Toward a Better Raft Model: Modulated Phases in the Four-Component Bilayer, DSPC/DOPC/POPC/CHOL

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ABSTRACT The liquid-liquid (Ld + Lo) coexistence region within a distearoyl-phosphatidylcholine/dioleoyl-phosphatidylcholine/palmitoyl-oleyl-phosphatidylcholine/cholesterol (DSPC/DOPC/POPC/CHOL) mixture displays a nanoscopic-to-macroscopic transition of phase domains as POPC is replaced by DOPC. Previously, we showed that the transition goes through a modulated phase regime during this replacement, in which patterned liquid phase morphologies are observed on giant unilamellar vesicles (GUVs). Here, we describe a more detailed investigation of the modulated phase regime along two different thermodynamic tie-lines within the Ld + Lo region of this four-component mixture. Using fluorescence microscopy of GUVs, we found that the modulated phase regime occurs at relatively narrow DOPC/(DOPC + POPC) ratios. This modulated phase window shifts to higher values of DOPC/(DOPC + POPC) when CHOL concentration is increased, and coexisting phases become closer in properties. Monte Carlo simulations reproduced the patterns observed on GUVs, using a competing interactions model of line tension and curvature energies. Sufficiently low line tension and high bending moduli are required to generate stable modulated phases. Altogether, our studies indicate that by tuning the lipid composition, both the domain size and morphology can be altered drastically within a narrow composition space. This lends insight into a possible mechanism whereby cells can reorganize plasma membrane compartmentalization simply by tuning the local membrane composition or line tension.

INTRODUCTION

The lipid raft model postulated that functional domains exist in the plasma membrane (PM), arising from nonrandom mixing of lipids and proteins (1). This lateral heterogeneity would serve to compartmentalize the bilayer and facilitate cellular processes that occur at the membrane, such as immune signaling and endocytosis. Evidence of heterogeneities in the cellular PM is found in many studies (2–4), with size scales of domains ranging from nanoscopic to microscopic depending on the experimental conditions and detection methods used. If these heterogeneities are related to coexisting liquid-disordered (Ld) and liquid-ordered (Lo) phases, they can be studied in chemically simplified, lipid-only model membrane systems.

Micron-sized domains have been detected in cell membranes after cross-linking (5,6). Soon after the lipid raft hypothesis was proposed, Harder et al. (4) demonstrated that raft elements at the PM can be cross-linked using antibodies or toxins. Their studies suggested that the PM could be phase-separated when segregation of raft and nonraft compartments was due to different lipid environments. In immunoglobulin E (IgE) receptor signaling, receptors are cross-linked by antigens, resulting in clustering of protein components in raft-like domains (7,8). In other studies, domains characteristic of Ld and Lo phases were observed in biomembrane preparations such as giant plasma membrane vesicles (9) and plasma membrane spheres (10) when cholera toxin-B was added. These studies collectively imply that macroscopic phase separation can be found in the cellular PM, but only when components are cross-linked with antigens, antibodies, toxins, or chemicals.

In unperturbed cells, micron-sized Ld + Lo domains are not observed. Instead, nanoscopic domains are detected via methods that are more sensitive to small length scales, such as fluorescence resonance energy transfer (FRET) (3,11), electron spin resonance (ESR) (12), and stimulated emission depletion (STED) far-field fluorescence nanoscopy (13). In particular, FRET (11) and ESR (12) have detected nanodomains that have Ld and Lo characteristics, indicating that liquid-liquid phase separation could occur at the nanoscopic scale in resting cells. A current view pictures lipid rafts as nanoassemblies enriched in long-chain saturated sphingolipids and cholesterol (CHOL), possibly of short lifetime, that can be induced when needed to form more stable platforms to promote enzymatic reactions at the membrane (2).

The physical-chemical basis for membrane rafts being small has remained unclear. Chemically defined model membrane mixtures are useful for studying underlying physical interactions that govern nonrandom mixing in the more-complex cellular PM (14). The first clear and direct observation of macroscopic phase-separated domain size and shape was made via fluorescence imaging of GUVs (15), and this method has proved to be an invaluable tool for studying model membranes. Many three-component mixtures consisting of a high-melting lipid, low-melting lipid, and CHOL readily display Ld + Lo phase coexistence (16–20). These include mixtures that contain only phosphatidylycerolines and CHOL, or mixtures that contain
sphingomyelin (SM). Usually, biologically rare lipids such as distearyl-phosphatidylcholine (DSPC), dioleoyl-phosphatidylcholine (DOPC) and diphytanoyl-phosphatidylcholine (DiPhyPC) are used to drive the phase separation. This is because not only are these lipids immiscible, the phase domains are macroscopic and clearly display interesting phase behavior that allows for the construction of thermodynamic phase diagrams that are useful for predicting the mixing behavior of other similar systems.

Although most three-component phase diagrams describe macroscopic phase behavior, a few mixtures with nanoscopic Ld + Lo regions have been reported. In fact, perhaps the first observation of nanodomains in lipid mixtures was made more than a decade ago in dipalmitoyl-phosphatidylcholine/dilauroyl-phosphatidylcholine/CHOL (DPPC/DLPC/CHOL) mixtures, by investigators using FRET and the dipyrene-PC excimer/monomer ratio to detect phase separation that did not show up in GUV imaging (21). Later, when more biologically common lipids such as palmitoyl-oleoyl-phosphatidylcholine (POPC) replaced the low-melting lipid DOPC, nanoscopic Ld + Lo domains were detected in DSPC- and SM-containing mixtures (22–24). These three-component mixtures that display nanodomains may be superior models for studying the physical properties of raft nanoassemblies in cellular PM. However, aside from cross-linking events that drastically change domain size in cells and model membranes (9,10,25), the nature of this nanoscopic-to-macroscopic size change is not well understood. A better model is needed to study the nano-to-macro transition of domain size (and shape) without extreme perturbations, and preferably under steady-state conditions. Equilibrium thermodynamics can provide a good starting point to make predictions about the behaviors of such systems.

In a previous study, we showed that equilibrium (i.e., constant temperature variation of lipid composition) is all that is required to change the phase domain size and morphology (26). As POPC is replaced gradually with DOPC, patterned phase domains (modulated phases) form in the DSPC/DOPC/POPC/CHOL system over a defined range of compositions. To our knowledge, this was the first report of liquid-modulated phases in free-floating bilayer systems, although modulated phases have been previously observed in, e.g., magnetic fluids and lipid monolayers (27). Modulated phase morphology occurs for two coexisting liquid phases when line tension drives the minimization of the domain perimeter while an opposing long-range interplay competes with line tension to break up the domain, typically into periodic patterns.

A long-range repulsive interaction can arise from various sources. For example, long-range dipole-dipole repulsion acts to stabilize superstructures in monolayers (28). Theoretical studies have shown that dipole repulsion may occur in cells between transmembrane proteins and lipids to maintain nanodomains (29). However, dipole repulsion in bilayers is only effective over distances of a few nanometers, so it is not likely to be responsible for the observed micron-scale periodic patterns (26,30). Lateral tension is another long-range and effectively repulsive force that could arise, for example, from adsorption of membranes onto a solid surface, thereby modulating the line energy and inducing the formation of striped patterns. This was observed on bilayer vesicles adsorbed on the surface of supported bilayers (31) and for Pb deposited onto Cu surfaces (32). In addition, osmotic swelling might also apply enough tension to GUVs to cause macroscopic phase separation (33). Moreover, patterned phase morphologies can also arise from critical fluctuations in bilayers, a dynamic process that occurs when line tension is low and close to the critical point (34,35). In all of these examples, competing interactions are required to stabilize periodic structures.

Recently, we modeled the formation of modulated phases on the surface of GUVs by conducting Monte Carlo simulations of a competing interactions model of line tension and curvature energies (36). Curvature energies have been shown in many studies to be a long-range repulsive force that can modulate coexisting liquid phases into periodic domains (30,35,37). Differences in phase properties, in particular the bending moduli, could impose constraints on a phase to preferentially assume areas of a particular curvature (38). Using interferometry coupled with fluorescence imaging, Kaizuka and Groves (30) distinguished different areas of curvature on modulated phase domains on quasiplanar lipid bilayers. At the macroscopic scale, competing interactions between curvature energies and line tension provide a plausible explanation for the stabilization of modulated phase patterns on unsupported bilayers.

In this study, we employed wide-field fluorescence microscopy of GUVs and systematically changed the mixture composition to observe that modulated phases are found within the Ld + Lo volume of the four-component mixture DSPC/DOPC/POPC/CHOL. The compositional range of this modulated phase regime varies depending on the location within the two-phase region. In particular, the greater the fraction of DOPC that is required for patterns to appear, the shorter is the tieline that connects the coexisting phases, and hence the more alike are the phases. We conclude that in the presence of the competing interaction from curvature energies, line tension exerts the main control over the occurrence of patterned domains in different compositions within the Ld + Lo region. Together, these observations are consistent with the notion that phase thickness mismatch controls the line tension, with the thickness of the Ld phase in particular being under strong control via its composition.

**MATERIALS AND METHODS**

**Materials**

Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL). CHOL was obtained from Nu Chek Prep (Elysian, MN). Fluorescent dye C12:0-DiI (1,1′-didodecyl-3,3′,3′-tetramethylindocarbocyanine...
perchlorate) was obtained from Invitrogen (Carlsbad, CA). Concentrations of phospholipid stocks were determined to <1% error via inorganic phosphate assay (39), and purity of >99% was checked by thin-layer chromatography of 30 μg samples. Briefly, lipids were spotted onto pre-washed and activated silica gel GHL Unplates (Analtech, Newark, DE). Plates were developed with chloroform/methanol/water (65:25:4). CHOL stock solution was prepared by standard gravimetric procedures to ~0.2%. Fluorescent dye concentrations were determined by absorption spectroscopy on an HP 8452A spectrophotometer (Hewlett-Packard, Palo Alto, CA).

Sample preparation

GUVs were prepared using the electroformation method (40) as described previously (17,26), with the following modifications: Lipid films were swelled at 55°C in either 100 mM sucrose or 100 mM glucose in an AC field of 5 Hz for 2 h to form GUVs, and then cooled to room temperature (23°C) over 12 h. Samples were harvested into microcentrifuge tubes (Fisher Scientific, Agawam, MA) using large orifice pipet tips (Fisher Scientific), and allowed to settle for at least 2 h before observation. Because the samples were not harvested into a solution of lower density as done previously, this longer settling time was required because microscopy observations could be made. To minimize perturbations from any osmotic gradient across the membranes, only identical glucose (or sucrose) solutions were used on both sides of the GUV, because changes in the osmotic pressure of the GUVs can influence the modulated phase morphologies. Glucose may be superior to sucrose for this purpose because it can equilibrate in a period of hours across membranes to relieve any osmotic gradients that might occur during sample annealing (41). Neither of the sugar solutions strongly affected the modulated phase window of the compositions examined or the yield of the samples.

Fluorescence microscopy and image analyses

Wide-field microscopy was performed on a Nikon Diaphot-TMD inverted microscope at 23°C using a 60× 1.4 NA oil immersion objective. To minimize light-induced artifacts, neutral density filters (ND = 1.0) were used, and GUVs were first located in bright-field mode before illumination for fluorescence. The samples contained 0.02 mol% C12:0 DiI, imaged with 535–550 nm excitation and 565–610 nm emission. Images were collected with a charge-coupled device camera (CoolSNAP; Photometrics, Tucson, AZ). All images were contrast-enhanced and analyzed using NIS Elements Basic Research Software (MVI, Inc., Avon, MA).

To determine the compositional range of modulated phases, the number of GUVs with various phase morphologies (uniform, patterned, or macroscopic-round domains) at each composition was examined (see Tables S1 and S2 in the Supporting Material). The fraction of GUVs that displayed modulated phases at each composition was plotted, and the mean ± standard error (SE) was determined (see Fig. 3).

The area fractions of the two phases on GUV images (see Fig. 6) were determined using Image J (42). The images were first converted into true binary images. Then, representative areas on the vesicles were selected for area fraction calculation. Calculated phase mole fractions were then adjusted for the Lo phase occupying 30% less area than the Ld phase (43,44). These Lo area fractions were compared with the expected area fractions of the compositions, which were calculated using the lever arm rule and the phase boundaries determined at ρ = 30% (T.M. Konyakhina, J. Wu, J.D. Mastroianni, F.A. Heberle, and G.W. Feigenson (hereafter referred to as T.M. Konyakhina et al.), unpublished).

Simulation model

To model the experimental observations, we used a Monte Carlo simulation of the competition between line tension and curvature (36). The model uses the Helfrich energy functional to describe the energetics of a two-phase membrane:

\[ H[\varphi, H, G] = \gamma L + \int_S \kappa(\varphi)[H]^2 dA + \int_S \tilde{\kappa}(\varphi) G dA \]

The three fields defined on the surface are the local phase (\( \varphi \)), defined to be zero in Ld and one in Lo, the mean curvature (H), and the Gaussian curvature (G). The first term is the line-tension contribution, the energy per unit length (\( \gamma \)) multiplied by the total length of the phase boundary (L), which favors the formation of macroscopic round domains to minimize the perimeter/area ratio.

The second term is the mean curvature (H) contribution, which couples to the energy through the bending modulus (\( \kappa \)), which varies depending on the local phase. This is integrated over the membrane surface (S). Similarly, the third term is the Gaussian curvature (G) contribution, which couples through the saddle-splay modulus (\( \tilde{\kappa} \)) that also depends on the local phase.

We represented the GUV in our simulation as a triangulated surface with spherical topology (36). Energy was minimized by a two-stage metropolis algorithm:

1) The phases on two randomly chosen vertices were exchanged, and this move was accepted or rejected based on the change in energy (with probability \( e^{-\Delta E/kT} \)).

2) A randomly chosen vertex was moved a small amount in a random direction, and this move was accepted or rejected based on the change in energy (with probability \( e^{-\Delta E/kT} \)).

After this process was iterated, the lattice eventually relaxed to a minimal energy state (to within thermal fluctuations.) We found that for certain values of the energetic parameters, the minimal energy configuration took on morphologies consistent with the modulated phases observed in the DSPC/DOPC/POPC/CHOL system.

RESULTS

We previously reported observations made in a limited number of compositions of a modulated phase regime in DSPC/DOPC/POPC/CHOL (26). Here, we systematically examined modulated phases within the Ld + Lo coexistence region in the four-component system. Starting from the three-component DSPC/POPC/CHOL mixture at a fixed DSPC/CHOL ratio, POPC was replaced with DOPC as described by the replacement ratio, ρ:

\[ \rho \equiv \frac{\chi_{DOPC}}{\chi_{DOPC} + \chi_{POPC}} (\% ) \]

where \( \chi_{DOPC} \) and \( \chi_{POPC} \) are mole fractions of DOPC and POPC. The experimental design is illustrated in Fig. 1. At a chosen DSPC/CHOL ratio starting from the POPC face of the tetrahedron, a series of samples was prepared traveling in composition space from one face to another of the tetrahedral phase diagram (ρ trajectory). Using well-determined phase boundaries and thermodynamic tie lines from previous studies (22) and unpublished results from the four-component mixture (T.M. Konyakhina et al., unpublished), we found modulated phases occurring at a range of ρ-values along two different approximated tie lines close...
to the previously measured tielines in the liquid-liquid coexistence region.

**Modulated phases occur at similar $r$-values along a given tieline**

The first tieline examined (tieline 1) is located slightly above the bottom phase boundary of the Ld + Lo region (Fig. 2). Six starting compositions with defined DSPC/CHOL ratios were chosen along tieline 1 to investigate the modulated phase compositional window (compositions T1A–T1F; Table 1). In all of the chosen compositions, the majority of GUVs appeared uniform at $r < 15\%$, where domains are nanoscopic (22).

Modulated phase patterns started to occur at $r = 15\%$ for composition T1A, located on the far right of tieline 1 (Fig. 3 A). At $r = 20\%$, patterned domains were observed in compositions T1A–T1D (Figs. 2 and 3 A). For compositions T1E and T1F, this modulated phase window began at $r = 25\%$. The fraction of patterned GUVs in compositions T1E and T1F, where the predominant phase had changed to Ld-rich, was smaller than that found for compositions T1A–T1D (Fig. 3 A), where the predominant phase was Lo-rich. In T1F, only $25\%$ of the GUVs analyzed displayed modulated morphologies; the other GUVs were either uniform or displayed large round domains (Fig. 3 A, Table S1). For all of the compositions examined on tieline 1, the modulated phase window ended at $r = 30–35\%$ (Fig. 3 A), with higher $r$-values showing rounded macroscopic domains. A typical progression of GUV morphologies from the onset to the end of a modulated phase window is shown for composition T1B in Fig. S1.

We observed that the occurrence and behavior of modulated phases followed rules that govern phase separation along thermodynamic tielines. On the far right of tieline 1, Ld is the minor phase, forming thin stripes, honeycomb, or stripe-like patterns that resembled two-dimensional (2D) bubbles on the predominantly Lo phase surface of the GUVs (Fig. 2, J–L). As the Ld phase fraction increases, either by moving toward the left side of the tieline or by increasing $r$, the thin stripes start to coarsen (Fig. 2, C and G), typically giving rise at higher $r$-values to larger dispersed Lo domains with irregular edges (Fig. 2, E and H; Fig. S1 F). The mole fractions of Ld and Lo on patterned GUVs follow the lever arm rule (see Fig. 6).

**TABLE 1 Compositions examined in modulated phase window studies**

<table>
<thead>
<tr>
<th>Composition</th>
<th>$X_{\text{DSPC}}$</th>
<th>$X_{(\text{DOPC} + \text{POPC})}$</th>
<th>$X_{\text{CHOL}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1A</td>
<td>0.487</td>
<td>0.25</td>
<td>0.263</td>
</tr>
<tr>
<td>T1B</td>
<td>0.45</td>
<td>0.30</td>
<td>0.25</td>
</tr>
<tr>
<td>T1C</td>
<td>0.413</td>
<td>0.35</td>
<td>0.237</td>
</tr>
<tr>
<td>T1D</td>
<td>0.375</td>
<td>0.40</td>
<td>0.225</td>
</tr>
<tr>
<td>T1E</td>
<td>0.30</td>
<td>0.50</td>
<td>0.20</td>
</tr>
<tr>
<td>T1F</td>
<td>0.225</td>
<td>0.60</td>
<td>0.175</td>
</tr>
<tr>
<td>T2A</td>
<td>0.395</td>
<td>0.30</td>
<td>0.305</td>
</tr>
<tr>
<td>T2B</td>
<td>0.283</td>
<td>0.45</td>
<td>0.267</td>
</tr>
<tr>
<td>T2C</td>
<td>0.17</td>
<td>0.60</td>
<td>0.23</td>
</tr>
</tbody>
</table>
In summary, modulated phases occurred at $\sim 20\% \leq \rho \leq 30\%$ for all compositions examined along tieline 1. However, this window narrowed to $25\% \leq \rho \leq 30\%$ for T1E, and a well-defined modulated composition range was not observed for T1F.

Increasing CHOL concentration moves modulated phase windows to higher $\rho$-values

Along tieline 1, the width of the modulated phase windows has a composition range of $\sim 10$–$15\%$ in $\rho$. We investigated the compositional values and width of the modulated phase window on a different tieline at higher CHOL concentration within the Ld + Lo region. We note that at higher CHOL concentrations, the compositions of the coexisting phases become more similar, as described by shorter tielines.

We examined three compositions along tieline 2 (Fig. 4; Table 1). In composition T2A, the modulated phase window began at $\rho = 30\%$, persisting up to $\rho = 65\%$ (Fig. 3 B). This compositional window is much broader than those found for compositions on tieline 1. The types of patterns observed for T2A (Fig. 4, I–L) are similar to patterns we find for tieline 1 Lo-rich compositions. Toward the left of T2A in composition T2B, the modulated phase window narrowed significantly, occurring at $40\% \leq \rho \leq 50\%$ (Fig. 3 B). In addition to stripes and honeycomb-like patterns, larger domains with uneven edges were also observed at T2B (Fig. 4 H). Finally, on the left side of tieline 2 at composition T2C, a smaller fraction (<10%) of the GUVs examined appeared patterned when $40\% \leq \rho < 50\%$ (Fig. 3 B, Table S2). As with T1F, we were not
able to determine a well-defined modulated phase window for T2C.

**Area fractions of phases influence the modulated phase compositional range**

We observed a narrowing of the modulated phase windows and a decrease in the fraction of patterned GUVs at compositions having Ld fractions > ~50% along tielines 1 and 2. In other words, even with the same composition of Ld and the same composition of Lo phases in equilibrium, a switch from the predominant phase being Lo to its being Ld changes the modulated phase morphology. This is not a change in percolation, which describes the connectivity of phases, because we observed that the Ld phase is the continuous phase when modulated phase morphology is present. Thus, we observed that the appearance/disappearance of modulated phases and the types of patterns that are found depend on which phase area fraction is greater.

The location of this changeover in area predominance could help us understand how modulated phase patterns evolve along a tieline. However, when modulated phase patterns are examined, it can be difficult to determine which phase area fraction is greater. Therefore, to find the changeover compositions, we left the modulated phase regime and used just slightly higher \( p \)-values where the domains were round. In this macroscopic regime, a changeover in phase area dominance is related to a change in phase percolation, which is the criterion we examined in the following experiments.

For tieline 1, samples were prepared at \( \rho = 40 \% \). This \( p \)-value lies just outside the modulated phase window for the compositions examined in Fig. 2. A series of 10 samples were prepared (Fig. 5). GUVs from compositions A–D displayed Ld phase connectivity (Fig. 5, A–D). In composition E in Fig. 5, both types of phase connectivity were observed (Fig. 5, E1–E3), indicating that this composition is very close to the changeover composition. Based on the estimated Ld + Lo boundaries at \( \rho = 40 \% \) (T.M. Konyakhina et al., unpublished), composition E is located at ~52 mol% Ld phase, which occupies an area fraction of ~61%. The predominant phase switches to Lo at compositions located to the right of the changeover point (Fig. 5, F–J). It should be noted that for compositions in the middle region of the tieline, where the area fractions of Lo and Ld phases are close to equal, the majority of GUVs displayed a single round Ld (or Lo) domain. This is an indication that the samples were well equilibrated, without multiple small kinetically trapped domains. The changeover point occurred when the mole fraction of Ld was roughly equal to that of Lo, but because Lo occupies ~30% less area than Ld (43,44), the Ld area fraction predominated at the changeover point.

Similar area changeover experiments were conducted along tieline 2, but at \( \rho = 70 \% \), where large round domains were found for the compositions examined (Fig. S2). The

![FIGURE 5 Percolation threshold along tieline 1 at \( \rho = 40 \% \). Domain morphologies of GUVs change along compositions A–J as shown in Fig. 5. GUVs displayed Ld percolation at A–D (open circles), Lo percolation at F–J (solid circles), and mixed percolation at E1–E3 (half-open circles). The Ld + Lo boundaries at \( \rho = 40 \% \) is shown, with the approximate position of the critical point (star) (T.M. Konyakhina et al., unpublished). GUV compositions DSPC/DOPC/POPC/CHOL: (A) 0.187/0.260/0.39/0.163, (B) 0.225/0.24/0.36/0.175, (C) 0.263/0.22/0.33/0.187, (D) 0.30/0.20/0.30/0.20, (E) 0.338/0.18/0.27/0.212, (F) 0.375/0.16/0.24/0.225, (G) 0.413/0.14/0.21/0.237, (H) 0.45/0.120/0.180/0.25, (I) 0.487/0.10/0.15/0.263, and (J) 0.525/0.08/0.120/0.275. C12:0-DiI (0.02 mol%) partitions into Ld. Scale bars: 10 \( \mu m \); temperature: 23 °C.](image)
changeover points on both tielines, such as T1E and T1F on tieline 1, and T2B and T2C on tieline 2, have narrower modulated phase windows compared with the Lo-predominant compositions on the right of the changeover points. Thus, we find a consistent influence of phase area predominance on the modulated phase behavior along a tieline.

**DISCUSSION**

**Three regimes of domain size and morphology**

We observe three separate regimes within the Ld + Lo region that differ in domain size and morphology, which we term nanoscopic, modulated, and macroscopic. Simply by tuning the ratio of DOPC/(DOPC+POPC), i.e., by increasing ρ, we obtain GUVs that are uniform by fluorescence microscopy but contain nanodomains, then modulated phases, and finally macroscopic, round liquid domains. These transitions occur at compositions that vary only slightly for ρ trajectories that start along the same tieline, but change significantly as CHOL concentration is increased, i.e., along a different tieline. For example, nanodomains occur up to ρ = ~15% along tieline 1, but can persist up to ρ = 40% at higher CHOL concentration (Fig. 3).

In almost all of the compositions examined, the transition from nanoscopic to macroscopic round domains went through a regime in which domains appeared patterned. The range of this modulated phase window hardly varied along tieline 1, but did change with CHOL concentration between tielines 1 and 2 within the Ld + Lo region. We observed a broader modulated phase window in composition T2A along tieline 2 (higher CHOL) than in composition T1A along tieline 1.

The transition of domain size from nanoscopic to macroscopic along a ρ trajectory was relatively well defined, even abrupt compared with the gradual change in all of the phase boundaries of this four-component system as the low-melting lipid changed from POPC to DOPC. The upper boundary of the Ld + Lo region shifted smoothly from 30% to 40% CHOL at ρ = 0–100% (22). In contrast, GUVs changed from uniform to patterned within a ~5% change in ρ. In addition, in many compositions within the two-phase region, the range of ρ-values at which modulated phases occurred was only ~10% in composition space. This could have implications for biological membranes, providing a means for cells to abruptly change the size and connectivity of membrane compartments simply by tuning the local membrane composition.

**Experimental observations are consistent with a competing interactions model of line tension and curvature energies**

In a system where liquid phases coexist, line tension drives the minimization of the domain boundary between the two phases, resulting in the formation of a single round domain, unless an additional term competes with line tension to maintain high domain perimeters. Previously, using Monte Carlo simulations, we showed that a competing interactions model of line tension and curvature energies could result in the formation of modulated phases in a liquid-liquid coexistence region (36). Low line-tension values (0.01 pN) and high bending-moduli ratios ([10–100] × 10⁻¹⁹ J) of the two phases are required to generate stripe-like and honeycomb patterns on the surface of simulated GUVs.

The experimental results in this study are consistent with this competing interactions model. At fixed line-tension and bending-moduli values, the domain morphologies on simulated GUVs changed with the area fraction of each phase (Fig. 6, E–H). Experimentally, we observed similar changes in modulated phase patterns on actual GUVs as we moved along tieline 1 at a fixed ρ = 30% (Fig. 6, A–D). A comparison of experimental and simulated GUV morphologies is shown in Fig. 6. When Lo is the dominant phase, thin stripes, honeycomb, and 2D-bubble-like patterns formed by the Ld phase are observed. The line widths of these patterns coarsen as the area fraction of Ld increases, until eventually, dispersed, uneven Lo domains on an overall Ld-rich GUV are found. Whereas phase patterns on simulated GUVs do not always exactly match the patterns observed on experimental GUVs, the types of patterns (i.e., stripe-like or 2D-bubble-like) are alike. Furthermore, the Lo area fractions used in the simulations in Fig. 6, E–H, are remarkably similar to the area fractions measured from the GUV images in Fig. 6, A–D, which in turn correspond approximately to the area fractions estimated from

**FIGURE 6** Phase fractions of modulated phase patterns along a tieline at ρ = 30% follow the lever arm rule. (A–H) A comparison of experimental (A–D) and simulated (E–H) GUVs along tieline 1 at ρ = 30%, with only the phase fractions of Ld and Lo varied. The Lo area fraction of GUVs A–D was measured to be 0.75, 0.56, 0.45, and 0.25, respectively. Images were processed and shown as described in Fig. 2. GUV compositions DSPC/DOPC/POPC/CHOL at 23°C: (A) 0.487/0.075/0.175/0.263, (B) 0.413/0.105/0.245/0.237, (C) 0.300/0.150/0.35/0.20, and (D) 0.225/0.18/0.42/0.175; C12:0-Dil (0.02 mol%) partitions into Ld. Scale bars: 10 μm; temperature: 23°C. (E–H) Simulation parameters for GUVs: γ = 0.015pN, κ_L = 10 × 10⁻¹⁹ J, κ_d = 150 × 10⁻¹⁹ J, σ_d = −10 × 10⁻¹⁹ J, GUV radius = 25 μm. Lo area fractions used: (E) 0.80, (F) 0.60, (G) 0.40, and (H) 0.20.
the location of these compositions along tieline 1 at $\rho = 30\%$ (see Materials and Methods for details). Thus, modulated phases appear to follow the lever arm rule.

Based on the changeover compositions obtained for both tielines, and the compositional range of modulated phases, we find that a change in which phase area fraction is predominant has a strong influence on the appearance of modulated phases. For example, at a fixed value of $\rho = 25\%$ along tieline 1, thin stripe-like and honeycomb-like patterns were found in Lo-rich compositions (Fig. 2, F, G, J, and K), whereas thicker line widths of honeycomb patterns (Fig. 2 C) and dispersed Lo domains with uneven edges (Fig. 2 B) occurred in Ld-rich compositions. In addition to different patterns, the relative phase area fractions could also influence the decrease (or absence) of modulated phases: a significantly smaller fraction of GUVs was patterned in the Ld-dominant compositions T1E and T1F at $\rho = 25\%$ compared with the Lo-dominant compositions on the right end of tieline 1 (Fig. 3 A). Similarly, no patterned GUVs were observed for T2C at $\rho = 30\%$ compared with T2A, and the fraction of patterned GUVs at $\rho = 40\%$ for T2C was also lower than that obtained for T2A and T2B (Fig. 3 B). One possible reason for a lower fraction of patterned GUVs having Ld-rich compositions is simply that the appearance of a distinct pattern is less obvious when the Lo domains are fewer and more sparsely distributed on the GUV. When Lo predominates, stripe-like and honeycomb-like phase patterns form favorably. When Ld predominates, Ld stripes coarsen and Lo domains become sparse, but the morphology of individual Lo domains remains relatively unchanged. A change in phase predominance to Ld abruptly changes phase morphology, resulting in narrower modulated phase windows in compositions located on the Ld-rich side of a tieline. Although fluorescence microscopy has proved to be useful for detecting modulated phases on GUVs, its resolution is diffraction-limited, and this may explain why patterned morphologies were not as readily detectable in compositions on the Ld-rich end of tielines, for example, when Lo domains were isolated and near or below the diffraction limit in an Ld matrix. Hence, we are currently exploring spectroscopic methods such as FRET and ESR as alternative ways to detect the modulated phase regime.

**Line tension determines the compositional range of modulated phases**

While both line tension and curvature energies are important, simulations show that the line tension is the controlling factor for the formation of modulated phases (36). Assuming that modulated phases occur within a fixed range of line-tension values, during the transition from nanoscopic to macroscopic round domains along a $\rho$ trajectory at fixed DSPC/CHOL ratios, the system may be going through three separate regimes of line-tension values (Fig. 7). These distinct line-tension regimes could occur at different $\rho$-values for compositions located on different tielines due to changes in the properties of the coexisting phases. For example, on a tieline closer to the critical point, Ld and Lo phase properties are more similar, hence the line tension would be smaller. In such cases, more DOPC would be required to increase the line tension to the value that yields modulated phases. Indeed, experimentally we observed a broader modulated phase regime for T2A on tieline 2 compared with the equivalent composition T1A on tieline 1, with the modulated phase window occurring at higher $\rho$-values along tieline 2. Even a very simple model can illustrate this effect, i.e., a linear variation of line tension with $\rho$ as shown in Fig. 7. An alternative model is perhaps more likely in light of recent small-angle neutron scattering studies in which domain size was found to vary linearly with bilayer thickness (F.A. Heberle, R.S. Petruzio, J. Pan, P. Drazba, N. Kučerka, R.F. Standaert, G.W. Feigenson, and J. Katsaras, unpublished), implying a squared dependence of line tension on $\rho$. Consistent with low line-tension values in the proximity of a modulated phase regime, we observed GUVs with uneven domain edges that sometimes moved during our observations in compositions near the modulated-to-macroscopic round transition (Figs. 2 H and 4 H; Fig. S1 F).

The competing-interactions model shows that modulated phases are thermodynamically stable, and that kinetic trapping only occurs when the line tension is high (36). Consistently, we observed that in compositions just outside the modulated phase regime (e.g., $\rho = 40\%$), where macroscopic round domains were observed but line-tension values were still relatively low, the majority of GUVs displayed a single Ld (or Lo) domain in the matrix of the opposite phase. If extensive kinetic trapping were present, the GUVs would display multiple round domains. This further
indicates that our experimental procedures produced equilibrium domain morphologies, consistent with the model of competing interactions.

Using the competing-interactions model, we found that as line tension is increased, the domains coarsen, coalesce, and eventually round up (Fig. 8, E–H). Increasing line tension in a simulation is equivalent to increasing \( \rho \)-values in GUV experiments. A similar evolution of domain morphologies was also observed on actual GUVs during the nano-to-macro transition along a \( \rho \) trajectory (Fig. 8, A and B, and Fig. S1). Simply by modeling our system as two coexisting liquids where the modulated phase behavior is driven by line tension, we obtained a remarkable correlation between the model and experimental observations in the modulated and macroscopic regimes when curvature energies were applied as the force opposing line tension. This model might be applicable to the nanoscopic regime if different or additional competing forces, such as dipole repulsion, could be operating to stabilize nanodomains. Understanding these interactions would be valuable for describing nanoheterogeneities that exist in cellular PMs.

CONCLUSIONS

In this study, we have shown that there are three regimes that vary in domain size and shape within the Ld + Lo liquid-liquid immiscibility volume of DSPC/DOPC/POPC/CHOL. By tuning the fraction of low melting lipid that is DOPC, we observe a transition from nanodomains to modulated phases, and finally to macroscopic round domains on GUVs. The modulated phase patterns can be modeled using competing interactions between line tension and curvature energies. We found that line tension is the main factor controlling the location of the modulated phase regime: the lower the line tension at \( \rho = 0 \), the more DOPC is required to form modulated phases by raising the line tension sufficiently.

This view that Ld + Lo phase morphology can be controlled by competition of line tension with an opposing interaction that favors small domains might extend to the nanodomain regime if there exist interaction(s) at that distance scale, such as lipid-lipid dipolar repulsion, that favor small domains. In that case, the plasma membranes of cells might exhibit a patterned phase morphology rather than the simple picture of small Lo rafts floating in a large Ld sea. Furthermore, as we observed for the mixture studied here, it may be that lower line tension leads to smaller phase domains, as well as to a higher \( \rho \) window where modulated phases are observed. If so, then the convenient use of fluorescence microscopy for determining the \( \rho \) window for a given mixture might be a substitute for the less convenient direct measurement of domain size at the nanoscale.

SUPPORTING MATERIAL

Two figures and two tables are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(13)00037-4.

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