# Saturated Phospholipids with High Melting Temperatures Form Complexes with Cholesterol in Monolayers

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Many mixtures of phospholipids and cholesterol form immiscible liquid phases in a monolayer at an air−water interface. As discovered recently, some binary mixtures of phospholipid and cholesterol exhibit *two* upper miscibility critical points. This phenomenon can result from the reversible formation of a chemically distinct liquid product or "condensed complex" between the phospholipid and cholesterol. The present work describes an empirical connection between high melting temperatures of saturated phospholipid bilayers and the appearance of two upper miscibility critical points in cholesterol−phospholipid monolayers. A rough correlation is also found between melting temperature and the composition of the complex. Bilayer melting temperature is a convenient measure of the tendency of the phospholipid acyl chains to order, and this same tendency is evidently important for condensed complex formation.

## Introduction

Understanding the biophysical chemistry of cholesterol in cell membranes is a formidable challenge.[1−3] In view of their simplicity and broad range of accessible molecular densities, studies of phospholipid monolayers containing cholesterol have the potential to uncover interactions important for understanding bilayer membranes.[4]

At low surface pressures, $\Pi$ , two coexisting liquid phases are often observed when phospholipids are mixed with cholesterol in monolayers at the air−water interface.[5−7] At higher surface pressures the two liquid phases merge into one liquid phase (e.g., Figure 1a−c). The highest pressure at which this occurs corresponds to the upper miscibility critical point. At the critical composition, domains in the two-phase region form stripes with equal areas of phospholipid-rich and phospholipid-poor phases.[8] The observation of stripes is characteristic of proximity to a miscibility critical point.[9]

Many studies of monolayers have used dihydrocholesterol (Dchol) rather than cholesterol since Dchol is more resistant to air oxidation. Control experiments show that the phase diagrams obtained with the two sterols are very similar. For example, critical pressures are the same to within ±1mN/m.[10]

Some mixtures of phospholipids and Dchol exhibit two upper miscibility critical points (e.g., Figure 1d).[11] The two two-phase regions are named $\alpha$ and $\beta$ in order of increasing Dchol concentration. This phase behavior can be explained if molecules of phospholipid and Dchol associate to form a chemically distinct "condensed complex".[11] The $\alpha$ region results from immiscibility between the complex and pure phospholipid, the $\beta$ region from the complex and pure Dchol.[11] The two components, C (cholesterol or Dchol) and P (phospholipid) are considered to react to form a complex $C_qP_p$,

$$qC + pP \rightleftarrows C_qP_p \qquad (1)$$

where $p$ and $q$ are relatively prime numbers.[12] For an oligomerization reaction,

$$nqC + npP \rightleftarrows C_{nq}P_{np} \qquad (2)$$

complex formation is more cooperative even though the relative stoichiometric composition ($q/(p + q)$) is the same.[13]

To ascertain why some but not all mixtures of phospholipids and Dchol show two upper miscibility critical points rather than one, phase diagrams were determined for a range of mixtures of phospholipids with cholesterol.

## Methods

Phase behavior of lipid mixtures at the air−water interface of a Langmuir trough was observed by epifluorescence microscopy as described previously.[5,7] Transition pressures are reported as dyn/cm (mN/m). A minimal amount, 0.4%, of Texas red dimyristoyl phosphatidylethanolamine (TR-DMPE, Molecular Probes, Eugene, OR) was used as a dye for contrast between liquid phases unless otherwise noted. Other dyes used were 3,3′-dihexadecylindocarbocyanine (diI C18(3)), Bodipy glucocerebroside, Rhodamine C18, and 22-(*N*-(NBD)-23,24-bisnor-5-cholen-3B-ol (NBD-chol), all from Molecular Probes. Lipids had two acyl chains, which are described as chain length: unsaturation, and had a variety of headgroups, including phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS). Unless otherwise noted, phospholipids have L stereochemistry (Avanti Polar Lipids, Alabaster, AL). Racemic phospholipids and Dchol were from Sigma (St. Louis, MO). All were used without further purification. Dchol was used rather than cholesterol to minimize air oxidation.[10] Under our experimental conditions, photooxidation increases the critical pressure on the order of 0.016 dyn/(cm·min).[9] Phase boundaries were typically determined within 15 min. At short times, the behavior of both sterols in monolayers containing phospholipids is very similar.[10] All experiments were conducted at room temperature, 23 ± 0.5 °C. All solid lines shown in the phase diagrams are drawn to guide the eye and are not explicit fits of the data.

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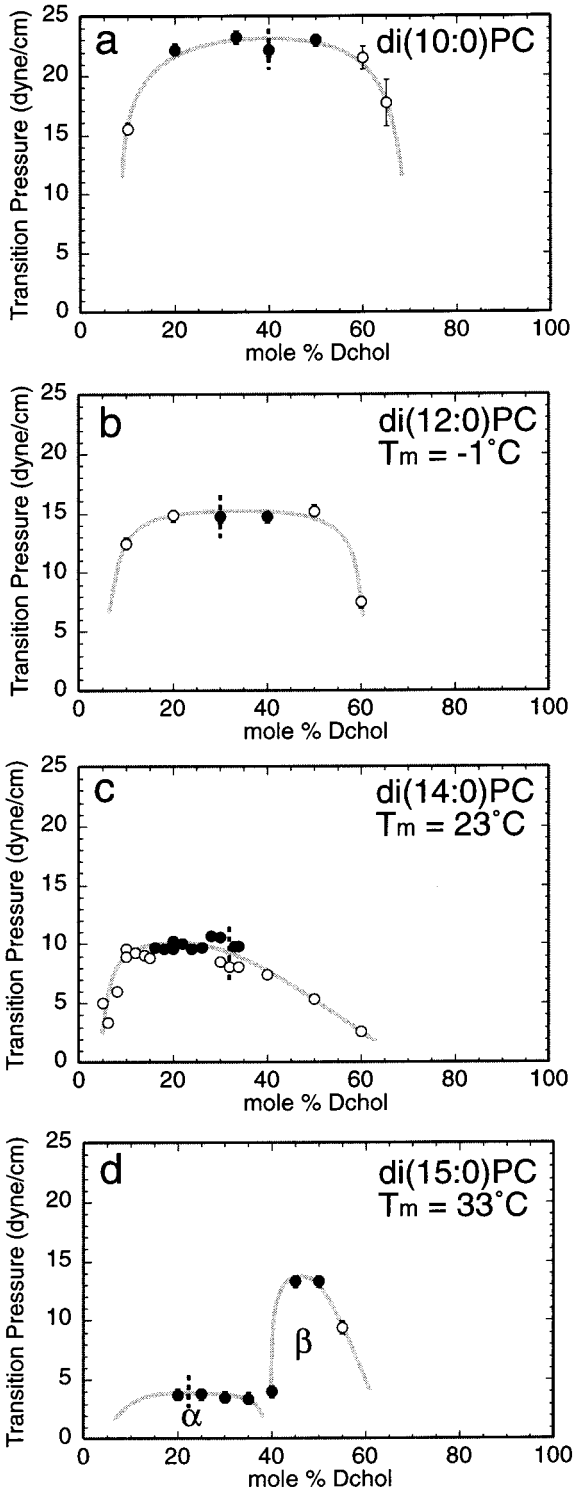


**Figure 1.** Phase diagrams for monolayers of Dchol and phosphatidylcholines. Phase boundaries are between two coexisting liquid phases at low surface pressure and one liquid phase at high pressures. Filled symbols vs open symbols denote the observation of stripe domains vs no stripe domains at the phase boundary where the two phases merge. Phase diagrams a−c show only one two-phase coexistence region, whereas phase diagram d has two. Phase diagram c is taken from ref 33. Critical concentrations for the α region are at (a) 40 ± 5, (b ) 30 ± 5, (c) 33 ± 1, and (d) 22.5 ± 2 mol % Dchol and are marked by dotted lines in the phase diagrams. The critical concentration for the $\beta$ region of phase diagram d is probably 47 ± 2 mol % Dchol.

Domains and critical points in the α region were indistinguishable from domains seen in systems with only one miscibility critical point, whereas domains in the $\beta$ region were small and difficult to see.[11] To determine whether stripes formed near the transition in the $\beta$ region, an electric field was used to fuse small domains.[13,14] In all cases, transition pressures were recorded when recognizable fluid domains nucleated from a uniform fluid background as the surface pressure decreased. Gas phases (at surface pressures <1dyn/cm) and solid phases (at elevated pressures) are not discussed here.

**TABLE 1: Correlation of Two Miscibility Critical Points with High Bilayer Melting Temperatures of Saturated Phospholipids**

| lipid | $\bar{T}_m$(°C) | $\Pi_{crit}$ | $\Pi_{crit}(\alpha)$ | $\Pi_{crit}(\beta)$ | source |
|---|---|---|---|---|---|
| Symmetric Unbranched Lipids | | | | | |
| di(10:0)PC | −33 | 22.9 | | | |
| di(12:0)PC | −1 | 14.4 | | | |
| di(14:0)PC | 23 | 7.6 | | | |
| di(12:0)PE | 29 | | 5.7 | 14.5 | |
| di(15:0)PC | 33 | | 3.8 | 13.3 | |
| di(14:0)PS | 35 | | 1.6 | 16.2 | 11,12 |
| di(16:0)PC | 41 | | 1.4 | | |
| Symmetric Branched Lipids | | | | | |
| diphytanoylPC | <−120 | 6.6 | · | | |
| Aymmetric Lipids | | | | | |
| (16:0−14:0)PC | 27 | ? | 5.6 | 14.8 | |
| (14:0−16:0)PC | 35 | ? | 4.3 | 12.9 | |
| Lipid Mixtures of Equal Chain l Length | | | | | |
| 2/1 di(14:0)PC/di(14:0)PS | 30 | · | 6.0 | 11.3 | |
| 1/2 di(14:0)PC/di(14:0)PS | 31 | · | 3.8 | 11.4 | 11,12 |
| 1/1 di(14:0)PC/di(14:0)PE | 37 | | 3.7 | 18.4 | |
| Lipid Mixtures of Unequal Chain Length | | | | | |
| 1/1 di(14:0)PC/di(12:0)PC | 22 | 15.4 | · | · | |
| 3/1 di(14:0)PC/di(16:0)PC | 28 | | 7.2 | 9.3 | |
| 2/1 di(14:0)PC/di(16:0)PC | 30 | | 6.2 | 10.3 | |
| 1/1 di(14:0)PC/di(16:0)PC | 32 | | 5.2 | 12.2 | 12 |
| 5/4/3 di(14:0)PC/di(14:0)PS/ di(16:0)PC | 32 | | 4.0 | 11.2 | |
| egg sphingomyelin | 37−40 | | 2.65 | 18.8 | 13 |
| 1/1 di(14:0)PC/ egg sphingomyelin | 30−32 | | 12 | 15 | |

Bulk melting temperatures, $T_m$, of pure phospholipid bilayers in water are taken from differential scanning calorimetry data[15,16] except for di(10:0)PC, which is from calculations.[17] For the mixtures given in Table 1, the temperatures $\bar{T}_m$ given are the averages of bulk melting temperatures of the pure phospholipids.

## Results

**Phase Behavior.** Figure 1 shows phase diagrams of Dchol mixed with a series of phosphatidylcholine lipids which have symmetric, saturated, unbranched acyl chains. As the acyl chain length increases, the bilayer melting temperature also increases, and the critical pressure decreases (Figure 1a to 1c). Lipids with high bilayer melting temperatures show two miscibility critical points (Figure 1d). The cusp in the phase diagram between the α and $\beta$ regions typically falls between 25% and 45% Dchol.[13] Even though the melting temperature of the phospholipid bilayer is high, all of the transitions described are from two coexisting *liquid* phases to one uniform *liquid* phase.

The correlation of two miscibility critical points with high bilayer melting temperatures of saturated phospholipids is documented more extensively in Table 1. When mixed with Dchol in monolayers, phospholipids with melting temperatures at or below 23 °C exhibit only one upper miscibility critical point, at critical pressure $\Pi_{crit}$. Phospholipids with higher melting temperatures exhibit *two* upper miscibility critical points, with critical pressures $\Pi_{crit}(\alpha)$ and $\Pi_{crit}(\beta)$. The trend also holds for several cases of phospholipids with asymmetric acyl chains and for mixtures of phospholipids. Care has to be taken in interpreting critical pressures across categories in Table 1. The critical

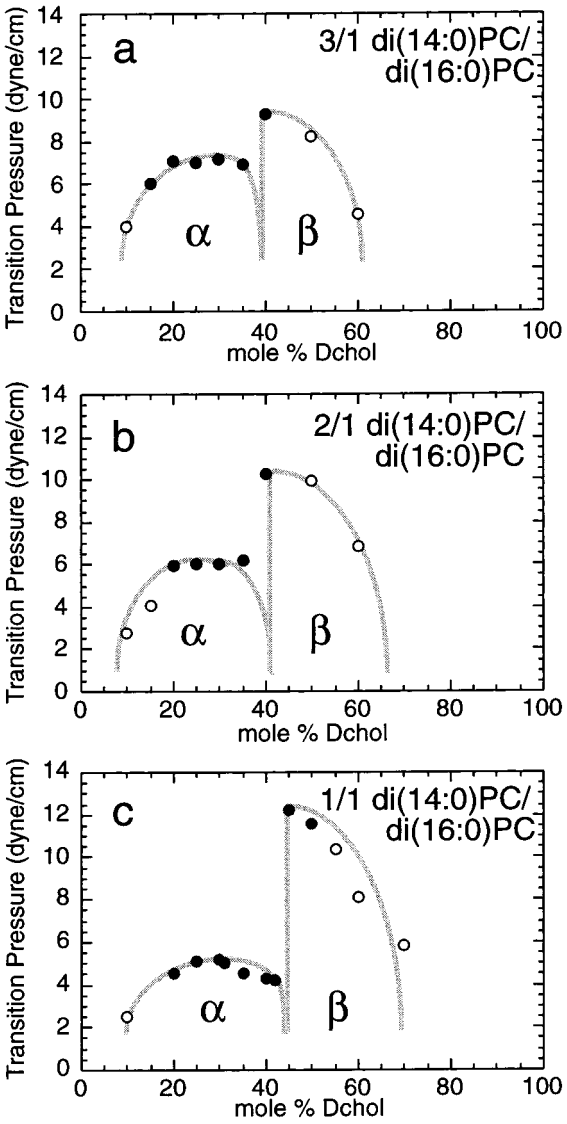


**Figure 2.** Phase diagrams for monolayers of Dchol with di(14:0)PC and di(16:0)PC systematically mixed in different proportions. Phase boundaries are between two coexisting liquid phases at low surface pressure and one liquid phase at high pressure. Filled symbols vs open symbols denote the observation of stripe domains vs no stripe domains at the phase boundary where the two phases merge. As the average of the bilayer melting temperatures of the phospholipids in the mixture increases (Figure 2a to 2c), $\Pi_{crit}(\alpha)$ decreases and $\Pi_{crit}(\beta)$ increases. Figure 2c is reproduced from ref 1.

pressure of Dchol mixed with lipids with asymmetric acyl chains, with mixtures of lipids of different chain lengths, or with mixtures of lipids with different degrees of unsaturation can be surprisingly high.[18] As yet, phase diagrams with only one critical point have been observed for mixtures of Dchol and unsaturated phospholipids.[19] Mixtures containing saturated phospholipids and low concentrations of unsaturated phospholipids can doubtless also show two critical points.

In general, for lipids that exhibit two upper miscibility critical points when mixed with Dchol in a monolayer, the higher the melting temperature of the phospholipid, the lower $\Pi_{crit}(\alpha)$. The miscibility of the complex is higher with the long-chain phospholipids (e.g., di(16:0)PC) than with Dchol. When two phospholipids are systematically mixed in different proportions with Dchol, a low $\Pi_{crit}(\alpha)$ is correlated with a high $\Pi_{crit}(\beta)$. For example, in Figure 2 $\Pi_{crit}(\alpha)$ decreases and $\Pi_{crit}(\beta)$ increases when di(16:0)PC is increased with respect to di(14:0)PC. Figure

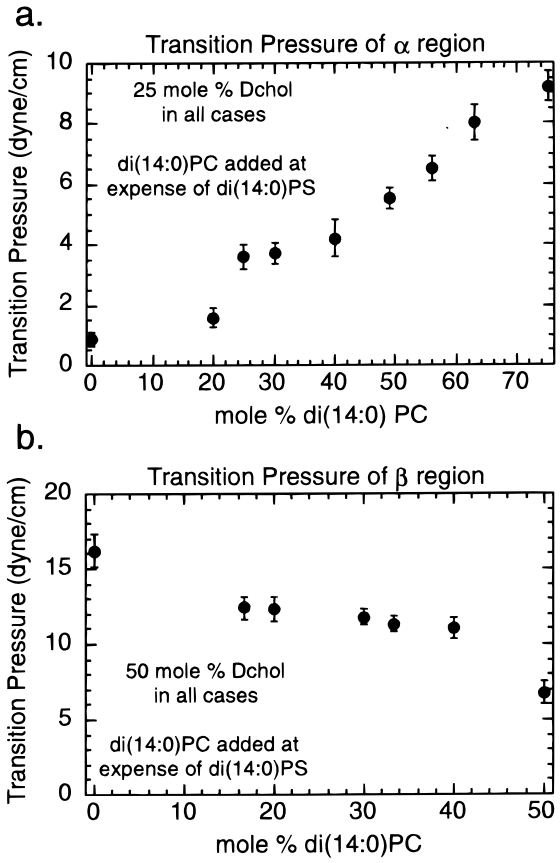


**Figure 3.** Transition pressures for the α region (3a) and $\beta$ region (3b) of Dchol−phospholipid monolayers with fixed Dchol concentration and varying ratios of di(14:0)PC and di(14:0)PS. Transitions were between two coexisting liquid phases at low surface pressure and one liquid phase at high pressure. The data correspond to roughly linear plots of transition pressure vs $\bar{T}_m$.

3 demonstrates the same result for a ternary mixture of di(14:0)PC, di(14:0)PS, and Dchol for which transition pressures of the α and $\beta$ regions were measured at 25 and 50 mol % Dchol, respectively. As di(14:0)PC was added at the expense of di(14:0)PS, the average of the bilayer melting temperatures of the phospholipid mixture decreased, $\Pi_{crit}(\alpha)$ increased (Figure 3a), and $\Pi_{crit}(\beta)$ decreased (Figure 3b).

There is also an approximate correlation between the $\bar{T}_m$ of the phospholipid component of phospholipid−Dchol mixtures and the cusp composition of the phase diagrams, as shown in Figure 4. The stoichiometric composition of the phospholipid−Dchol complexes ($q/p + q$) is closely related to the cusp composition. Theoretical studies show that for binary mixtures where one complex is formed, the cusp composition is almost exactly the stoichiometry of the complex, whereas in multicomponent mixtures with more than one phospholipid and more than one complex, the relationship may be less well-defined.[20] Figure 4 shows that the approximate correlation between the $\bar{T}_m$ and the cusp composition holds equally for mixtures containing one or more phospholipid. So, it is reasonable to assume that the $\bar{T}_m$ also correlates with the stoichiometric composition ($q/p + q$) of the complex. For higher $\bar{T}_m$, less cholesterol is needed to order the phospholipid acyl chains, which corresponds to lower values of both the cusp composition and the complex stoichiometry.

The complexes between phospholipids and Dchol which give rise to two upper miscibility critical points are termed "condensed" complexes because the average molecular area often is a minimum at a Dchol concentration near the cusp in the

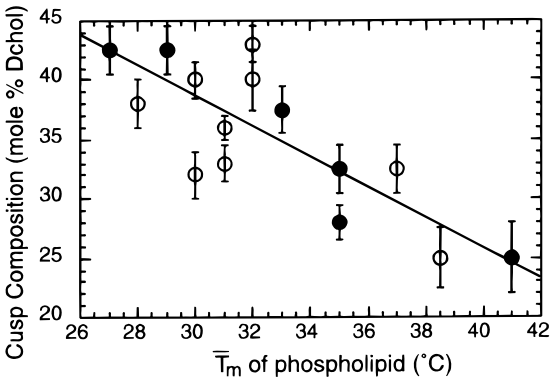


**Figure 4.** Concentration of Dchol at which a cusp is observed between the α and $\beta$ two-phase regions in the phase diagrams vs melting temperatures for various phospholipid mixtures obtained from Table 1. Filled circles represent mixtures containing Dchol and just one phospholipid, while the open circles represent mixtures containing Dchol and more than one phospholipid.

phase diagram between the α and $\beta$ regions. The concentration of complex is a maximum near the cusp.[12] Sharp breaks in the molecular area are seen in Figures 5c and 5d for Dchol mixed with either of the asymmetric, saturated phospholipids (14:0−16:0)PC or (16:0−14:0)PC. A minimum in the molecular area was not seen for Dchol and di(15:0)PC (data not shown). In general, a minimum in average molecular area as a function of composition requires that the molecular area of the complex be small relative to other components, and that the concentration of the complex be large and peaked at the stoichiometry of the complex. Failure to observe a minimum experimentally is most likely due to a combination of these factors. Figure 6 displays more phase diagrams of Dchol mixed with phospholipids. Only one upper miscibility critical point is observed in monolayers of Dchol and diphytanoylPC (Figure 6a), whereas two are observed for Dchol and di(12:0)PE (Figure 6b), and 1/1 di(14:0)PC/di(14:0)PE (Figure 6c).

A mixture of Dchol with 1/1 racemic di(14:0)PC and racemic di(16:0)PC has a phase diagram indistinguishable from that of the corresponding L enantiomeric phospholipids in Figure 2c. Note that Figure 2c is very similar to Figures 5a and 5b. The phospholipids in Figure 2c are a 1/1 mixture of di(14:0)PC and di(16:0)PC, whereas in Figures 5a and 5 they are asymmetric lipids with chain lengths of 14:0 and 16:0 in either the *sn*-1 or *sn*-2 position, respectively. As discussed earlier, the concentration of Dchol at which the cusp between the α and $\beta$ regions is observed relates to the stoichiometry of the phospholipid and Dchol in the complex (see eq 1 and refs 12 and 20). The cusp composition of Dchol is most similar in Figures 2c and 5b. If complex formation is stronger between Dchol and a 16:0 acyl chain than a 14:0 acyl chain, then the shifting of the cusp composition suggests that the ability of a phospholipid to form a complex with Dchol is more strongly influenced by the lipid's *sn*-1 chain than by its *sn*-2 chain. At the very least, comparison of Figures 5a and 5b leads to the conclusion that the stoichiometry of the complex in binary phospholipid−Dchol mixtures is a sensitive function of molecular structure.

**Microscopy.** Domains found in the middle of the α region are large and have good contrast. Assuming that the α region arises from immiscibility between the phospholipid and complex, white domains are rich in phospholipid and TR-DMPE, and black domains rich in complex.[13] For low concentrations of Dchol, black domains on a white background are observed, whereas at high concentrations of Dchol the contrast is reversed. At extremes of Dchol concentration, domains become small and

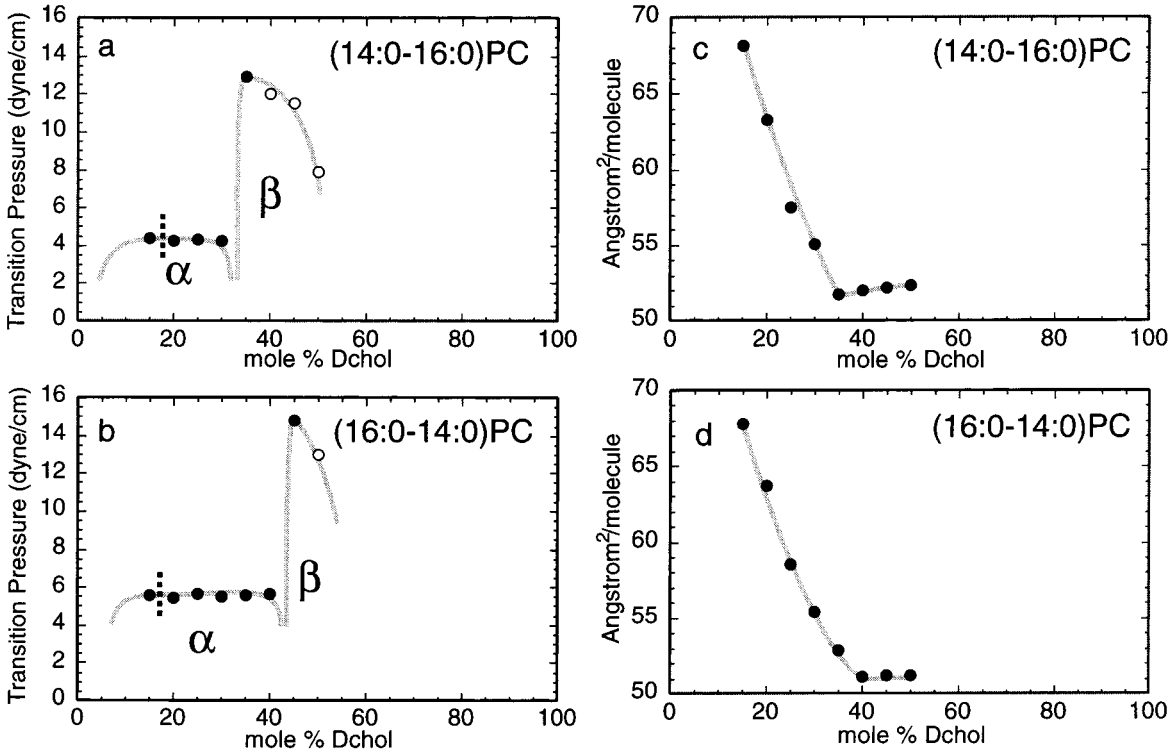


**Figure 5.** Phase diagrams for monolayers of Dchol with (a) (14:0−16:0)PC and with (b) (16:0−14:0)PC. Phase boundaries are between two coexisting liquid phases at low surface pressure and one liquid phase at high pressure. Filled symbols vs open symbols denote the observation of stripe domains vs no stripe domains at the phase boundary where the two phases merge. For the α regions, critical points are at Dchol concentrations 17.5 ± 2% for both (a) and (b), and are marked by dotted lines in the phase diagrams. For the $\beta$ regions, the critical compositions are presumably (a) 35 ± 2 and (b)45 ± 2 mol % Dchol. Figures (c) and (d) show average molecular areas for the lipid mixtures in (a) and (b) at a pressure of 2 dyn/cm.

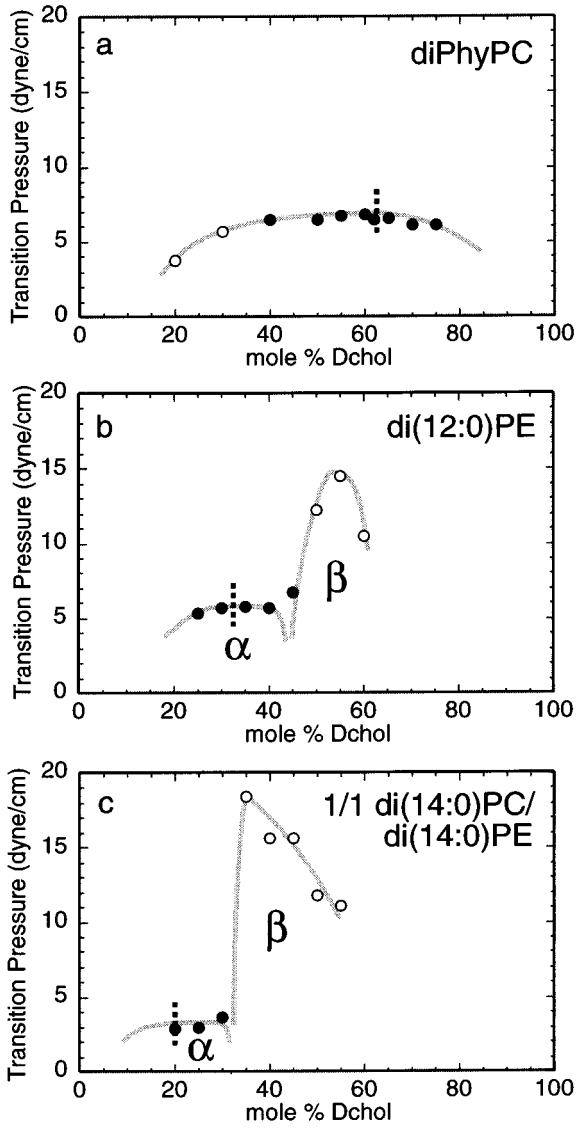


**Figure 6.** Phase diagrams for monolayers of Dchol and (a) diphytanoylPC, (b) di(12:0)PE, and (c) 1/1 di(14:0)PC/di(14:0)PE. Phase boundaries are between two coexisting liquid phases at low surface pressure and one liquid phase at high pressure. Filled symbols vs open symbols denote the observation of stripe domains vs no stripe domains at the phase boundary where the two phases merge. Phase diagrams in (b) and (c) show two upper miscibility critical points. For the α regions, critical concentrations are at (a) 63 ± 1, (b) 32.5 ± 2, and (c) 20 ± 4 mol % Dchol and are marked by dotted lines in the phase diagrams. For the $\beta$ regions, the critical compositions are difficult to determine. In (a), the concentration of Texas Red dye used varied from 0.06 to 0.2 mol % whereas in (b) and (c) it was set at 0.4 mol %.

difficult to see. At the critical point of the α region, the areas of black and white phases are equal. It should be stressed that although the domains are always in shape equilibrium, only when the system is close to the critical point is the stripe width uniform.[9]

The $\beta$ region often appears quite different from the α region. Domains are much smaller and have poor contrast.[13] The contrast deteriorates as the surface pressure increases, as would be expected from the narrow peaks of the $\beta$ region phase diagrams. Assuming that the $\beta$ region arises from immiscibility between the complex and Dchol, white domains are thought to be rich in complex and TR-DMPE, and black domains rich in Dchol.[13] Given this, we would expect that for low concentrations of Dchol within the $\beta$ region that there should be a majority of white domains. Instead, for the lipid systems listed in Table 1,

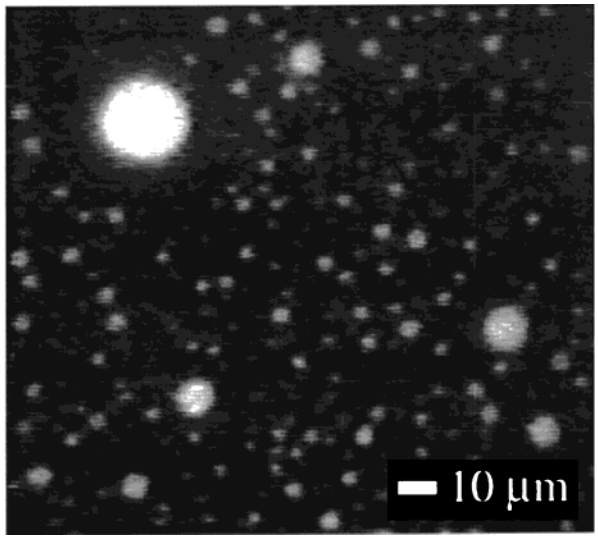


**Figure 7.** Micrograph captured from video of the $\beta$ region for a lipid mixture of 1/1 di(14:0)PC/di(14:0)PE with 35% Dchol. As seen in Figure 6c, at 35% Dchol the lipid mixture is barely within the $\beta$ region.

a majority of white phase is never observed in the $\beta$ region. For example, Figure 7 is a micrograph from the low-Dchol edge of the $\beta$ region of 1/1 di(14:0)PC/di(14:0)PE with 35% Dchol, as seen from the phase diagram in Figure 6c. Even at the low-Dchol edge of the $\beta$ region, the black background dominates over small white domains. We presume that many of the white domains are too small to be resolved. In general, white domains of the $\beta$ region are largest, brightest, and most numerous near the left boundary of the $\beta$ region and at an intermediate surface pressure.

Since the $\beta$ region always consists of small white domains on a black background, and since contrast deteriorates with increasing Dchol concentration, it can be difficult to distinguish the $\beta$ region from the high-Dchol part of the α region unless the critical pressure of the $\beta$ region is greater than that of the α region. It may be the case that when mixed with Dchol, phospholipids with intermediate melting temperatures (e.g., di(14:0)PC) produce phase diagrams which consist of an α region on the left and a $\beta$ region with a low critical pressure on the right rather than only one skewed two-phase region as in Figure 1c.

Additional experiments were performed to confirm the assignment of which lipids are prevalent in the white and black domains. An electric field gradient was applied in the plane of the monolayer as described previously.[13,14] In all cases, the white phase is attracted to a positive electrode. This holds if the dipole density of Dchol > complex > phospholipid. Calculations have shown that experiments are consistent with dipole densities of 2.9 D/100 Å$^2$ for Dchol, 2.7 D/100 Å$^2$ for the complex, and 1 D/100 Å$^2$ for phospholipid.[13]

Some dyes are thought to partition preferentially into condensed or gel phases of bilayers and therefore may partition preferentially into the condensed complex rather than into the phospholipid-rich phase.[21,22] The dye TR-DMPE was replaced by either diI C18(3), rhodamine C18, Bodipy gluco-cerebroside, or NBD-chol, and the phase behavior was investigated for lipid systems which had either one miscibility critical point (di(14:0)PC and Dchol) or two miscibility critical points (1/1 di(14:0)PC/di(16:0)PC and Dchol). In all cases, the distribution of white and black phases was just as it was for the dye TR-DMPE. As before, the white phase was always attracted to a positive electrode. Hence, the condensed complex accommodates very little impurity and the phase rich in Dchol accommodates even less.

## Discussion

The appearance of two upper miscibility critical points in mixtures of phospholipid and Dchol correlates with a high melting temperature of the corresponding phospholipid bilayer. Melting temperature is a convenient measure of the tendency of the phospholipid acyl chains to order. The significance of this result is in the trend rather than the numerical value of the bilayer melting temperature.

Assuming that the phase behavior of two upper miscibility critical points results from formation of a condensed complex, then the gain in free energy from complex formation increases as the melting temperature of the phospholipid increases. Indeed, the van der Waals contacts between cholesterol and the C2−C9 segment of the phospholipid chains are thought to be strongest when the lipids are saturated.[23,24] Experiments measuring the oxidation rate of cholesterol suggest that it is sequestered more tightly with longer chain saturated lipids.[25]

The cusp composition (which relates to the stoichiometry of the complex) also correlates with the phospholipid $\bar{T}_m$. The stoichiometry is sensitive to the phospholipid $\bar{T}_m$ since it is determined by the amount of cholesterol required to condense the phospholipid acyl chains. Thus, the $\bar{T}_m$ of the phospholipid correlates with both the formation and composition of these Dchol−phospholipid complexes.

The observations associated with condensed complexes in monolayers have similarities to those discussed in connection with the superlattice model and liquid ordered phases in bilayers. A more detailed discussion is found in reference 12 and references therein. The superlattice model has been used to interpret the observations of sharp changes in membrane behavior at distinct stoichiometries.[26] Liquid ordered phases have been reported in membranes containing cholesterol and are likely to contain lipids with high melting temperatures.[27] Similarly, "detergent-resistant membranes" or rafts harbor saturated lipids and proteins attached to long-chain saturated lipids with high melting temperatures.[28−32]

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