Sterol-lipids enable large-scale, liquid-liquid phase separation in membranes of only 2 components

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Abstract

Despite longstanding excitement and progress toward understanding liquid-liquid phase separation in natural and artificial membranes, fundamental questions have persisted about which molecules are required for this phenomenon. Except in extraordinary circumstances, the smallest number of components that has produced large-scale, liquid-liquid phase separation in bilayers has stubbornly remained at three: a sterol, a phospholipid with ordered chains, and a phospholipid with disordered chains. This requirement of three components is puzzling for two reasons: (1) the Gibbs Phase Rule states that only two components are necessary, and (2) only two components are required for liquid-liquid phase separation in lipid monolayers, which resemble half of a bilayer. Inspired by reports that sterols interact closely with lipids with ordered chains, we tested whether phase separation would occur in bilayers in which a sterol and lipid were replaced by a single, joined sterol-lipid. By evaluating a panel of sterol-lipids, we discovered a minimal bilayer of only two components (PChemsPC and diPhyPC) that demixes into micron-scale, liquid phases. In this system, the sterol-lipid behaves as a 3:1 ratio of cholesterol to phospholipid. Our system gives the computation and theory community a two-component membrane that maps directly onto simplified theories and that can be used to validate simulation force fields. It suggests a new role for sterol-lipids in nature, and it gives experimental communities a membrane in which tie-lines (and, therefore, the lipid composition of each phase) are easily determined and will be consistent across multiple laboratories.

Significance Statement

A wide diversity of bilayer membranes, from those with hundreds of lipids (e.g., vacuoles of living yeast cells) to those with very few (e.g., artificial vesicles) phase separate into micron-scale liquid domains. The number of components required for liquid-liquid phase separation has been perplexing: only two should be necessary, but more are required except in extraordinary circumstances. What minimal set of molecular characteristics leads to liquid-liquid phase separation in bilayer membranes? This question inspired us to search for single, joined “sterol-lipid” molecules to replace both a sterol and a phospholipid in membranes undergoing liquid-liquid phase separation. By producing phase-separating membranes with only two components, we mitigate experimental challenges in determining tie-lines and in maintaining constant chemical potentials of lipids.

Introduction

Liquid-liquid phase separation in lipid membranes is important across scientific fields, from fundamental biology (e.g., understanding how living yeast survive nutrient reduction (1)) to fundamental physics (e.g., measuring 2-dimensional dynamic critical exponents (2)). Underlying these grand applications is a mystery. Except in extraordinary circumstances (3, 4), all lipid bilayers that have been found to phase separate into large-scale, coexisting liquid phases have been comprised of at least three lipids: a sterol like cholesterol, a lipid with a high melting temperature (T_{melt}), and a lipid with a low melting temperature (Fig. 1) (5–7).

The requirement of *three* components is perplexing. Phase separation of only *two* components is theoretically allowed by the Gibbs Phase Rule in systems where temperature and pressure freely vary. Moreover, lipid monolayers (which, at their simplest, are half of a bilayer) require only two components to demix into micron-scale liquid domains (8, 9), even at higher surface pressures (10, 11). Monolayers account for one of the extraordinary circumstances: when two monolayers are deposited on a substrate to form a bilayer, domains from each monolayer can persist (3). The other exception arises when cholesterol is chemically altered through the addition of a hydrophobic group on its acyl tail. The resulting hydroxysterol is found in multiple configurations (parallel, anti-parallel, and perpendicular to the other lipids in the bilayer, in crystals, and possibly in hydrogen-bonded clusters) (4). If these configurations interconvert quickly on the time scale of experiments, then all hydroxysterol molecules in the bilayer act as one component. If they interconvert slowly, then each configuration can represent a separate component.

There are many reasons to be excited about a truly minimal, 2-component membrane for liquid-liquid phase separation. Theorists could treat it exactly. Modelers could use it as a test case for simulations. Experimentalists could be confident that membranes produced in their labs demix along the same tie-line and produce phases of the same lipid composition as those in any other laboratory at the same temperature.

If we were to search for binary lipid compositions that phase separate into micron-scale liquid ordered (Lo) and liquid disordered (Ld) phases in bilayers, where would we start looking for them? Lo phases in bilayers are enriched in high-T_{melt} lipids and, to a lesser extent, sterols. These molecules experience Van der Waals interactions and have the potential to hydrogen bond if the high-T_{melt} lipid is a sphingomyelin (12–14). If membrane phase separation is at least partially driven by close interactions between the sterol and the high-T_{melt} lipid, then it is reasonable to surmise that the two molecules could be replaced by a single, joined sterol-lipid, which would decrease the number of components.

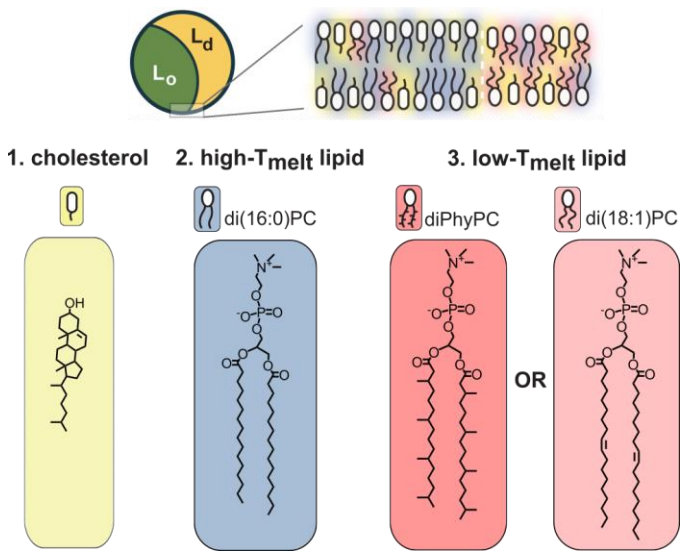

Figure 1: Typically, bilayers that undergo liquid-liquid phase separation contain at least three lipid components. The Lo phase is enriched in high-T_{melt} lipid and cholesterol, whereas the Ld phase is enriched in low-T_{melt} lipid.

To that end, we produce vesicle membranes from binary mixtures of a phospholipid with one of the four sterol-lipid molecules in Fig. 2, and we test if the membranes undergo liquid-liquid phase separation to form micron-scale domains. The sterol-lipids represent a range of structures in which a sterol is linked to a hydrocarbon chain. A strong advantage of these structures is that the sterol should be in only one configuration: parallel to the surrounding lipids. The lack of additional configurations of the sterol means that the systems we investigate are truly 2-component.

- In **Sterol-lipid A (PChemsPC)**, cholesterol replaces one of the saturated hydrocarbon chains of a phospholipid.

- **Sterol-lipid B (BbGL-1**) is similar but lacks a phosphate group. Instead, cholesterol and a hydrocarbon chain are β-linked via a galactose moiety. Sterol-lipid B is found in *Borrelia burgdorferi*, the bacterium responsible for Lyme Disease (15).

- **Sterol-lipid C (CPG)**, which is found in *H. pylori*, is composed of a phospholipid α-linked to cholesterol via glucose, a sugar in a ring form (16). Unlike the other sterol-lipids in our set, it has the same 2:1 ratio of acyl chains to sterol group as the two molecules it replaces.

- In **Sterol-lipid D (OChemsPC)**, an unsaturated hydrocarbon chain replaces the saturated chain of Sterol-lipid A. This molecule serves as a control because it has a low-T_{melt} carbon chain rather than a high-T_{melt} chain.

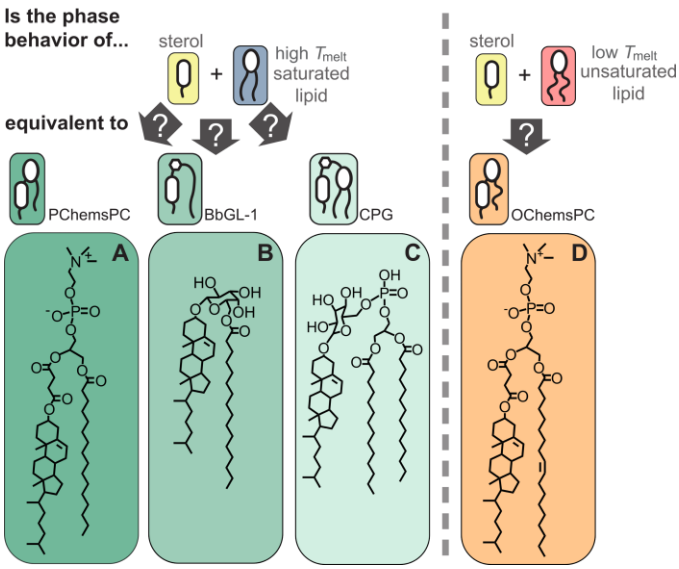

Figure 2: We test if micron-scale, liquid-liquid phase separation occurs in membranes with only two components, one of which is a sterol-lipid. The acyl chains of the sterol-lipids we use are either saturated, as in Sterol-lipid A, B, and C (known as PChemsPC, BbGL-1, and CPG, respectively) or unsaturated as in Sterol-lipid D (OChemsPC). The acyl chain lengths of Sterol-lipids A, B, C, and D are 16, 16, 14, and 18, respectively.

It seems easier to speculate about whether a mythical centaur accurately represents both a human and a horse than to predict whether a sterol-lipid can replace both cholesterol and a high-T_{melt} lipid in a phase-separating membrane. One reason to be skeptical that binary lipid membranes with sterol-lipids could form micron-scale liquid phases is that most phase-separating membranes contain different ratios of cholesterol to high-T_{melt} lipid in their Lo and Ld phases (17), whereas this ratio is necessarily constant in a sterol-lipid. Moreover, it is not clear if membranes containing high fractions of sterol-lipids should be stable. From their structures in Fig. 2, sterol-lipids appear to be cone-shaped, with larger tails than headgroups; they may assemble into curved inverted hexagonal (H_{II}) structures rather than flat lamellae. On the other hand, there are also good reasons to hypothesize that binary membranes of phospholipids and sterol-lipids will demix into liquid domains. From a theoretical standpoint, a sterol-lipid could correspond to a complex between cholesterol and a reactive phospholipid (18). From an experimental standpoint, some sterol-lipids have been incorporated into ternary or multi-component membranes at moderate concentrations, where they can substitute for cholesterol and allow micron-scale liquid-liquid phase separation to occur (19). For example, multi-component, phase-separating membranes have been made from mixtures of sterol-lipids (one of which is Sterol-lipid B) and phospholipids or from mixtures of a single sterol-lipid (CAG) with all of the PE lipids extracted from *H. pylori*, which encompass a range of acyl chains (19, 20).

Results and Discussion

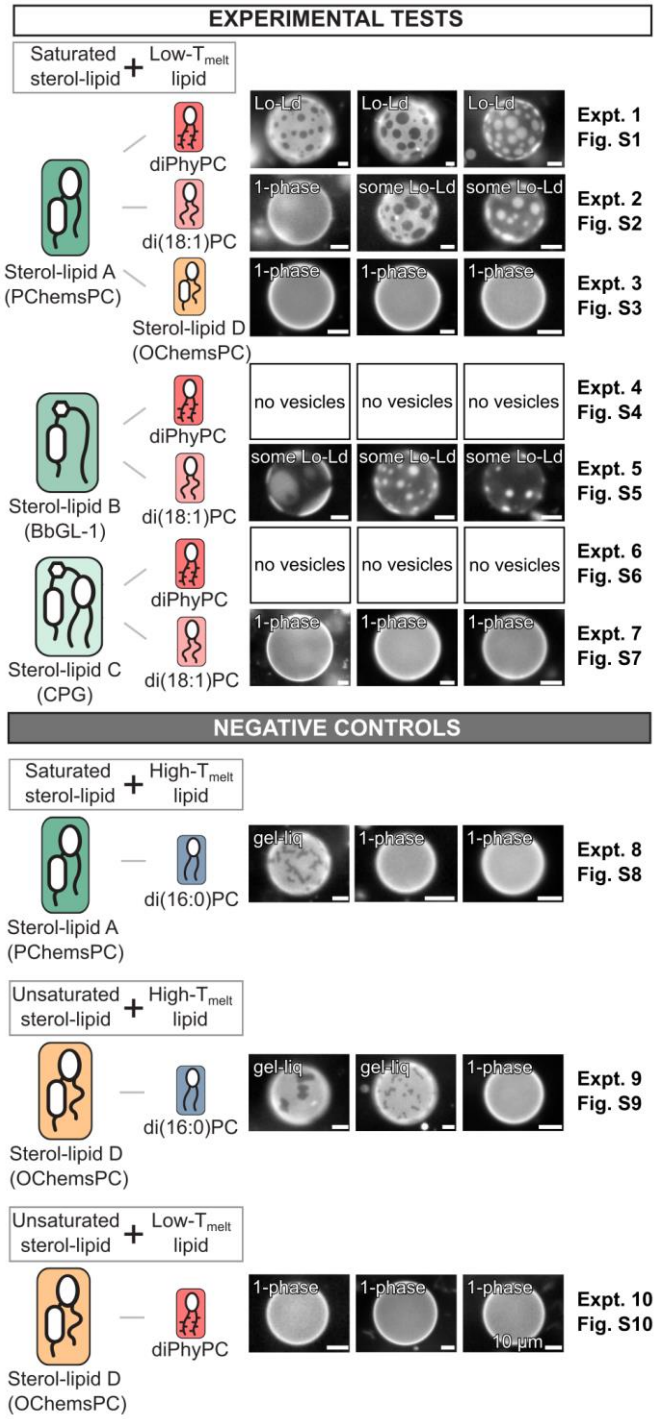

Figure 3: Seven experiments (top) and three negative controls (bottom) to determine if liquid-liquid phase separation occurs in vesicles composed of binary lipid mixtures in which at least one component is a sterol-lipid. Robust liquid-liquid phase separation is observed only in Experiment 1. The designation "some Lo-Ld" means ≤ 50% of vesicles displayed liquid domains in slightly hyperosmotic solutions, and that no vesicles phase separated without osmotic pressure. Other vesicles displayed only gel-liquid coexistence or one uniform liquid phase. For some combinations, no vesicles formed. The scale bar on all fluorescence micrographs is 10 µm. Wide fields of view are in supplementary Fig S1-S10.

Robust liquid-liquid phase separation in binary membranes

It is well known that lipid membranes comprised of a sterol, a high-T_{melt} lipid, and a low-T_{melt} lipid can undergo liquid-liquid phase separation (5–7). Here, we tested whether two of these three components could be replaced by a single, joined sterol-lipid (Fig. 2). Specifically, we hypothesized that we would observe micron-scale, liquid domains in giant vesicles composed of binary mixtures of saturated sterol-lipids and low-T_{melt} lipids. Fig. 3 summarizes all experiments and controls in this study

Our central result, shown in Experiment 1 of Fig. 3, is that binary lipid membranes containing a sterol-lipid do indeed undergo liquid-liquid phase separation. Demixing is most robust when one lipid is saturated Sterol-lipid A (PChemsPC) and the other is a phospholipid with a very low T_{melt} (diPhyPC). Characteristic of liquid phases, the membrane domains are circular (Fig. 4A), and they coalesce quickly when they collide (Fig. S11 and Movie S2). In taut vesicles, domains merge until only one domain of each phase remains, so domain size is a function of the time elapsed since domains nucleated (21) and tension in the membrane (22). At lower temperatures, nearly 100% of vesicles in Experiment 1 demix. The miscibility transition temperature, T_{mix}, is defined at the half-max (Fig. 4B).

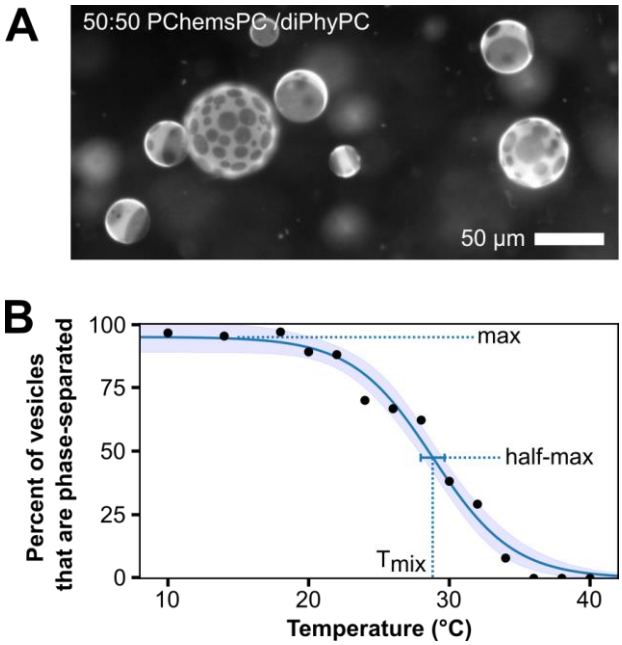

Figure 4: (A) Fluorescence micrograph of micron-scale liquid domains in membranes of vesicles of a 50:50 mixture of a sterol-lipid (PChemsPC) and a low-T_{melt} lipid (diPhyPC) at 10˚C. Bright regions are Ld phase and dark regions are Lo phase. (B) Roughly 100 vesicles were observed at each temperature to determine the miscibility transition temperature, T_{mix}. The shaded region is the 95% confidence interval of the fit.

Our hypothesis was that a single, joined sterol-lipid can behave as a combination of a sterol and a high-T_{melt} lipid in a phase-separated bilayer. This hypothesis leads to the expectation that the Lo phase of binary membranes is enriched in sterol-lipid, just as the Lo phase of ternary membranes is enriched in sterols and high-T_{melt} lipids (23). Consistent with that expectation, the area fraction of dark, Lo phase increases in vesicles as the mole fraction of sterol-lipid increases (Fig. 3).

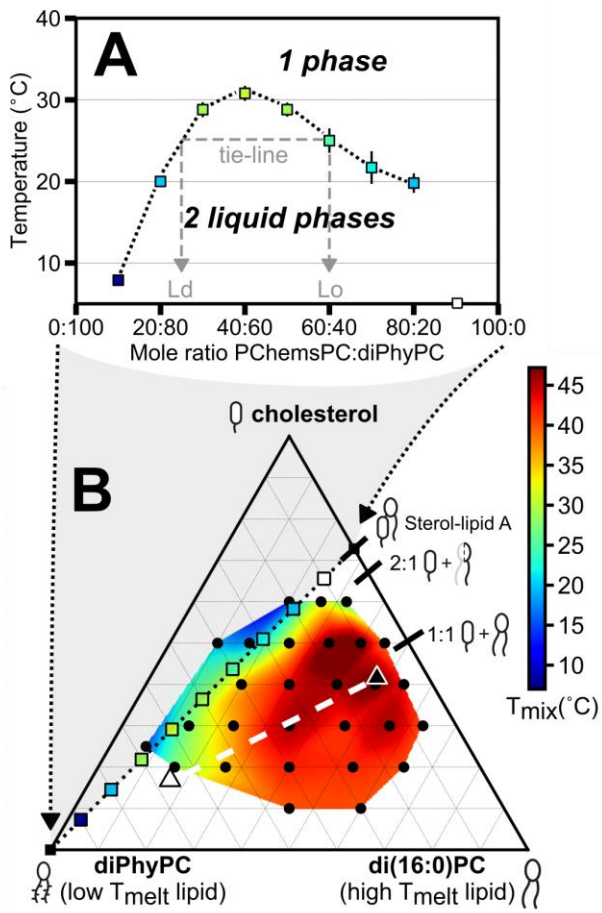

Figure 5: (A) Miscibility transition temperatures for vesicle membranes composed of Sterol-lipid A (PChemsPC) and a low-T_{melt} lipid (diPhyPC). The transition is reversible with temperature. Uncertainties in T_{mix} are 95% confidence intervals for individual experiments, as in Fig. 4B. The white square at 90% sterol-lipid denotes that those membranes are uniform down to temperatures of ≤ 8˚C, the lower limit of our experiments. At 25˚C, endpoints of the tie-line fall at roughly 25:75 and 60:40 ratios of sterol-lipid to high-T_{melt} lipid; these are the compositions of the Ld and Lo phases at 25˚C. (B) Ternary phase diagram of membranes comprised of a sterol (cholesterol), a high-T_{melt} lipid (di(16:0)PC) and a low-T_{melt} lipid (diPhyPC) from (24), used with permission. All possible ratios of the three components fall within the triangle. Miscibility transition temperatures from Panel A are plotted along the black dotted line. The white, dashed line is a known tie-line through the point 30% cholesterol, 35% di(16:0)PC and 35% diPhyPC at 27˚C (24). Colors in both panels refer to the temperature scale.

Binary phase diagrams are delightfully straightforward to interpret. In Fig. 5A, we compile the T_{mix} values we measured for binary mixtures of Sterol-lipid A and a phospholipid. Several aspects of this phase diagram make it particularly appealing. 1) The membrane is either in one uniform phase or in two coexisting liquid phases, over the broad range of 10-90 mol% sterol-lipid. The sterol does not separate into crystallites at higher temperatures or mole fractions as in (4). 2) The membrane has a clear upper miscibility critical point. The highest transition temperatures correspond to 30-50 mole% sterol-lipid. Indeed, for membranes with 50% sterol-lipid, domain shapes fluctuate, characteristic of low line tension near a critical point (supplementary Fig. S1 and Movie S1) (2). 3) Binary phase diagrams greatly simplify the task of evaluating tie-lines. Finding the ratio of each lipid in the Ld and Lo phases at 25˚C is as easy as drawing a horizontal line across the diagram at 25˚C and noting that it intersects the transition to one uniform phase at roughly 25% and 60% sterol-lipid, the compositions of the Ld and Lo phases at that temperature.

Sterol-lipid A corresponds to 3:1 sterol to lipid

The observation of liquid-liquid coexistence in Fig. 4 indicates that Sterol-lipid A acts as a combination of a sterol and a high-T_{melt} lipid, but what ratio of those molecules does the sterol-lipid emulate? The answer lies in the ternary phase diagram (Fig. 5B) for the low-T_{melt} lipid (diPhyPC), the sterol (cholesterol), and the high-T_{melt} lipid (di(16:0)PC). Because the sterol-lipid has only a single chain, a working hypothesis is that the sterol-lipid is equivalent to one sterol and one-half of a high-T_{melt} lipid, in a ratio of 2:1. This ratio is close to the best fit of the binary data within the ternary diagram, which occurs at 73:27 (approximately 3:1). The slope of this line is consistent with a known tie-line in the ternary mixture at 25˚C (specifically, it does not cross the white, dashed line in Fig. 5B).

What structural elements of the low-T_{melt} lipid in Experiment 1 (diPhyPC) allow it to phase separate so robustly from Sterol-lipid A (PChemsPC)? The lipid diPhyPC is unusual in having multiply methylated chains, which give it an extremely low melting temperature, below -120˚C (25). For comparison, another common low-T_{melt} lipid, di(18:1)PC, melts at -18 ± 4˚C (26). Indeed, when we replace diPhyPC with the alternative low-T_{melt} lipid, phase separation is less robust: liquid domains appear in, at best, ~50% of vesicles at 8˚C, implying the compositions are close to a phase boundary, but not within it (Experiment 2, Figs. 3 and S2). The conclusion that the lipid bilayers in Experiment 2 lie close to a phase boundary is consistent with our conclusion that Sterol-lipid A approximates a 3:1 ratio of cholesterol to high-T_{melt} lipid. This result is best seen in Fig. 6, where the dashed line at 3:1 skirts the outer edge of the published 2-phase region, which includes data down to 10˚C (23).

Having concluded that Sterol-lipid A corresponds to a 3:1 ratio of cholesterol to high-T_{melt} lipid, it makes sense that the binary membranes in Experiment 3 do not phase separate even though they contain all three components necessary for liquid-liquid phase separation: chains with high-T_{melt}

values, with low-T_{melt} values, and sterols. Both components of the membrane are sterol-lipids: Sterol-lipid A (PChemsPC) and Sterol-lipid D (OChemsPC) (Figs. 3 and S3). If both sterol-lipids correspond to a 3:1 ratio of cholesterol to lipid, then the membranes containing these molecules lie along the grey dashed line in Fig. 6, far from any known miscibility phase transition in the corresponding ternary membranes.

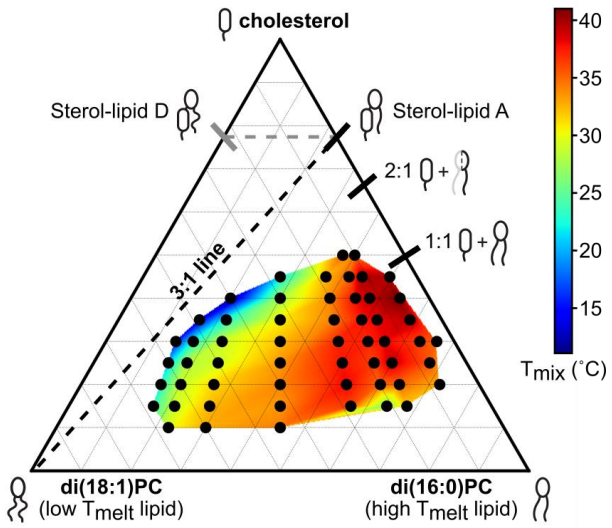

Figure 6: Ternary phase diagram of membranes comprised of a sterol (cholesterol), a high-T_{melt} lipid (di(16:0)PC) and a low-T_{melt} lipid (di(18:1)PC) from (23), used with permission. All possible ratios of the three components fall within the triangle. Miscibility transitions occur at black dots, at temperatures indicated on the color scale, at temperatures down to ~10˚C. The black dashed line corresponds to a 3:1 ratio of cholesterol to high-T_{melt} lipid. The gray dashed line corresponds to mixtures of Sterol-lipid A and Sterol-lipid D.

Alternative lipids and negative controls

The highly disordered chains of diPhyPC, which helped membranes phase separate in Experiment 1, render this lipid a poor choice when mixed with other sterol-lipids. No stable vesicles form in binary mixtures of diPhyPC with either Sterol-lipid B or C (BbGL-1 or CPG; Expts. 4 and 6), likely because all three molecules are cone shaped. The problem is so severe that no vesicles formed from 100% Sterol-lipid B. diPhyPC's conical shape is reflected in its large cross-sectional area (*e.g.*, 80.5 ± 1.5 Å^2 at 30˚C, compared with 67.4 ± 1 Å^2 for di(18:1)PC (27, 28); values for diPhyPC under different conditions are compiled elsewhere (27, 29)). A conical shape of diPhyPC is consistent with shallow membrane defects and low chain order in molecular dynamics simulations of diPhyPC membranes (30, 31)

We mitigated, but did not entirely solve, the problem of unstable membranes due to cone-shaped lipids by switching to the alternative low-T_{melt} lipid, di(18:1)PC. Transition temperatures of binary membranes of Sterol-lipid B and the alternative lipid appear to lie below the lowest temperatures accessible in our experiments. Specifically, fewer than half of the vesicles phase separate in slightly hypoosmotic conditions at ~8˚C, and no vesicles phase separate without an osmotic pressure difference (Expt. 5, Figs. 3 & S5). Based on previously observed trends (32), we hypothesized that replacing di(18:1)PC with lipids with shorter chains would allow coexisting liquid phases to persist at higher temperatures. However, binary membranes of Sterol-lipid B and either di(16:1)PC or di(14:1)PC did not undergo robust liquid-liquid phase separation over our experimental temperatures. Similarly, binary membranes of Sterol-lipid C and the alternative low-T_{melt} lipid do not phase separate (CPG and di(18:1)PC, Expt. 7, Figs. 3 & S7). This was a surprise given that, at first glance, Sterol-lipid C looks most like a 1:1 ratio of a sterol and a two-tailed phospholipid. Moreover, membranes of 100% Sterol-lipid C exhibit high order (20). Chemical alterations to Sterol-lipid C that might allow it to phase separate in a binary membrane might include lengthening its chains to 16 carbons.

Last, we performed a battery of negative controls. At minimum, we expect a sterol-lipid to behave as a sterol, whether it has a saturated or unsaturated chain. When viewed by fluorescence microscopy, binary membranes comprised of cholesterol and phospholipids exhibit either one liquid phase (if the lipid has a low T_{melt}) or gel-liquid coexistence (if the lipid has a high T_{melt}) (33). In line with these expectations, we observe only one liquid phase in some of the binary membranes (Sterol-lipid D with di(18:1)PC; Expt. 10, Fig. S10), and coexisting gel and liquid phases in the others (Sterol-lipid A or Sterol-lipid D mixed with di(16:0)PC; Expts. 8 & 9, Figs. S8 & S9). The last negative control (Sterol-lipid D & di(16:0)PC) supports the assertion that preferential interactions between saturated lipid tails and cholesterol are necessary for liquid-liquid phase separation.

Outlook

In addition to the sterol-lipids investigated here, others could be chemically synthesized. Structural components that can be varied include the length and unsaturation of the sterol-lipid's acyl chain, the orientation of its sugar (α-linkage vs. β-linkage affects lipid orientations (34) and its charge (zwitterionic for Sterol-lipid A and Sterol-lipid D, neutral for Sterol-lipid B, and charged for Sterol-lipid C). Sterol lipids are also extracted from natural sources. For example, Huang et al. used an ACGal mixture of sterol-lipids (one of which is Sterol-lipid B) (19). One interest of those authors was the potential application of sterol lipids in drug delivery (34).

Additional applications might lie in engineering minimal cell systems to function with only two classes of lipids (35); if one of those classes were a sterol-lipid, it would be interesting to test if the cell membrane phase separated. Micron-scale, reversible, liquid-liquid phase separation has previously

been observed in more complex membranes of living cells (1, 36), where it has biological importance. For example, in yeast, phase separation of vacuole membranes promotes docking of lipid droplets during periods of nutrient restriction (37–39). Furthermore, yeast actively modify their lipid compositions to maintain their vacuole membranes near a miscibility transition (40). Our discovery that sterol-lipids can substitute for both a sterol and a high-T_{melt} lipid suggests a new role for sterol-lipids in biological membranes where they are found. In addition to functioning as a sterol (19, 20), sterol-lipids may enable liquid-liquid phase separation in membranes that otherwise lack sufficient saturated lipids. Our results also open new directions for researchers conducting research *in silico*. Our minimal membrane presents a new system for researchers to validate force fields and leverage recent advances in atomistic simulations of membranes undergoing liquid-liquid phase separation (41–43).

More fundamentally, 2-component bilayers that undergo liquid-liquid phase separation solve challenges in determining tie-lines in 3-component membranes. Tie-lines contain essential information: their endpoints reveal the ratio of lipids in each phase, and they provide a path along which chemical potentials of membrane components are equal and constant. In a system with only *two* components, tie-lines are straightforward to determine – only one tie-line is possible at each temperature. As a result, tie-lines in two-component systems are trivial to traverse in the laboratory: the amount of only one type of lipid needs to be tuned. Two-component systems are also special because their tie-lines are parallel at different temperatures. In contrast, in a system with *three* components, multiple challenges arise:

I) the direction of each tie-line must be experimentally determined for every new set of molecules (33);
II) the directions of tie-lines found by measuring area fractions may only approximate the directions found by measuring mole fractions (44);
III) when the amount of only one component is changed, the system lies on an entirely new tie-line; and
IV) the direction of the tie-line changes with temperature (17).

As a result, when different labs seek to compare results in lipid bilayers, it is easy for them to verify that their systems lie along the same tie-line when they use 2-component membranes, and difficult when they use 3-component membranes. One caveat is that a membrane composed of only two kinds of molecules may not qualify as a 2-component membrane if the molecules are in different configurations that do not interconvert quickly on time scales of the experiment. Similarly, if one of the components crystalizes (as hydroxysterols do in membranes (Kamal 2023)), it is unclear how to assign lipid compositions at one end of the tie-line.

Conclusion

To summarize, we observe micron-scale, liquid-liquid phase separation in bilayer membranes of only two components, one of which is a sterol-lipid. The resulting phase diagram is an archetype of an upper miscibility critical point, a nearly textbook example of two liquid phases at low temperature, critical behavior at the highest transition temperature, area fractions of phases consistent with the lever rule, and only one liquid phase otherwise. This clean system makes interpretation of results remarkably straightforward. Moreover, it gives us a window into what molecular features might be important. Independent of whether liquid-liquid phase separation occurs in a standard 3-component membrane or in a 2-component membrane with sterol-lipids, the membrane apparently must contain carbon chains with low order, chains with high order, and a sterol, where the sterols closely interact with the ordered chains. Here, we find that one of the sterol-lipids behaves as a 3:1 ratio of cholesterol to a high-T_{melt} lipid, di(16:0)PC.

Materials and Methods

Chemicals

The lipid di(14:0)-cholesteryl-6-O-phosphatidyl-α-d-glucopyranoside (CPG) was synthesized as previously described (45).The lipids 1-palmitoyl(16:0)-2-cholesterylhemisuccinoyl-sn-glycero-3-phosphocholine (PChemsPC), 1-oleoyl(18:0)-2-cholesterylhemisuccinoyl-sn-glycero-3-phosphocholine (OChemsPC), cholesteryl 6-O-palmitoyl-β-D-galactopyranoside (BbGL-1), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), dipalmitoyl(18:1)-phosphocholine (DPPC), diphytanoyl(16:0-4Me)-phosphocholine (diPhyPC), and dipalmitoyl(16:0)-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rhod-DPPE) were purchased from Avanti Polar Lipids (Alabaster, AL). All lipids have only one length of acyl chain. To disambiguate the sterol-lipids in this study from others in the literature, ACGal, or 6-O-acyl-β-D-galactopyranoside, refers to a mixture of sterol-lipids with different acyl chains, one of which is BbGL-1 (19). Similarly, CAG, or cholesteryl-6-O-tetradecanoyl-α-D-glucopyranoside differs from BbGL-1 by having only 14 carbons (rather than 16) in its acyl chain and in the orientation of the sugar (α-linked rather than β-linked); both are found in *H. pylori* (16). Sucrose (ACS grade) was from Fisher Chemical, and vacuum grease (MOLYKOTE High Vacuum Grease) from DuPont. All chemicals were used as supplied, without further purification. Avanti Polar Lipids quotes purities at 99%.

Electroformation

A total of 0.125 mg lipids was mixed in stock chloroform solutions. Lipid ratios included 0.8 mol% Rhod-DPPE to fluorescently label the liquid-disordered phase. The lipid mixture was spread at 60˚C across an ITO-coated glass slide (Delta Technologies, Loveland, CO) with the side of a glass

pipette, leaving a thin, even lipid film. The lipid-coated slide was then placed in a vacuum desiccator for ≥ 30 min to evaporate residual chloroform. Next, a chamber was formed as follows: vacuum grease was used to attach two 1-mm-thick Teflon spacers near the edges of the slide, and a second ITO-coated slide was placed on top of the spacers. The chamber was then filled with 100 mM sucrose in 18 MΩ-cm water, sealed with more vacuum grease, and placed in a 60˚C oven. An AC field of 1 V at 10 Hz was applied across the two ITO surfaces at 60˚C for 1 hr.

Imaging

Vesicle solutions were imaged within 2 hrs. of production. They were removed from electroformation chambers, diluted, sandwiched between coverslips, sealed with vacuum grease, and imaged on a home-built temperature-controlled stage with a Nikon Eclipse ME600L upright epifluorescence microscope and a Hamamatsu C13440 digital camera**.**

Dilution entailed mixing 20 µL of vesicle solution with 130 µL of 100 mM sucrose (for equal osmotic pressures) or, more commonly, 80 mM sucrose (for a slight osmotic difference that minimized vesicle tubulation). Newly electroformed vesicles containing sterol-lipids are flaccid, unlike the ternary membranes they are designed to replace. Although membranes in these flaccid vesicles can phase separate, tubules and other non-spherical shapes often appear with changes in temperature, making transitions difficult to measure (Fig. S1). Tubulation may be exacerbated in our experiments because the constituent lipids likely have conical shapes and because the lipid mixtures do not include free sterols, which can mitigate membrane asymmetries via flip flop (46). To address this problem, we applied a small osmotic pressure difference sufficient to prevent most tubulation (100 mM sucrose inside, 80 mM outside). At higher concentration differences (100 mM inside, 50mM outside), some vesicles cyclically ruptured (47), and T_{mix} was ~3˚C higher. Membrane tension, whether due to osmotic pressure or other sources, is known to shift T_{mix} (48–50).

Measuring T_{mix}

To measure miscibility transition temperatures (T_{mix}) of membranes, vesicles were cooled stepwise from 40˚C to 8˚C. Images of ~100 vesicles were collected from multiple fields of view every 2˚C near the transition temperature and at least every 4˚C otherwise. To confirm reversibility of the transition, temperature was incrementally raised back from 8˚C to 40˚C.

The percentage of phase-separated vesicles at each temperature was manually recorded by a single user. Uncertainty in T_{mix} occurs due to slight variation in lipid composition from vesicle to vesicle. To find T_{mix}, a non-linear least squares regression was used to fit a sigmoidal curve with the form: $\textit{Percent phase Separated} = \textit{Max}\ (1 - (1 + \exp(-(T - T_{mix}) / w)))^{-1}$), where *Max* is the maximum percent of vesicles that phase separate, T is temperature, and w is the width of the sigmoidal curve. 95% confidence intervals were calculated from variances of fit parameters.

Author Contributions

H.Q.N. and J.G.H. synthesized the CPG sterol-lipid. K.W. and S.L.K. designed the subsequent research plan. K.W. performed research and analyzed data. K.W. and S.L.K. wrote the manuscript and produced the figures.

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Declaration of Interests

The authors declare no competing interests.

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