

# Mixing Temperatures of Bilayers Not Simply Related to Thickness Differences between $L_o$ and $L_d$ Phases

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**ABSTRACT** Micron-scale coexisting  $L_o$  and  $L_d$  liquid phases can appear in lipid bilayers composed of a ternary mixture of a low-melting temperature lipid, a high-melting temperature lipid, and cholesterol. A priori, temperatures at which membranes demix,  $T_{\text{mix}}$ , are not simply related to differences in thicknesses,  $\Delta h$ , between  $L_o$  and  $L_d$  phases. Here, we use fluorescence microscopy to measure  $T_{\text{mix}}$  and we use atomic force microscopy at 22°C to measure  $\Delta h$  for a series of bilayers composed of different ratios of the three components. Our data illustrate cases in which a change in  $T_{\text{mix}}$  or  $\Delta h$  does not result in a change in the other parameter. The data provide a context in which to evaluate recent reports of a correlation between  $T_{\text{mix}}$  and  $\Delta h$ .

Model lipid membranes composed of a high-melting temperature ( $T_{\text{melt}}$ ) lipid, a low- $T_{\text{melt}}$  lipid, and a sterol can phase-separate into liquid-ordered ( $L_o$ ) and liquid-disordered ( $L_d$ ) phases (1). Typically, the  $L_o$  phase is thicker than the  $L_d$  phase (2–4), and is sometimes referred to as the “raft phase” by researchers who associate membrane phases with the lipid raft hypothesis (5).

Two important parameters characterizing this phase separation are  $T_{\text{mix}}$ , the mixing temperature below which  $L_o$  and  $L_d$  phases appear in the membrane, and  $\Delta h$ , the thickness difference between  $L_o$  and  $L_d$  phases at a given temperature. Previous studies have shown that a mismatch between  $L_o$  and  $L_d$  thicknesses affects protein sorting (6), and that bilayer thickness affects protein function (7,8).

Fig. 1 frames possible relationships between  $\Delta h$  and  $T_{\text{mix}}$ : the variables can vary independently (line A or B) or be correlated (curve C). Previous work by García-Sáez et al. (2) reported that  $T_{\text{mix}}$  increases with increasing  $\Delta h$  (as in Fig. 1 C) for bilayers at a fixed 40:40:20 mol ratio of low- $T_{\text{melt}}$  lipid/high- $T_{\text{melt}}$  lipid/cholesterol. In their work, the high- $T_{\text{melt}}$  lipid was stearyl-sphingomyelin. Their low- $T_{\text{melt}}$  lipids were a series of phosphocholines (PCs) with carbon chains that systematically lengthened from 14:1 (largest  $T_{\text{mix}}$  and  $\Delta h$ ) to 22:1 (smallest  $T_{\text{mix}}$  and  $\Delta h$ ). They reported  $T_{\text{mix}}$  proportional both to  $\Delta h^2$  and to line tension.

The results of García-Sáez et al. (2) have been used to support statements that hydrophobic length mismatch between

lipid types promotes their segregation in phase-separated membranes (6) and drives domain formation by increasing line tension (8). However, the quantitative result of García-Sáez et al. (2) holds only for the particular ratio and types of lipids for which it was reported. For a broader class of membrane systems,  $T_{\text{mix}}$  is not related to  $\Delta h$  (or to line tension) in a simple way.  $T_{\text{mix}}$  is determined by the shape of the phase boundary and depends on the ratio and type of lipids. Consider an analogy of an ant crawling over a bowl placed upside-down on the floor. Specifying the type of bowl (the types of lipids in the membrane) limits the ant’s highest possible elevation (the highest  $T_{\text{mix}}$ ), but does not specify the ant’s current elevation (the membrane’s  $T_{\text{mix}}$ ) unless the shape of the bowl and the ant’s latitude and longitude across the floor (the ratio of lipids in the membrane) are known.

Moreover, the highest  $T_{\text{mix}}$  can be difficult to predict for a membrane composed of an arbitrary ternary set of lipids (as opposed to the series of García-Sáez et al. (2)). We have found no monotonic relationship between the highest  $T_{\text{mix}}$  of a ternary membrane and the estimated thickness difference between the  $L_o$  and  $L_d$  phases at room temperature (4).

Now imagine two ants crawling over two nested bowls. The bowls may be nonaxisymmetric and their centers may be offset. Even if the bowl depths are simply related (e.g., the highest possible elevation of ant 1 is half that of ant 2), and the ants share the same latitude and longitude with respect to the floor, the difference between the ants’ elevations is nontrivial to find. This scenario corresponds to the nested phase diagrams in Fig. S1 in the Supporting Material. To give concrete examples, here we use data to illustrate cases in which a change in  $\Delta h$  or  $T_{\text{mix}}$  does not result in a change in the other parameter, as schematically depicted in Fig. 1, lines A and B.

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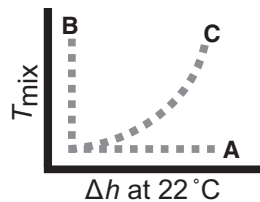
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**FIGURE 1** Possible trends between thickness mismatch ( $\Delta h$ ) and miscibility temperatures ( $T_{\text{mix}}$ ) for membranes that demix into  $L_o$  and  $L_d$  phases. Data as in curve C appear in García-Sáez et al. (2) and may correspond to Fig. S1, a and b. Here we present data as in lines A and B.

To gather data as in lines A and B in Fig. 1, we used tactics applicable to any membrane that demixes into  $L_o$  and  $L_d$  phases. All possible ratios of the three lipid types in a membrane can be plotted in a plane. By definition, a tie-line passes through the overall lipid ratio of any particular membrane. The ends of that tie-line fall at the lipid ratio of that particular membrane's  $L_o$  phase, and of its  $L_d$  phase. The length and orientation of the tie-line within the plane varies with temperature (9). Two membranes at the same temperature that have different overall ratios of the same lipid types demix into exactly the same  $L_o$  and  $L_d$  phases if their overall lipid ratios fall on the same tie-line. Along the line, relative areas of the  $L_o$  and  $L_d$  phases vary.

To observe the trend in line A of Fig. 1, our goal was to measure  $\Delta h$  for a series of membranes with lipid ratios that follow an isotherm in  $T_{\text{mix}}$ . We chose each lipid ratio to fall on a different tie-line. Based on previous work, we hypothesized that membrane compositions that lie on longer tie-lines would produce larger values of  $\Delta h$  (9–11).

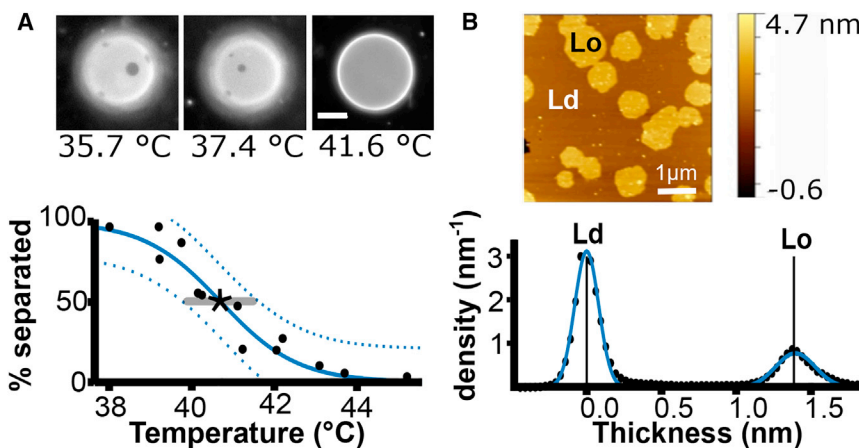
To observe the trend in line B, our goal was to measure  $T_{\text{mix}}$  for a series of membranes with lipid ratios that follow a previously determined tie-line at 22°C (9). Because

the composition of the  $L_o$  and  $L_d$  phases are constant along this line, all of these membranes should share the same  $\Delta h$ .

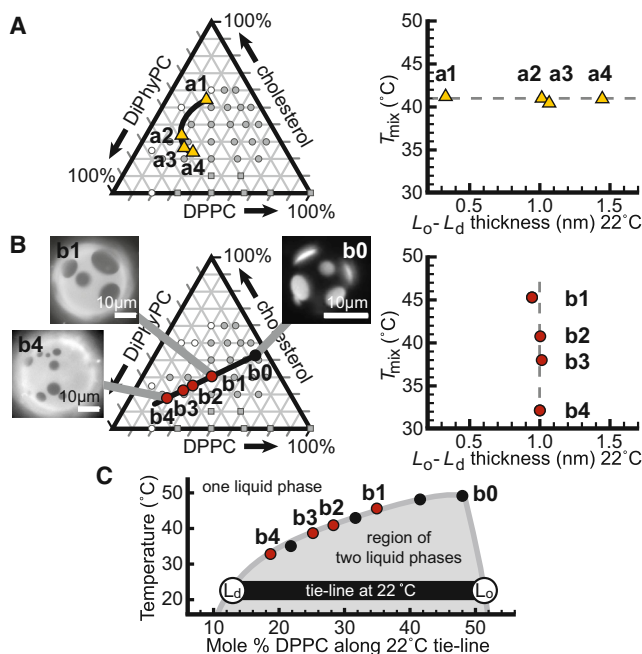
We determined  $T_{\text{mix}}$  as the temperature at which 50% of giant unilamellar vesicles (GUVs) demix, as in Fig. 2 A. GUVs were composed of mixtures of DPPC (dipalmitoyl-PC), DiPhyPC (diphytanoyl-PC), and cholesterol. They were labeled with 0.8 mol % Texas Red, which partitions preferentially to the  $L_d$  phase. GUVs were electroformed and imaged as previously noted in Veatch and Keller (11). We determined  $\Delta h$  from atomic force microscopy (AFM) measurements of supported bilayers. Small unilamellar vesicles were deposited on mica at 60°C in a solution of 5 mM  $\text{CaCl}_2$  (10). The resulting bilayer was rinsed with 18 M $\Omega$ -cm water by pipette and scanned in an atomic force microscope at 22°C. AFM images were flattened in Gwyddion (12). Height histograms were exported to MATLAB (The MathWorks, Natick, MA) and fit with Gaussian peaks as in Fig. 2 (4). Further details of Materials and Methods are in the Supporting Material.

Our central result, namely that  $T_{\text{mix}}$  and  $\Delta h$  can vary independently, is shown in Fig. 3. Points a1–a4 in Fig. 3 A lie on an isotherm in  $T_{\text{mix}}$ . Points b1–b4 in Fig. 3 B lie on a tie-line. Because our result follows from general attributes of phase diagrams, it applies to any ternary membrane that demixes into  $L_o$  and  $L_d$  phases.

Our result reveals difficulties inherent in quantifying relationships between  $T_{\text{mix}}$  and  $\Delta h$  for membranes made from different lipid species unless full phase diagrams and tie-lines are known. Generalizing from data collected at just one ratio of lipids is meaningful only when phase boundaries for the two systems are related by a simple geometric scaling and are centered at the same ratio of lipids. In addition, either the phase boundary must be hemispherical or the direction of the tie-lines must not change



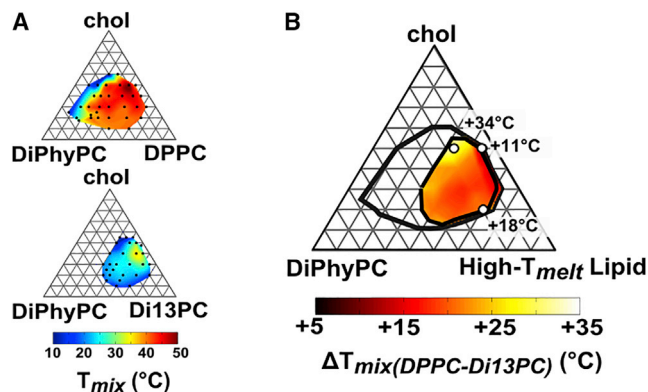
**FIGURE 2** Measured values of  $T_{\text{mix}}$  and  $\Delta h$  for 48:32:20 DiPhyPC/DPPC/chol bilayers. (A) Fluorescence micrographs for a single GUV (top) and a graph of percent demixed GUVs versus temperature for a population of GUVs (bottom). The solid blue line is a sigmoidal fit of the black points. Dashed lines bound the 95% confidence interval. The star lies at  $T_{\text{mix}}$ , and the bar spans the uncertainty; here  $T_{\text{mix}} = 40.7 \pm 0.3^\circ\text{C}$ . (B) AFM scan of a supported lipid bilayer at 22°C (top) and height histogram of the same scan (bottom). The blue line follows two Gaussian peak fits of the black data points. For a single scan,  $\Delta h$  is reported as the distance between the mean thicknesses of the two peaks, and uncertainty is reported as the standard deviation of the bootstrap fit, here  $\Delta h = 1.39 \pm 0.02$  nm. Values of  $T_{\text{mix}}$  and  $\Delta h$  are listed in Table S2 in the Supporting Material. To see this figure in color, go online.



**FIGURE 3** Measured values of  $T_{\text{mix}}$  versus  $\Delta h$  at 22°C for bilayers of DiPhyPC/DPPC/Chol that fall (A) on a  $T_{\text{mix}}$  isotherm and (B) on a tie-line. Ternary phase diagrams at 22°C are from Veatch et al. (9), where shaded circles denote two liquid phases, open circles denote one liquid phase, and squares denote that a gel phase is present. Micrographs show that area fractions of the  $L_d$  phase increases from point  $b_0$  to  $b_4$ . Dashed lines are not fits. Tables S2–S9 list values and uncertainties. (C) Phase boundary along the tie-line in (B). Solid points were determined in this study. The underlying shaded curve is not a fit. To see this figure in color, go online.

with temperature (see Fig. S1). We know of no two membrane phase diagrams discovered to date that meet these criteria.

Fig. 4 illustrates this point for two ternary membrane systems for which the high- $T_{\text{melt}}$  lipid species differ in the lengths of their acyl chains. Fig. 4 A plots  $T_{\text{mix}}$  values for membranes of DiPhyPC/DPPC/chol and DiPhyPC/Di13:0-PC/chol, where Di13:0-PC has three fewer carbons in each of its two chains than DPPC. The membrane containing DPPC demixes over a broader range of lipid ratios at all temperatures, and its  $T_{\text{mix}}$  is higher at any lipid ratio at which both membranes demix. A broader range of ratios means longer tie-lines, which can translate into larger  $\Delta h$  (10). If we arbitrarily choose any ratio of the three membrane components, we would expect some positive correlation between values of  $T_{\text{mix}}$  and  $\Delta h$ . However, because the phase boundaries and the tie-lines differ in the two systems, a different choice of this ratio will result in a different quantitative relationship between  $\Delta h$  and  $T_{\text{mix}}$ . Inherent in this discussion is the perspective that demixing of membranes into phases of different compositions gives rise to  $\Delta h$  and line tension (with values that vary across the phase diagram), and that the value of  $\Delta h$  or line tension at a single



**FIGURE 4** (A) Phase diagrams for which the high- $T_{\text{melt}}$  lipid is either DPPC (with 16 carbons in its acyl chain, top) or Di13:0-PC (bottom). Black dots are experimental values of  $T_{\text{mix}}$  including values from Veatch et al. (9). The top diagram has higher values of  $T_{\text{mix}}$  at any lipid ratio and a larger phase boundary at any temperature. (B) Difference in  $T_{\text{mix}}$  (denoted  $\Delta T_{\text{mix}}$ ) for GUVs of the two ternary lipid mixtures in (A). To see this figure in color, go online.

lipid ratio and temperature does not determine the shape of the full phase boundary.

Given that Fig. 3 shows how a single ternary membrane can produce a horizontal or vertical line in a graph of  $T_{\text{mix}}$  versus  $\Delta h$ , various functional forms relating  $T_{\text{mix}}$  and  $\Delta h$  for two ternary membranes are then possible, subject to the choice of lipid ratio. Extending this idea, it is possible to imagine future discoveries of membrane systems that produce a negative slope on a graph of  $T_{\text{mix}}$  versus  $\Delta h$ , as in Fig. S1.

Separately, we can test the correlation between domain size and  $\Delta h$  reported by Heberle et al. (13). They used neutron scattering to find that small, 60-nm vesicles with low  $\Delta h$  values had more unmerged domains. They report  $<2$  domains/vesicle for  $\Delta h = 0.97$  nm and 23 domains/vesicle for  $\Delta h = 0.64$  nm. These  $\Delta h$  values are in line with AFM values from similar lipid systems (2). In our GUVs, domains of the same liquid phase merge until only one domain of each phase remains, even when  $\Delta h$  is as small as 0.32 nm. Our results imply that a small  $\Delta h$  value alone does not determine the number of stable, liquid domains.

In conclusion, our results demonstrate challenges of quantitatively establishing how  $\Delta h$  relates to  $T_{\text{mix}}$ . There is no reason to question the validity of the correlation between  $\Delta h$  and  $T_{\text{mix}}$  reported by García-Sáez et al. (2) for the systems they studied. Nevertheless, Fig. 4 illustrates why other groups are likely to find different values if they employ different ratios of lipids. We also illustrate a method that follows directly from known thermodynamic principles to identify lipid ratios for which  $T_{\text{mix}}$  and  $\Delta h$  vary independently. We present this method to support future studies of how lipid structure affects  $T_{\text{mix}}$  and  $\Delta h$ .

## SUPPORTING MATERIAL

Supporting Materials and Methods, one figure, and nine tables are available at [http://www.biophysj.org/biophysj/supplemental/S0006-3495\(16\)30276-4](http://www.biophysj.org/biophysj/supplemental/S0006-3495(16)30276-4).

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