Interleaflet Coupling Mechanisms in Bilayers of Lipids and Cholesterol

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ABSTRACT Whereas it appears to be generally believed that the leaflets of a phospholipid/cholesterol bilayer interact with each other in some way, the exact mechanism remains undetermined. Various suggestions have been invoked, including chain interdigitation and rapid translocation of cholesterol. There is little, if any, direct evidence supporting or excluding these hypotheses. In this letter, I examine a few different possibilities. Chain interdigitation is unlikely to be significant. Cholesterol translocation meets some, though not all, of the relevant criteria, and probably plays an important role. The simplest explanation is that the layers interact at the midplane in the same way that the ordered and disordered liquid phases common in these systems interact at their interfaces. A quick estimate of that interfacial energy shows that this is a very likely candidate. The consequences of such an energy in biological systems are briefly considered.

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It is by now widely known that ternary mixtures of lipids and cholesterol spontaneously self-segregate into two or more phases over a wide range of compositions and temperatures. The primary requirement appears to be that the two lipids have substantially different chain melting temperatures T_m (1). In this letter, I will focus on the coexistence of two liquid phases, generally known as the liquid-ordered and liquid-disordered phases, and in particular, how one leaflet might ''know'' about the presence of one or the other phase in a leaflet opposite the bilayer midplane from itself. These phases are invoked to explain the putative cellular rafts thought to be important in various cellular processes at the membrane (1–3). The issue is complicated by the fact that many cellular membranes, including the plasma membrane, are chemically asymmetric. While bilayers formed from outer-leaflet-like lipids spontaneously phase-separate, bilayers intended to model the inner leaflet do not. Motivated by this, we recently set out to study the phase behavior of model asymmetric lipid bilayers (4). Our observations implied a strong interleaflet interaction, although in tandem with theoretical work (5,6), our observation of three distinct phases limits how strong that interaction can be relative to interactions between lipids in the same monolayer—a fact to which I will return below. It is not, however, this letter's purpose to review that work, but rather to understand what sorts of predictions would be made by different coupling models, in the hope that some of these can be excluded.

There are two important observations that any model of interleaflet interactions must reproduce. The first is the apparent fact that the two leaflets are chemically aware of each other; that is, that each leaflet is able to influence the chemical potentials in the other sufficiently to induce or suppress phase separation. This has been demonstrated by us (4) and also in polymer-cushioned supported asymmetric bilayers (7), although a full composition-temperature phase diagram has not yet been completed, and there are some anomalies yet to be resolved. The second, perhaps related, fact is that to date no unsupported lipid bilayer system has ever displayed what I term an ''overhang,'' where, say, some region of ordered liquid in one leaflet makes contact with a region of disordered liquid at the midplane. (Imagine in each leaflet a circular ordered liquid region where the circles have different centers in the plane of the bilayer; thus, there is a part of the ordered liquid in one leaflet that overhangs that part in the other.) We require a strong interaction capable of inducing phase separation, and a local interaction that disfavors ordered and disordered liquids contacting at the bilayer midplane. It remains to be seen whether these are necessarily the same interaction.

Proposed mechanisms for leaflet interactions include chain interdigitation (8) and cholesterol translocation between leaflets (9,10). Interdigitation is an unlikely prospect for a variety of reasons (11–13), despite its intuitive appeal. Essentially, interdigitation is weak except in those cases in which there are lipids whose two chains have significantly different length. Even then, the interdigitation appears to be obliterated by the presence of cholesterol.

Translocation of cholesterol ensures that the cholesterol concentrations in the two leaflets will quickly reach equilibrium, so that the chemical potential of cholesterol, μ_c, is the same in both leaflets. In contrast, there is no such constraint for phospholipids in cellular membranes, where their concentrations are maintained out of equilibrium by special proteins, or in model asymmetric membranes, where the lipids equilibrate across the bilayer slowly (4). This model, where cholesterol is itself the mechanism of communication

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between the two leaflets, has intuitive appeal, so let us consider the model's consequences.

As a simple example, consider a monolayer of phospholipids and cholesterol, which separates into two liquids over some range of temperatures. Now to this first leaflet appose a second leaflet consisting only of phospholipids, which we shall presume for the moment form a homogenous liquid. The leaflets exchange cholesterol, ensuring that μ_c is the same everywhere in the system. Cholesterol will thus flow from the first to the second leaflet. As cholesterol is depleted from the first leaflet, its density drops, and cholesterol's chemical potential in that leaflet should decrease. In the second leaflet, the density increases and μ_c rises. As the two systems reach equilibrium, μ_c takes on one value everywhere. In a vesicle, the areas of the two leaflets are fixed, assuming no pressure changes or leakage into or out of the vesicle. As a result, the increased number of cholesterol molecules in the second leaflet will lead to crowding and a larger lateral pressure. The changes in lateral pressure and composition of the two leaflets bring them to new points in their respective phase diagrams, where, presumably, there could be one, two, or perhaps more coexisting phases. But this says little about what happens if we should bring two leaflets together whose cholesterol chemical potentials are already the same.

Now consider a symmetric bilayer. By symmetry, the chemical potentials of cholesterol in the two leaflets are identical, irrespective of the magnitude of any coupling or rate of translocation. Thus, the mechanism just described is moot. As a result, the two leaflets are really unaware of the other if translocation is the only mechanism of coupling. As far as one leaflet is concerned, the other appears homogenous, because all it ''knows'' of the second leaflet is the chemical potential of cholesterol. An important consequence is that there is no free energy penalty for overhang fluctuations. An ordered domain in one leaflet can float past a similar domain in the second without a care in the world.

The missing ingredient, which aligns the edges of domains in the two leaflets, is a surface tension between the two. Just as there is a quasi-one-dimensional interface between the ordered and disordered liquids (whose line tension is ~1–10 pN (14)), there is a two-dimensional interface between the two leaflets—the bilayer midplane. If two different liquids should contact each other at this interface via overhang, we would expect this to incur a free energy penalty over the state without overhang, just as extending the line-like interface between the liquids in one leaflet incurs a penalty. Indeed, unlike the line interface, this midplane interfacial energy scales with area of the interface, so that it can in principle be large enough to actually influence the phase equilibria. Again, this energy favors having the liquids on either side of the midplane being identical at all points in the plane of the bilayer. In that case, there should be minimal surface tension, so this interfacial energy must depend on the local compositions of the two leaflets. Satisfyingly, such a surface tension takes on the approximate form one finds in recent Landau models of interleaflet coupling (e.g., (15)).

The explanation is simple: the interface between two leaflets is one of hydrocarbon chains. As such, it must be grossly similar to the interface between two liquids in one of the monolayers. This latter interface has a line tension of ~5 pN, but it is not really a line tension (just as the bilayer is three-dimensional but often pictured as two-dimensional). The interface has an area, which I take to have a height of ~2.5 nm—just the hydrocarbon region. So in terms of the area of the interface, the tension is ~2 pN/nm, or ~0.5 kT/nm^2. While not enormous, this is now a significant free energy per unit area. (The entropy s of phase separation will be $\sim kT\ln 2$ per lipid, each of which has an area of ~0.6–0.7 nm^2, so $s \sim kT/nm^2$.)

Typical fluctuations will have energy $\sim kT$, so that any overhang fluctuations will be vanishingly, probably undetectably, small—of the order of just a few lipids' area. I have assumed that the familiar line tension is entirely due to chain-chain interactions, although it certainly contains contributions from headgroup interactions and deformations due to hydrophobic mismatch (e.g., (16)) so my estimate is necessarily very rough. Still, even if the midplane surface tension was 10-times smaller, a typical overhang fluctuation would still only have an area ~20 nm^2; very roughly 30 or 40 lipids total, or approximately the area of a typical small membrane protein. Of course, near a critical point this interfacial energy should vanish, just as the line tension of the domains will do. Thus, such overhang fluctuations should become larger near a critical point, assuming the usual concentration fluctuations do not render the domains unrecognizable.

In some sense, this is quite underwhelming, but it is important. In the presence of an interaction dependent on the local composition of the two leaflets, the two compositions are no longer independent of each other. This rules out the possibility of macroscopic overlap of ordered liquid in one leaflet and disordered liquid in the other. The associated interfacial energy is large enough not just to bring preexisting domains into alignment, but also to perturb the compositions of those domains and change the equilibrium phase behavior of the system. Under no circumstances can the lowest energy state have a liquid of one composition in one leaflet that is simultaneously opposite two liquids of different composition in the apposed leaflet—the two liquids in the second leaflet would perturb the chemical potentials of the liquid in the first leaflet differently, meaning that the system would be inherently out of equilibrium. Instead, the system adjusts compositions and area fractions, reverts to a homogenous phase, or in some cases spawns a third phase. It is not necessary for there to be only one kind of raft in biological membranes, which have many components and most importantly are asymmetric.

All of this is notably in the absence of proteins: any putative raft in a cellular membrane must span the bilayer, independent of proteins that may or may not be present. As has been

discussed theoretically (5,6) and demonstrated in supported bilayers (7) and in unsupported membranes in our lab (4), this leads to a rich new phase behavior which will presumably grow more rich as we examine ever more complicated model systems.

There are at least two other important points about biological membranes that I have not yet considered: intrinsic monolayer curvature, and the presence of an electrostatic potential across the membrane. Thus far, experiments (4,7) have included neither curvature nor electric fields, so it is hard to say much about their effects. Both are known to affect the properties of the lipid hydrocarbon chains. Curvature can lead to lipid sorting, potentially altering the coupling in highly curved regions of membrane. Electric fields tend to thin membranes—accumulated charges on either side attract each other. Any effect this has probably depends on the membrane's elastic properties, also manifest in the lipids' intrinsic curvature. One might imagine that changes in electrostatic potential could alter the coupling and change the raft-forming properties of a membrane. Also, due to nonlinearities in the energy stored in a membrane-as-capacitor, the membrane prefers to thin out wherever it can, even at the expense of thickening elsewhere (e.g., (17)). This too can act as a domain coupling mechanism, but has not been explored experimentally. As such, curvature and electrostatics cannot be invoked to explain the coupling thus far observed.

Whether midplane surface tension is biologically relevant remains to be seen. There is a clear relationship between the number of phases observed and the relative strength of the coupling (5), and a small increase in coupling strength can eliminate one kind of raft without affecting the others substantially. Experimental evidence suggests that the coupling is strong enough to be important to the phase behavior, but not so strong as to limit the number of possible phases to just two. Any mechanism which changes the midplane surface tension, or any monolayer-monolayer interaction, will certainly affect the phase behavior of the membrane. This presents new ways in which domain formation could be used to sensitively detect changes in the cell's environment, say by detecting molecules intruding in the bilayer midplane. But in real cell membranes, proteins actively control the chemical makeup of the two lipid monolayers. It is conceivable that those proteins actively suppress the sort of surface tension I have discussed. Alternatively, these proteins may enhance or transiently modify that surface tension to suit the cell's needs. We are just beginning to explore this issue, and much remains to be learned.

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