Published in final edited form as:
Annu Rev Biophys Biomol Struct. 2007 ; 36: 63–77. doi:10.1146/annurev.biophys.36.040306.132721.

Phase Boundaries and Biological Membranes

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Abstract

Bilayer mixtures of lipids are used by many researchers as chemically simple models for biological membranes. In particular, observations on three-component bilayer mixtures containing cholesterol show rich phase behavior, including several regions of two-phase coexistence and one region of three-phase coexistence. Yet, the relationship between these simple model mixtures and biological membranes, which contain hundreds of different proteins and lipids, is not clear. Many of the model mixtures have been chosen for study because they exhibit readily observed phase separations, not because they are good mimics of cell membrane components. If the many components of cell membranes could be grouped in some way, then understanding the phase behaviors of biological membranes might be enhanced. Furthermore, if the underlying interaction energies between lipids and proteins can be determined, then it might be possible to model the distributions of lipids and proteins in a bilayer membrane, even in complex mixtures.

Keywords

multicomponent mixture; phase diagram; ternary mixture; thermodynamics of mixing

INTRODUCTION

The thermodynamics of mixing is a powerful way to understand and to predict behaviors of molecules in mixtures. The nonrandom mixing of components in a mixture directly reflects differences in the interaction energies between molecules and the tendency of each component to react or to bind to other molecules. Because biological membranes are physical mixtures of lipids and proteins, we might benefit from the potential power of a thermodynamic treatment. But with hundreds of lipids and hundreds of proteins, the complexity of biological membranes is daunting.

First we briefly consider the nature of nonrandom mixing within the milieu of cell membranes. Then we consider what types of experiments on mixtures can make use of the power of thermodynamics. The goal of discovering which molecules cluster together, including the size of the clusters and even macroscopic phase separation, can be achieved by finding the experimentally determined phase boundary in chemically simple model mixtures. We will see that this area of research is at an early stage, especially regarding the thermodynamic analysis, but at this time sufficient data have indicated some important characteristics of lipid mixing.

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CELL MEMBRANE HETEROGENEITY: FUNCTION, MOTION, AND STRUCTURE

For many years now, investigators have concluded that animal cell membranes show compositional heterogeneity over the membrane surface. A number of recent reviews support this conclusion (1a,10,11,15,22-24,26,39). In one type of experiment, the question asked is whether membranes of living cells reveal any sort of compositionally distinct domains to proteins, as measured by protein functional assays. The answer is that changing the anchoring motif of a membrane-bound protein by removing acylation (19), changing the type of acylation (34), or substituting a transmembrane peptide anchor for acylation (19) results in loss of function. These observations are consistent with, but do not prove, the finding that the changed protein anchor binds to a compositionally distinct area of the membrane.

In another experiment with cells, translational diffusion of labeled proteins or lipids is examined. For several decades, investigators had observed that the proteins of animal cell plasma membranes move in the two-dimensional plane of the membrane in non-Brownian ways. At timescales of milliseconds to seconds, nonrandom molecular movements show up as a fraction of the molecules that seem not to diffuse, as a dependence of the diffusion coefficient on the size of the area studied, or as “transient confinement zones” (2,9,24). The molecular-level explanation for these nonrandom movements improved significantly when Kusumi and coworkers (22,23) achieved microsecond time resolution for tracking the diffusion of both proteins and lipids. They found that membrane-bound molecules spend some time and undergo some translational motion within small areas, followed by similar restricted motion in an adjacent small area. They termed this hop diffusion. The size of the areas depends upon cell type and ranges from 5 to 200 nm in diameter. The finding that a labeled lipid is transiently confined in this way might imply that each small patch of membrane is partially isolated from adjacent areas, at least with regard to lipid translational motion, but perhaps also partially insulated with regard to lipid phase behavior. An implication is that an “average lipid composition” of a biological membrane could be misleading and that an ensemble of patches having different compositions and different phase behaviors should be considered.

Direct measurement of compositional heterogeneity in animal cell plasma membranes shows up on a distance scale of tens of nanometers in fluorescence resonance energy transfer (FRET) measurements of labeled proteins (25). Particular proteins formed relatively well-defined small clusters in separated domains. Using electron microscopy, several researchers have directly observed gold-labeled proteins clustering together in distinct domains (30,49).

In summary, observations on cells of protein function, of protein and lipid translational diffusion, and of protein-protein colocalization are all consistent with plasma membranes having numerous regions that are compositionally distinct. One contribution to this heterogeneity might come from the nonrandom mixing of the lipids and the proteins. One way to explore the mixing is to study chemically well-defined mixtures.

THE PROBLEM OF MANY COMPONENTS

Because of both experimental and theoretical difficulties, only simple mixtures of three or four components can be treated rigorously. Following this practical way of thinking, some investigators have emphasized the small number of “main lipid components” in each leaflet of a biomembrane (12,36-38,42-46). In this manner, the outer leaflet of an animal cell plasma membrane could be regarded as a mixture of predominantly sphingomyelin (SM), phosphatidylcholine (PC), and cholesterol (chol); the inner leaflet as a mixture of predominantly phosphatidylethanolamine (PE), phosphatidylserine (PS), and cholesterol. Yet, we should examine whether the simplification of studying a small number of components can

capture the essential features of a complex mixture: Those who work with lipids know that just one double bond or two methylene groups make a world of difference in lipid behaviors. The complexity of cell membranes is great—van Meer (41) has described the cell's “lipidome” as having over a thousand different lipids.

Once we explicitly recognize the large number of components of a biological membrane, how might we treat such a complex system? An ambitious, long-term goal, framed in terms of the thermodynamics of mixing, would be to create a physical picture of the membrane proteins and lipids that makes clear all significant patterns of contacts of like and unlike molecules.

ANALYSIS STRATEGY

In order to formulate an overall strategy for treating a mixture of hundreds of components, we follow the lead of the geologists. These researchers wrestled long ago with this very problem of complexity in mixtures having many components, because rocks—whose composition and phase states can be determined—manifest the phases of complex multicomponent mixtures that formed under particular conditions of composition, temperature, time, and pressure (28). In the earlier years, some geologists argued that rocks are far too complex to be represented simplistically, as mixtures of a small number of components. Yet the success of physical chemists was abundantly clear in routinely representing phase behavior of three-component mixtures by use of triangular phase diagrams. Geologists soon found that if rocks of granite or basalt were modeled as far more simple mixtures than they actually are, then the processes of rock formation could be understood much better. A key simplification in the model phase diagrams was to group related classes of the components so that the phase behavior could be described as that of a pseudo-three-component or, more rarely, four-component mixture (28).

Perhaps we can apply this same approach to the study of biological membranes. The phase diagrams for various three-component lipid bilayer mixtures can be used to characterize the phase behavior of particular mixtures. As to the useful groupings of the many different lipids, we would like to know which lipid groups give rise to the same characteristic type of phase behavior. So far, there have been few achievements along this line. As an example of the problem, even with the same lipid headgroup of glycerophosphocholine, the PCs exhibit vastly different phase behaviors, alone or in mixtures, depending upon whether the acyl chains have one double bond or none, or 12 or 14 carbons. The rules for grouping lipids by mixing and phase behaviors are a work in progress. In this review, we attempt to organize phase data obtained so far on three-component lipid mixtures containing cholesterol.

This use of phase diagrams, although but one aspect of a larger area of phase science, has a special role in its application to understanding biological membranes, a role that is not filled by other useful studies of membrane phases. This role is to provide the framework for treating even very complex mixtures of both lipids and proteins, on the basis of the additive property of the free energies. This scheme is familiar, for example, from tables of standard reduction potentials, which enable a relatively small number of equilibrium thermodynamics measurements to be combined in pairs to describe many combinations of the component reactions. Other, different aspects of phase science can be important for understanding biological membranes, for example, whether the detailed nature of a phase transition is first order or continuous (14). Or, the detailed characterization of phase properties such as translational diffusion and bending modulus might well be the keys to answering some questions, but these studies contain information that is different from that of the phase diagrams (7,26,27,46).

EXPERIMENTAL ISSUES

Finding the phase boundaries is especially challenging for lipid bilayer samples because coexisting phases in a bilayer membrane cannot be physically separated from each other and then analyzed for chemical composition, as is the usual procedure for bulk system phase studies (13). Instead, new kinds of experiments have been required for finding phase boundaries in bilayer lipid mixtures. The key experimental technique that led to much of the recent phase diagram results was fluorescence microscopy imaging of giant unilamellar vesicles (GUVs), which in favorable cases reveals both the existence of any coexisting phases and their phase identity (1,3,21). Still, fluorescence microscopy has some deficiencies, including failure to detect domains that are too small or that show too little contrast with a particular dye; difficulty to detect some boundaries accurately (disappearance of a small amount of the minor phase); poor equilibration, especially for samples containing large fractions of solid phase; artifactual light-induced domains (4) (see Sidebar); and difficulty to determine the relative fractions of any coexisting phases.

For constructing phase diagrams, the experimental measurements require near-equilibrium conditions. In practice, phase regions that include a solid can be slow to equilibrate. Whereas cooling rates of ~5°C h^{-1} are slow enough for the liquid phases, even a 10-fold-slower cooling rate can trap nonequilibrium behavior of solids. Some investigators might choose to ignore the solid phases as biologically irrelevant, focusing instead on the interesting coexistence of the two immiscible fluid bilayer phases, Lα + Lo. This is unfortunate, because then any phase regions that do contain a solid are more likely to be wrongly mapped on the diagram, for example, wrongly included in regions labeled as just the two coexisting phases, Lα + Lo.

An overarching issue is that for aqueous suspensions of lipid bilayers, lipids initially dissolved in organic solvent must have the solvent removed and be transferred into water to form the bilayer phases without trapping any slow-to-equilibrate states. The most notorious such nonequilibrium state gives rise to the artifactual precipitation of crystals of cholesterol monohydrate (17). Three approaches to solving this problem are to heat the lipid during solvent removal (27), remove a volatile organic solvent rapidly in the presence of the aqueous phase (so-called rapid solvent exchange) (5), or lyophilize a lipid mixture from solid chloroform (17). Other common problems of sample preparation and equilibration include trapping ill-defined solid states that exclude fluorescent dyes and thus show up as black regions in GUVs, light-induced lipid cross-linking leading to artifactual lipid immiscibility (4), and failure to measure sample concentrations in stock solutions (relying instead on nominal concentrations from the lipid supplier).

EXPERIMENTAL STRATEGY

Phase diagrams for three-component lipid mixtures can show rich behavior, or they can be featureless in every direction in composition space. Well-defined phase boundaries are a must, since simulation of phase boundaries is the link between a model that describes how the components are distributed and the thermodynamic data. Already, some mixtures have been studied over all composition space, and many others at only a few compositions. Given what we currently know, we might be ready to understand a significant range of lipid types, including even many-component membranes. At the very least, sufficient lipid mixture phase data have been reported to make an overview useful.

Simple lipid mixtures can show several different compositional regions of phase coexistence. This is not to imply that such mixtures are a priori good models for animal cell plasma membranes. Indeed, such interesting mixtures are chosen for detailed study not because the lipid components are the best representative molecules from a biological perspective, but rather because they present us with some key phase boundaries that are especially easy to detect.

Measuring distinct phase boundaries is an important practical consideration, because reliable data interpretation for many different sample compositions is needed in solving rich behavior. So far, the methods that have been used with success to solve the three-component phase diagrams include fluorescence microscopy imaging of GUVs, NMR spectroscopy, FRET, X-ray diffraction, and dilute dye fluorescence spectroscopy (12,42-46). Other methods of proven usefulness for characterizing lipid phase behavior, such as electron spin resonance (ESR) spectroscopy, calorimetry, and fluorescence quenching, are likely to provide data for solving the three-component phase diagrams.

The phase behavior of several three-component mixtures containing cholesterol has been thoroughly studied, and the phase diagrams have been solved. These are the mixtures dipalmitoylphosphatidylcholine (DPPC)/dilauroylphosphatidylcholine (DLPC)/chol, DPPC/dioleoylphosphatidylcholine (DOPC)/chol, DPPC/diphytanoyl-PC/chol, and distearoylphosphatidylcholine (DSPC)/DOPC/chol (12,42-46; J. Zhao, J. Wu, P. Klawitter, T.T. Mills, G. Huang, et al., manuscript submitted). In addition, a number of other three-component mixtures have been studied less extensively but still provide useful phase information. In the most reliable phase studies, at least two different methods have been employed and found to be in agreement. When using fluorescence microscopy, the most decisive experiments employ two dyes in each sample, each dye with different phase-partitioning properties. The use of two dyes having complementary phase-partitioning behavior facilitates recognition of nonequilibrium domains and adventitious binding of lipid to the GUVs, each of which can be mistaken for phase separation (12).

BILAYER ARTIFACTS MIGHT BE USEFUL

Domains appear as artifacts when samples are cooled too quickly, exposed to light and oxygen, or after cross-linking of the lipids, implying that these mixtures that contain cholesterol, even when apparently a single phase, are not far from phase separation. Such nonequilibrium behaviors are not a proper subject for analysis by use of equilibrium thermodynamics. Yet there might well be information in this plethora of artifacts: Many simple lipid mixtures seem ready to phase separate, perhaps with just a little help. One might reasonably infer that biological membranes in cells, although not at equilibrium, might themselves be subject to influences that would induce phase separation.

GROUPING OF PHASE BEHAVIORS

Upon review of the phase studies of three-component lipid mixtures containing cholesterol, a striking result emerges: The phase behavior observed so far for a number of different mixtures can be placed onto only two different types of phase diagrams. Consider the schematic phase diagrams in Figure 1. With regard to macroscopic phase separations, and with a caveat that not all of the phase studies have covered all of composition space, there are two different patterns of observations of the many different three-component mixtures. The question naturally arises as to whether there is a particular, well-defined sort of interaction between the lipids that is controlling the phase behavior.

THE TYPE I PHASE DIAGRAM

These mixtures show no region of macroscopic Lo phase domain separation. Type I behavior is observed for the following mixtures: DPPC/DLPC/chol, DPPC/palmitoyl, oleoyl-phosphatidylcholine (POPC)/chol, DSPC/DLPC/chol, DSPC/POPC/chol, DSPC/stearoyl, oleoyl-phosphatidylcholine (SOPC)/chol, and brain-SM/POPC/chol (12; G.C. Hunt, C. Barry, G. Costanza & N. Jain, unpublished observations). For all of these mixtures, a region of Lα + Lβ coexistence is observed at the lower cholesterol mole fractions from χ_{chol} 0 to ~0.16. The

macroscopic phase coexistence then terminates abruptly at a cholesterol concentration χ_{chol} ~0.16, with this upper boundary being nearly horizontal. The flat appearance of this boundary is consistent with the two-phase coexistence region Lα + Lβ being interrupted by the appearance of a new phase, which in this case would be Lo. However, no macroscopic domains of Lo phase are observed in fluorescence microscopy.

FRET measurements using donor C18:2-DiO and acceptor C20:0-DiI indicate that at cholesterol concentrations χ_{chol} from 0.16 to 0.25, the dyes separate from each other. This FRET behavior was found in three cases, DPPC/DLPC/chol, DPPC/POPC/chol, and brain-SM/POPC/chol, but not examined for the other mixtures (12; G.W. Feigenson & G.C. Hunt, unpublished observations). These observations imply that nanoscopic domain separation occurs, even though macroscopic phases are not visible by optical microscopy.

In these Type I mixtures, cholesterol has essentially the same solubility in the coexisting Lα and Lβ phases. A slight preference for Lβ to Lα was measured by ESR spectroscopy (6). This finding of similar solubility of cholesterol in the two phases corresponds to thermodynamic tielines that are nearly horizontal when the phase diagram triangle is oriented with cholesterol at the top, and indicates that the interaction of cholesterol with its neighboring lipids is not different in energy for chol-DPPC compared with chol-DLPC or chol-POPC, or for chol-brain-SM compared with chol-POPC.

For a variety of PCs and for brain-SM, cholesterol monohydrate crystals separate from Lo phase at a cholesterol concentration $\chi_{chol} = 0.67$ (17,32; J.T. Buboltz, unpublished observations). This corresponds to the upper region of two-phase coexistence.

THE TYPE II PHASE DIAGRAM

These mixtures exhibit more regions of macroscopic immiscibility than do the Type I mixtures, with at least three regions of two-phase coexistence and one region of three-phase coexistence. The mixtures showing Type II behavior are DPPC/DOPC/chol, DPPC/diphytanoyl-PC/chol, DSPC/DOPC/chol, brain-SM/DOPC/chol, stearoyl-SM/DOPC/chol, palmitoyl-SM/DOPC/chol, DPPC/di-C10:0-PC/chol, diC20:0-PC/DOPC/chol, and egg-SM/DOPC/chol (42-46; J. Zhao, J. Wu, P. Klawitter, T.T. Mills, G. Huang, et al., unpublished observations). The first six of these mixtures have been examined at many different compositions, so at least the general form of the phase behavior has been reliably determined.

A general characteristic of these mixtures, revealed in the shape of the low-cholesterol region of Lα + Lβ coexistence, is that cholesterol is less well accommodated in the fluid lipid Lα phases that are rich in di-phytanoyl-PC, DOPC, or di-C10:0-PC than in the coexisting Lβ phases that are rich in DPPC, DSPC, SM, or di-20:0-PC. For example, in the DOPC-rich Lα phase compared with the coexisting DSPC-rich Lβ phase, cholesterol is approximately 2.5 times more concentrated in the Lβ phase, corresponding to a 2.5-fold-higher chemical potential in the Lα phase. This higher cholesterol chemical potential in the DOPC-rich Lα phase is consistent with the observations in these mixtures of a separated phase of DOPC-poor Lo. We infer that the DOPC-chol interaction is unfavorable compared with, for example, the DSPC-chol interaction or the POPC-chol interaction.

These Type II phase diagrams show that as cholesterol is added to the mixture the two-phase region of Lα + Lβ coexistence is interrupted by the formation of the Lo phase, giving rise to the region of three-phase coexistence, Lα + Lβ + Lo. What is happening in this region is that the Lo phase is forming at the expense of the Lβ phase as cholesterol concentration increases: When sufficient cholesterol has been added, the Lβ phase is gone, and only the two phases Lα and Lo remain. In terms of the molecular interactions, a phase diagram with a closed-loop

miscibility gap is not different from the Type II diagram; it simply results from the melting of the solid phase at the higher temperature (42).

Some published reports describe a region of two-phase coexistence of Lβ + Lo in binary mixtures of PC/chol or SM/chol (47), whereas others have found the Lβ phase to change to Lo without a first-order transition (14). A deeper understanding of the nature of these phase transitions might provide insight as to whether the composition of a patch of a cell membrane could change abruptly or instead continuously. Moreover, most of these model studies, including those reported here, involve symmetrical bilayers, in which each leaflet has the same composition. A better model for cell membranes would be asymmetric, with each leaflet having a different composition. However, experimental difficulties have not all been solved for preparation and study of asymmetric bilayers (31,33,50).

ANALYSIS OF PHASE BOUNDARY DATA

One strength of the thermodynamic point of view is that free energies can be tabulated and combined to provide predictive power—here, the power to predict the way in which mixture components stay apart, cluster, or even separate into large domains. For such a complex mixture as a biological membrane, which contains hundreds of different proteins and hundreds of different lipids, we would benefit from an approach that provides a systematic way to handle complexity. For a lipid component of a mixture, the necessary information lies partly in the structure of the molecule of interest. In addition, crucial information is in the nature of the other mixture components and their concentrations. For these reasons, analysis by means of Monte Carlo simulations is attractive: The basic unit of information is the energy assigned to each intermolecular interaction, and the method inherently takes into account the properties of both a particular molecule and its neighbors (18). We are only at the early stages of treating complex mixing observations with Monte Carlo analysis.

The phase behavior of a mixture is determined by a summation of all of the interaction energies, with the equilibrium distribution having the lowest free energy. In a Monte Carlo simulation, each interaction is assigned an energy, which determines the probability of that interaction occurring in the lowest free-energy distribution of the components. For a given mixture, the sum of all the interaction energies depends upon the concentration of the components. A systematic way to keep account of the sum of all the interactions is to picture each lipid, or each lipid chain, on a triangular lattice, with six nearest neighbors in the case of phospholipids (16). In a case in which the energy depends only upon which two molecules (or acyl chains) are in contact, a so-called pairwise interaction, the calculation is not difficult, even for a three-component mixture. For example, for DSPC/DOPC/chol there are three like-like interactions (which do not contribute to the nonrandom aspects of the mixing), DSPC-DSPC, DOPC-DOPC, and chol-chol. Then there are the three pairwise unlike interactions, DSPC-DOPC, DSPC-chol, and DOPC-chol.

In a comparison of the two related mixtures, Type II DSPC/DOPC/chol and Type I DSPC/POPC/chol, we can begin to consider how to assign energies to each of the pairwise interactions in order to explain the different phase diagrams. The like molecule interactions, none of which contribute to nonrandom mixing, are DOPC-DOPC, DSPC-DSPC, POPC-POPC and chol-chol. In a relative and simplistic way, we could describe the unlike interactions: DOPC-DSPC is unfavorable compared with POPC-DSPC; DOPC-chol is unfavorable compared with POPC-chol; the other pairwise interaction, DSPC-chol is the same in the two mixtures. We then see a driving force to diminish the unfavorable interactions in DSPC/DOPC/chol by formation of DOPC-rich and DOPC-poor phases in this Type II mixture, but less driving force to form separated phases in the Type I mixture DSPC/POPC/chol. Thus, considering only the pairwise

interactions, we see a diminished driving force to form a POPC-rich phase and a POPC-poor phase, compared with the DOPC-containing mixtures.

Recently, Juyang Huang has performed Monte Carlo simulations of the phase behavior of DSPC/DOPC/chol (J. Huang, unpublished observations). He found that assigning appropriate energies to all of the pairwise interactions could yield two-phase coexistence of Lα + Lo. In the simulations, large, DOPC-rich domains formed, in equilibrium with domains poor in DOPC but rich in DSPC and chol. However, by assigning energies only to the pairwise interactions, the upper phase boundary of the Lα + Lo region could not be matched well to the experimentally determined boundary location. Experimental and simulated boundaries could be made to coincide if so-called multibody interactions were recognized and assigned their own energy. For example, acyl chain-acyl chain interaction energy depends upon whether the neighbors of an acyl chain are exclusively other acyl chains or a cholesterol (16). In such a case, three rather than two sites on the triangular lattice are involved. Similarly, chol-chol interaction energy depends strongly upon whether the cholesterol is completely surrounded by acyl chains or whether it has other cholesterol neighbors.

One goal of Monte Carlo simulations is to construct a model for any given mixture of lipids and proteins that would show the likelihood of every sort of clustering, ordering, and phase separation. This is the very subject of the thermodynamics of liquid mixtures, and we seek a picture of the nonrandom mixing on every relevant size scale, from dimers to phase separation of unlimited size. We want to predict how any clusters or phase separations change upon change of membrane composition; for example, do particular proteins tend to stay apart in some compositions and come together in others? Can rules about protein clustering behavior be inferred from their structure? Are the lipids separated into compositionally distinct regions? If so, how and what size?

STRATEGY FOR STUDY OF ADDITIONAL LIPID COMPONENTS

Mapping the phase behavior of a three-component mixture over all of composition space requires a great deal of labor. A plan to do this much work for every four-component mixture of interest would greatly limit the number of mixtures that could be studied.

Instead, an alternative strategy might be feasible: (*a*) Start with the solved phase diagram for, e.g., DSPC/DOPC/chol. (*b*) Examine the behavior of the fourth component of interest at a fixed mole fraction of the total mixture, low enough so that no new phase is introduced but high enough to perturb the phase boundaries of the DSPC/DOPC/chol. (*c*) One of the phase boundaries is especially easy to measure precisely—the upper (high cholesterol concentration) boundary of the two-phase coexistence of Lo + Lα. With samples containing DSPC/DOPC/chol, together with the small concentration of the fourth component, find the new location of this phase boundary in composition space.

With this strategy, we do not require that the initial formulation of the pairwise plus multibody interaction energies for the three-component mixture be an absolutely correct, unique description of the energetics. Rather, we have the more modest aim to have a “good” description of the interactions in this region of composition space for DSPC/DOPC/chol used as a reference mixture. Then, relative to the energies found in the reference mixture, the pairwise interactions are evaluated in order to describe the observed changes in upper phase boundary caused by the addition of a small concentration of a fourth component.

Note that for fourth components that give rise to Type I phase behavior in mixtures with cholesterol plus another phospholipid, little is learned about their interactions with the other mixture components by mapping yet another Type I phase diagram. Instead of mapping the full phase behavior, we measure the influence of an additional component on the upper phase

boundary of Lα + Lo in a Type II reference mixture that has clear, first-order phase transitions, we might recover the set of interaction energies.

HOW TO TREAT MEMBRANE PROTEINS

Some useful studies that treat the interactions of particular membrane proteins with lipid phases have been described (11). Whereas the study of lipid-only phase behavior is fundamentally well-defined in terms of the nature of the experiments and their interpretation, clear treatment is not the rule for approaching the long-standing question of a general approach to treat the phase behavior of the proteins in a bilayer. Sometimes the approach to this question is framed as an almost philosophical choice to either (*a*) determine the phase behavior of the lipids and then measure the behavior of a dilute protein in bilayer mixtures to find out the protein phase preferences (35), or (*b*) focus on the protein as though it can control and change its own lipid environment (2). Such a dichotomy is not necessary. Instead, we can treat the biological membrane as a physical mixture of lipids and proteins, with the detailed nature of the nonrandom mixing controlled by the interaction energies of molecules in contact with each other. The lipid-centric and the protein-centric views are then reconciled by an approach of measuring and then combining the energies for all molecular contacts, even in complex mixtures. For some mixtures, protein-protein and protein-lipid interactions would dominate the clustering and phase behavior, whereas in other mixtures, various lipid-lipid interactions would have the larger role.

Whereas our general understanding of the phase behaviors of lipid-only mixtures has progressed rapidly, the same cannot be said for lipid-protein mixtures. In particular, the influence of various types of membrane proteins on the positions of the phase boundaries of lipid mixtures is not understood. Some researchers even argue that studies of lipid-only mixtures are not relevant to biological membranes because the proteins might be in control of phase behavior (2). An especially interesting problem directly relevant to biological membranes is to determine the phase behavior of lipid-protein mixtures in the presence of high concentrations of proteins. One well-known problem is that increasing the protein or peptide concentration can lead to the disruption of the bilayer phase before any information appears as to the concentration at which protein precipitates, which would be a most useful measurement (8,20), greatly limiting the protein concentration range that can be studied. In stark contrast, many lipids can be examined over their entire concentration range, from mole fraction 0 to 1.0. As an example, increasing the cholesterol concentration all the way up to its separation from the bilayer as crystals of the monohydrate leads to robust bilayers in the Lo phase and stoichiometric precipitation of crystals (at cholesterol mole fractions of 0.50, 0.57, and 0.67), apparently without phospholipidcholesterol complex formation, leading to improved understanding of the thermodynamics of this important membrane lipid (17,18).

One strategy measures how the protein shifts a boundary of coexisting phases previously measured in a lipid-only mixture. This is a well-known method but so far used only for the most simple binary lipid mixtures that do not have sharp, first-order phase transitions and that are not good models for biological membranes (11). An example of how this method might be used (applied to cholesterol rather than to a protein) was to determine the partitioning of cholesterol between coexisting solid and fluid by the shift in the lipid melting temperature (40). For multi-component protein-lipid mixtures, the phase boundaries will also shift in a manner that would depend upon the nature of the various protein-lipid interactions as well as on the protein-protein interactions. The complexity of the interactions is inevitable. The important question whether a particular interaction, or only a small number of interactions, dominates the phase behavior has not yet been explored. The overall goals of such protein-lipid phase studies would include finding the energy of the protein interaction with each type of lipid in a mixture and also the number of energetically significant interactions of the protein

with different lipid neighbors. If patterns are observed, it might be possible to find some of the rules that relate structural features of the protein to lipid binding in the membrane. Moreover, it would be valuable to understand how protein-protein interactions change as a function of the protein concentration and the lipid composition.

RELATIONSHIP OF MODEL STUDIES TO CELL MEMBRANES

The value of reliable experimental determination of phase boundaries is that these can be simulated by finding the lowest free-energy distribution of the mixture components. The value of reliable phase boundaries in model mixtures is not that the boundary locations can be extrapolated to biological membranes. This is premature, especially because the mixtures most often chosen for study are carefully selected for having macroscopic phase separation. We do not yet know whether actual phase separation or merely highly non-random mixing is a good description of biological membranes.

The experimental mixtures that have been studied that yielded the phase boundaries that we describe as Type I or Type II are highly simplified, having three or at most four components, so their direct application to predict the behavior of the vastly more complex biological membranes would require more justification than has yet been convincingly adduced. Rather, at this time, the model mixtures provide only the possibility to obtain, step by step, the underlying interaction energies for many types of lipids and proteins, and only when this is achieved can we derive a picture of the behavior of such a complex mixture.

The first-order phase transition of a lipid bilayer holds a special interest because it is a dramatic, nonlinear response to a small change in composition or temperature. Biology shows us many cases in which a relatively small and defined change results in a dramatic response in structure or function: a change in hemoglobin oxygen affinity when blood cells approach tissue of slightly lower pH; a general reason why histidines are often located in an enzyme active site; and the addition of a single phosphate moiety to a serine or to a tyrosine, turning on the activity of a protein kinase or turning on the binding to a scaffold protein complex. In terms of a small or defined change giving rise to dramatic change of structure or function, the continuous phase transition would seem to provide only a modest change for amplification or for communication over a distance of many lipids. We can imagine that the difference between the first-order and the continuous phase transitions, especially given the fluctuations in energy associated with temperature in the physiological range, might encompass a span of behaviors up to macroscopic phase separation but including clusters having a range of sizes.

We also want to have a complete and general picture of the mixing of all the components of a biological membrane—the hundreds of different lipids and different proteins. Surely this more complete picture could involve phase transitions that are not first-order, so we must say what we can about these. Most generally, we seek a complete picture of the nonrandom mixing, including but not limited to macroscopic first-order phase separations, involving whatever sorts and sizes of clusters.

SUMMARY POINTS

1. The various phase diagrams for several different three-component lipid mixtures containing cholesterol can be grouped into just two categories.
2. A thermodynamic treatment of the phase boundaries could provide the means for simulating the behavior of even many-component mixtures, including mixtures containing membrane proteins.

FUTURE ISSUES

1. On and near the binary mixtures of cholesterol with the solid-type phospholipid, Type I and Type II phase behaviors are not yet fully characterized. Many published reports describe a region of two-phase coexistence of Lβ + Lo, whereas others have found the Lβ phase to change to Lo without a first-order transition.
2. Type I phase behavior has an additional complication: The phase behavior is not simply to form a single phase at cholesterol concentrations higher than those in the well-defined two-phase region of Lα + Lβ. FRET and pyrene excimer/monomer studies are consistent with nanoscopic domains, but this description should be regarded as incomplete without further characterization.
3. These three-component model mixtures of Type I and Type II are symmetrical bilayers, in which two leaflets of the bilayer have the same lipid composition and phase behavior. So far, systematic studies have not been done to describe the phase behavior of asymmetric lipid bilayers, in which each leaflet has different composition. For example, bilayers having one leaflet of SM/DOPC/chol and the other leaflet of PE/PS/chol would be a better model for an animal cell plasma membrane than the Type I or Type II bilayers, but experimental difficulties in preparation of asymmetric bilayers have not been overcome.

ACKNOWLEDGMENTS

The author thanks J. Zhao, J. Wu, and F. Heberle for data in advance of publication. This work was supported by grants from the National Science Foundation (MCB-0315330) and the American Chemical Society (PRF-38464-AC7) to G.W.F.

Glossary

SM, sphingomyelin
PE, phosphatidylethanolamine
PS, phosphatidylserine
GUV, giant unilamellar vesicle
Lα, lamellar phase with disordered acyl chains and rapid translational diffusion
Lo, lamellar phase with ordered acyl chains and rapid translational diffusion
Composition space, the entire range of concentrations of all mixture components
DPPC, dipalmitoylphosphatidylcholine
DLPC, dilauroylphosphatidylcholine
DOPC, dioleoylphosphatidylcholine
DSPC, distearoylphosphatidylcholine
POPC, palmitoyl, oleoylphosphatidylcholine
SOPC, stearoyl, oleoylphosphatidylcholine
Lβ, lamellar phase with ordered acyl chains and slow translational diffusion
Tieline, a line drawn onto a compositional phase diagram that joins the compositions of coexisting phases
Monte Carlo, a general method for calculating probabilities
Triangular lattice, two-dimensional array in which each point in the array has six equally spaced nearest-neighbor points
Pairwise interaction, between two nearest neighbors
Multibody interaction, a grouping of more than two adjacent molecules

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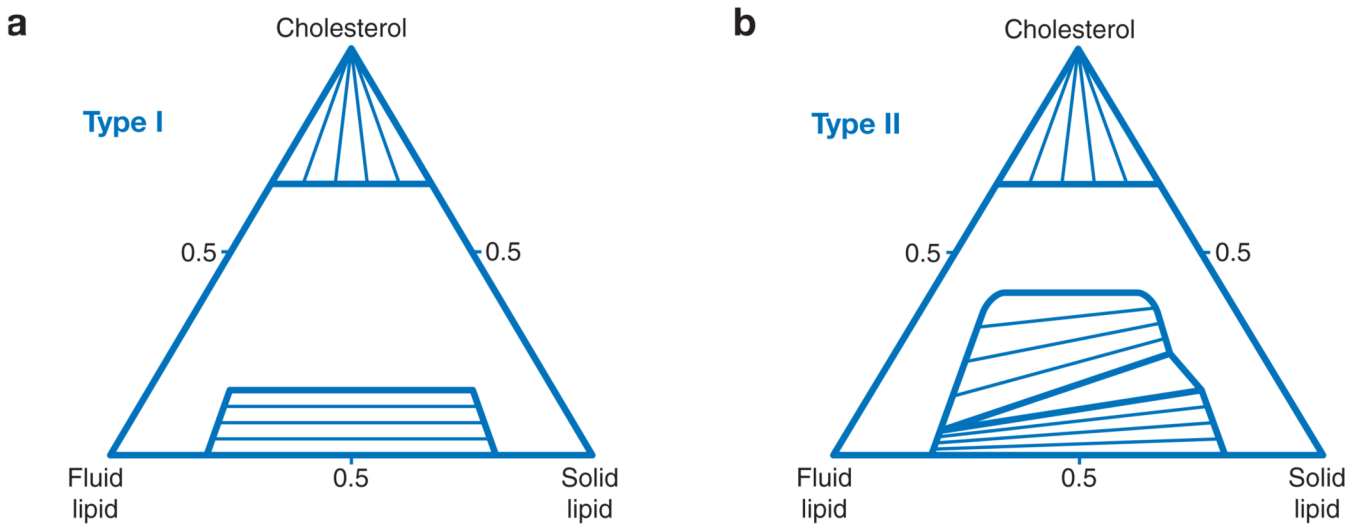

Figure 1.

Only two general types of phase diagrams are needed to describe all of the phase behavior observed so far for three-component lipid mixtures containing cholesterol that show phase coexistence. In addition to cholesterol, the other two components are a lipid whose fully hydrated bilayers are in a gel phase (Lβ or Lβ′) at the measurement temperature (solid lipid), and a lipid whose fully hydrated bilayers are in a liquid-disordered phase (Lα) at the measurement temperature (fluid lipid). (*a*) The Type I phase diagram has two regions of macroscopic two-phase coexistence. At high cholesterol concentrations, crystals of cholesterol monohydrate coexist with the liquid-ordered phase, Lo. At lower cholesterol concentrations, Lα and Lβ phases coexist. Proposed tielines are shown. (*b*) The Type II phase diagram has three regions of macroscopic two-phase coexistence, and one region of three-phase coexistence. In addition to the same two-phase regions seen in the Type I diagrams, there is a large two-phase coexistence region where Lα + Lo phases coexist. In addition, a triangle of three-phase coexistence is also seen in which Lα Lo + Lβ phases coexist. Tielines are shown in all of the regions of two-phase coexistence.