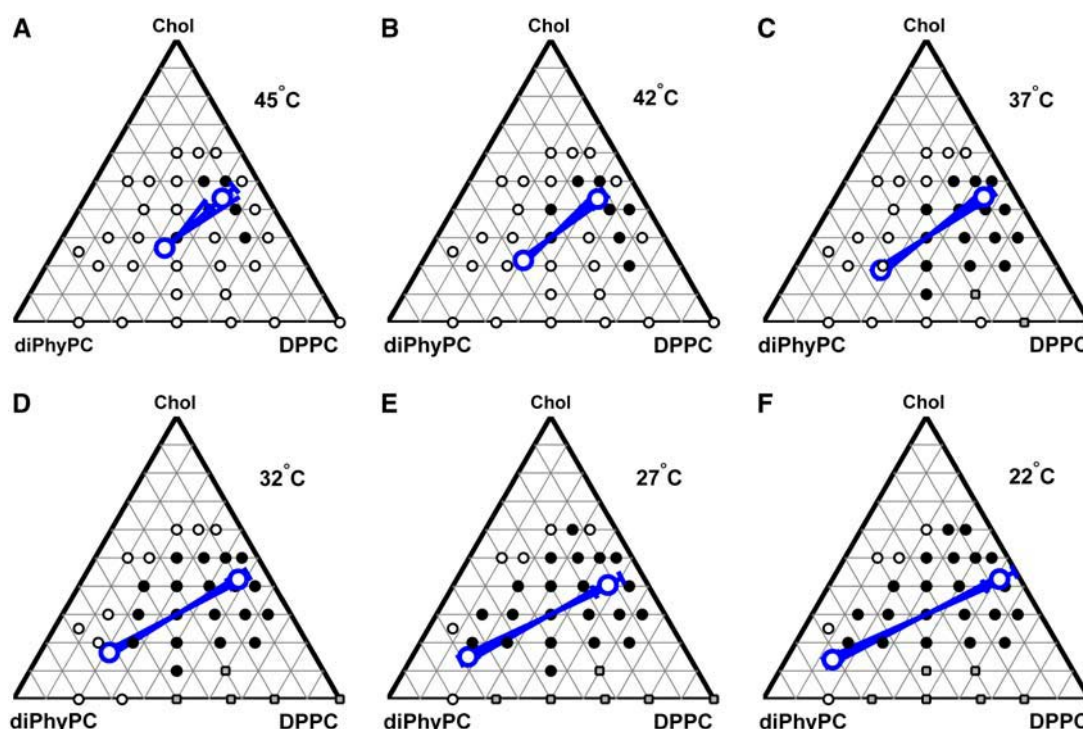


FIGURE 7 Tie-lines calculated from ^2H NMR spectra through the composition 1:1 diPhyPC/DPPC + 30% cholesterol at different temperatures are shown as thick blue lines. Calculated endpoints are represented by large open circles. Errors in the calculated endpoints are represented by the splay of the blue line, and tick marks around each endpoint. In most cases, calculated errors are on the order of the size of the large open circles. Tie-lines are superimposed on fluorescence microscopy data recording the phase behavior of GUVs of 34 different lipid compositions. *Black circles*, coexisting liquid phases; *open circles*, one uniform liquid phase; *gray squares* indicate coexistence of solid and liquid(s). ^2H NMR tie-line temperatures are shifted $+2.5^\circ\text{C}$ to correct for lower transition temperatures in perdeuterated lipid samples (20).

tie-line slope, and tie-lines can assume any orientation with respect to limiting binary mixtures, including an orientation perpendicular to the DPPC/Chol axis (as in Fig. 8 B).

In our experiments, we observe that tie-lines indeed run roughly perpendicular to the binary DPPC/Chol edge, even for membranes with small amounts of diPhyPC. It is unlikely that this tie-line orientation could be achieved if phase separation were present in the binary mixture. Our results suggest that membranes of DPPC and cholesterol do not form coexisting L_o and L_α phases above the DPPC chain melting temperature for binary mixtures in the vicinity of our ^2H NMR tie-lines. Instead, we conclude that these binary membranes form a single uniform liquid phase at these temperatures.

CONCLUSIONS

We observe coexisting liquid phases in ternary mixtures of diPhyPC, DPPC, and cholesterol by both fluorescence microscopy and deuterium NMR. Using both experimental methods, we find that this system is unusual because liquid

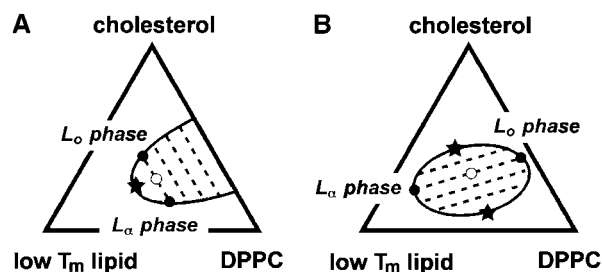


FIGURE 8 (A) If two liquid phases exist in vesicles containing binary mixtures of DPPC and cholesterol above the DPPC chain-melting temperature, then tie-lines through compositions close to the DPPC/cholesterol binary axis lie roughly parallel to that axis. For example, the composition at the open circle demixes along the dashed tie-line to form two liquid phases with compositions at the solid circles. Only one critical point (*star*) is expected. (B) In contrast, two critical points (*stars*) are found for a closed-loop miscibility gap, and tie-lines are allowed to run roughly perpendicular to the DPPC/cholesterol axis (as observed in the diPhyPC/DPPC/Chol system).

TABLE 1 Miscibility transition temperatures for mixtures of diPhyPC/DPPCd62/Chol

Lipid mixture	Transition temperature	
	Microscopy	^2H NMR
1:1 DiPhyPC/DPPCd62 + 30% Chol	$42 \pm 2^\circ\text{C}$	$43 \pm 2^\circ\text{C}$
1:2 DiPhyPC/DPPCd62 + 40% Chol	$43 \pm 2^\circ\text{C}$	$42 \pm 2^\circ\text{C}$
1:4 DiPhyPC/DPPCd62 + 40% Chol	$43 \pm 2^\circ\text{C}$	$43 \pm 2^\circ\text{C}$

TABLE 2 L_o and L_α phase compositions for tie-lines that pass through the point 1:1 diPhyPC/DPPC + 30% cholesterol

	L_o endpoint				L_α endpoint				Tie-line slope	
	diPhyPC	DPPC	Chol	σ diPhyPC	diPhyPC	DPPC	Chol	σ diPhyPC	Slope*	σ Slope
22°C	6	51	43	12	72	14	14	6	0.77	0.07
27°C	12	47	41	11	68	17	15	6	0.84	0.10
32°C	10	48	42	7	62	21	17	4	0.98	0.10
37°C	10	45	45	8	55	27	18	5	1.39	0.26
42°C	14	42	44	7	47	31	22	4	1.84	0.53
45°C	14	42	44	12	41	33	26	4	2.00	1.51

Data in table correspond to representation in Fig. 7. Tie-lines were determined by fitting ^2H NMR spectra as in Fig. 2. Tie-line errors are presented as standard deviations in both diPhyPC concentration (roughly equivalent to changes in tie-line length) and in tie-line slope, and are given by σ diPhyPC and σ slope, respectively. ^2H NMR tie-line temperatures are shifted +2.5°C to correct for lower transition temperatures in perdeuterated lipid samples (20).

*Slope = $(\phi_o^{\text{Chol}} - \phi_\alpha^{\text{Chol}})/(\phi_o^{\text{DPPC}} - \phi_\alpha^{\text{DPPC}})$.

domains persist well above the DPPC chain melting temperature, and coexisting phases are observed over a very wide range of lipid compositions. We directly observe critical fluctuations by fluorescence microscopy for long time periods. Ternary mixtures containing diPhyPC are striking in that mono-unsaturated lipids can function as the high T_m component as in diPhyPC/POPC/Chol and diPhyPC/SOPC/Chol.

We use a novel spectral subtraction method to calculate quantitative tie-lines directly from ^2H NMR spectra, independent of fluorescence microscopy results. We find that one liquid phase is rich in diPhyPC and the other liquid phase is rich in DPPC. The direction of tie-lines and the location of the phase boundary are in excellent agreement with independent fluorescence microscopy results. We report that observed tie-line orientations imply an absence of liquid immiscibility in binary DPPC/Chol mixtures at temperatures above the DPPC chain melting temperature of 41°C.

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