

An Unexpected Driving Force for Lipid Order Appears in Asymmetric Lipid Bilayers

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ABSTRACT: An ordered phase in one leaflet of an asymmetric bilayer can induce a precisely superimposed *induced order domain* in the apposed leaflet. Order is induced in such simple lipid compositions as dioleoylphosphatidylcholine/cholesterol (DOPC)/chol which is expected to be a uniform and disordered lipid mixture. Dye partitioning can be used to label and identify coexisting liquid-disordered (Ld), liquid-ordered (Lo), or gel-ordered ($L\beta$) molecules in a phase-separated leaflet. In the other leaflet of an asymmetric bilayer, dye partitioning also labels and identifies any induced order domains created by an Lo or gel phase domain in the apposed leaflet as well as the state of disorder of the lipid surrounding the induced ordered region. We explore a molecular level mechanism by which a disorder-prone uniform mixture of DOPC/chol = 0.8/0.2 would spontaneously separate into ordered regions coexisting with disordered regions. A redistribution of cholesterol seems to take place in the regions apposed to the ordered phase. The precision of the superposition of Lo or gel domains with their induced order domains implies a strong energy penalty that would be incurred if *order/disorder interfaces* were to form at the bilayer midplane. We conclude that the energy penalty for Lo/Ld or gel/Ld contact in the bilayer midplane is sufficient to drive disorderly DOPC/chol into an ordered state that reduces unfavorable order–disorder contacts at the bilayer midplane interface.

INTRODUCTION

Previous work with asymmetric bilayers reveals a puzzling finding. Asymmetric lipid bilayers have different lipid compositions in each of the two leaflets. Thus, one of the leaflets can be an ordered phase or can have coexisting ordered and disordered phase domains. An ordered phase in one leaflet of an asymmetric bilayer has previously been found to “induce” an exactly apposed ordered region in the other leaflet, which would otherwise be a uniform and disordered phase.^{1–9} Induced order domains were first imaged by the group of Lukas Tamm, using gently supported monolayer leaflets of different composition, sandwiched to create an asymmetric bilayer.¹ Here we seek a physical-chemistry-based understanding of how an ordered phase in one leaflet of a bilayer can “induce” an ordered region exactly across the bilayer. We examined the induced order phenomenon by use of especially simple lipid compositions and by use of untethered bilayers of free-floating asymmetric giant unilamellar vesicles (aGUVs). Previous studies by other researchers, together with the work we present here, make possible an explanation of how membrane order can be induced.^{5–8} Because natural cell membranes are asymmetric lipid bilayers, and in particular, we model here the cell’s outermost or plasma membrane, induced order would be important for understanding the membrane behavior of living cells.

Background. All natural lipid bilayers are mixtures of lipid components. We want to know, as an example, how changes in these components could change the phase state of a model for a natural bilayer. A simple but useful approximation of the energetics of component mixing of symmetric bilayers is to treat all of the energy of mixing to be from pairwise and

additive interactions between nearest neighbor lipids. This approximation is modestly successful, for example to describe interactions that lead to phase separation, and to estimate the magnitude of line tension between coexisting liquid-disordered and liquid-ordered phases.¹⁰ Other energy terms, for example, multibody and higher order interactions, could improve upon these pairwise additive simple models, which are nonetheless useful starting points for understanding lipid mixing and phase separations in symmetric bilayers.

Here, we describe another and quite different type of improvement for the description of the mixing of lipids that is important only for the case of asymmetric bilayers, that is, where each leaflet is composed of different lipid types. As previously introduced by other researchers,^{11–14} in the center of the bilayer at the midplane between the two monolayer leaflets there is contact between the apposing leaflets’ methyl/methylene groups at a two-dimensional interface. A lipid phase type such as Lo, Ld, or gel has certain characteristic phase properties, density being among these phase properties. The density at the bilayer midplane, which is mainly a density of methyl/methylene groups, is of special importance for asymmetric bilayers because the midplane is where the two different leaflets make contact and thus can directly influence

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each other. As pointed out by White, the phospholipids, together with any cholesterol that might be in the mixture, create a characteristic density of methyl/methylene groups at the bilayer midplane.¹²

We present a simple model for lipid mixing in an asymmetric bilayer where the mixing energetics has a large component from the pairwise additive interactions within a leaflet, as is the case for symmetric bilayers.¹⁰ For asymmetric bilayers that model cell plasma membranes, we now include an additional interaction that arises from the contact of the leaflets in their midplane area. This midplane component of the energetics has quite a different character compared to the pairwise additive energies of neighboring lipids, in part because this midplane energy scales with the area of phases in contact. It is a type of interfacial tension.^{13,14}

As to a mechanism for induced order, Collins was the first to identify midplane surface tension to be a missing component for understanding coupling of bilayer leaflets.¹⁵ Later, May (2009) also examined bilayer midplane surface tension as a key for understanding the coupling of the bilayer leaflets. May identified and estimated possible factors that could influence the midplane surface tension.¹⁵ To avoid confusion with the aqueous surface of a bilayer, we describe the energy per unit area at the midplane as an interfacial rather than a surface tension.

We speculate below about the molecular-level origin of induced order, which is distinct from that recognized previously. We emphasize that this is a new type of order found in lipid bilayers, an ordering of a monolayer that is induced by an intrinsically, that is, thermodynamically stable, ordered phase in the monolayer of the apposed leaflet within an asymmetric lipid bilayer. Any induced order is coupled to this apposed ordered phase, which we have found can be either Lo or gel. The induced order region is found to coexist in the same leaflet with regions of lipids in a disordered state.⁶

An advantage of imaging experiments for the study of asymmetric bilayers is that use of different color dyes in each leaflet enables separately observing order and disorder in each leaflet, as we do here. We seek to understand the molecular mechanism of the striking observation of induced order formation in what are otherwise expected to be disordered regions of phospholipid mixtures, caused by an apposed ordered phase, which can be either Lo or gel.⁶ By use of our system of making asymmetric GUVs by hemifusion,⁵ the inner leaflet can be labeled with, for example, a red dye, while the outer leaflet can be exchanged with lipids together with, for example, a green dye from a supported lipid bilayer (SLB).

In order to clarify our thinking about mechanism, we created especially simple experimental lipid bilayer systems, namely asymmetric GUVs having inner leaflet distearoylphosphatidylcholine (DSPC)/DOPC/cholesterol and outer leaflet DOPC/cholesterol, or else inner leaflet brain sphingomyelin (bSM)/DOPC/cholesterol with outer leaflet DOPC/cholesterol, as described in more detail.^{5–8} Figure 1A shows image data from confocal fluorescence microscopy of a symmetric GUV containing the red dye DiD. To create the asymmetric or aGUV shown in parts B and C, lipids from the symmetric GUV outer leaflet were replaced by SLB lipids DOPC/cholesterol = 0.8/0.2 together with green lipid dye, TopFluor-PC. The aGUVs formed in this way contain both red and green dyes, Figure 1B and C.

Behaviors characteristic of asymmetric bilayers are observed for these simple mixtures. An inner leaflet can exhibit thermodynamically stable phase separation of Ld + Lo because

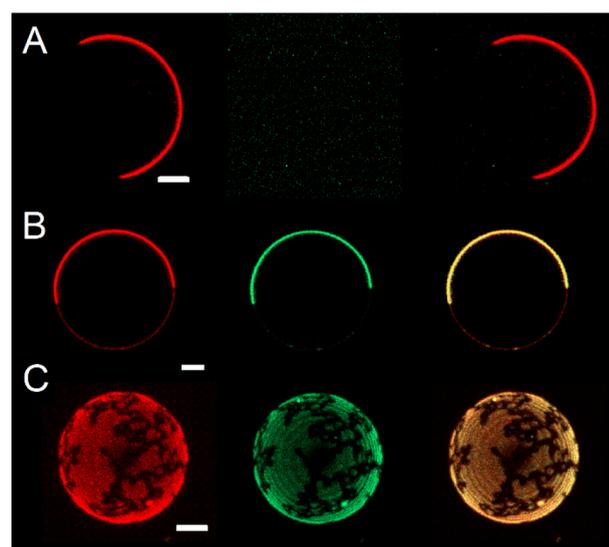


Figure 1. Order from either Lo or gel can induce domains in the apposed leaflet. Confocal microscopy in GUVs reveals domains of both the inner and outer leaflets. DiD fluorescence red, TFPC green, merged images in right column. (A) Equatorial plane of symmetric GUV of DSPC/DOPC/cholesterol = 0.39/0.39/0.22 shows a red Ld domain and a dark Lo domain in both leaflets; (B) A GUV similar to that in (A) has its outer leaflet replaced by DOPC/cholesterol = 0.8/0.2. The new outer leaflet has a round disordered domain (green) superimposable with the red inner leaflet Ld phase and a round induced order domain (dark); (C) z-stack of an aGUV forming a gel phase. Red DiD shows the Ld phase, and dark shows the gel phase of the inner leaflet. Green TFPC labels disordered regions in the outer leaflet, and induced solid-like domains are dark. Scale bar 5 μm , temperature 20 $^{\circ}\text{C}$. 1C adapted, with permission, from ref 6. Copyright 2022, Elsevier.

of its lipid composition, with an outer leaflet being DOPC/cholesterol = 0.8/0.2, Figure 1B. The striking observation in Figure 1B is the creation of induced order domains in the outer leaflet: the aGUV outer leaflet of DOPC/cholesterol reorganizes into two visibly distinct coexisting regions, one enriched with a disorder-preferring green dye across from the inner leaflet Ld phase domain; the other region lacking the green dye is across from the inner leaflet Lo phase domain.^{5–8} Similarly, $L\beta$ gel ordered domains are observed to induce solid-like domains, Figure 1C. In 1C many solid-type ordered domains are induced in the outer leaflet by the many gel phase domains in the phase-separated inner leaflet.

Wagner, Loew, and May used a mean field treatment to examine monolayer–monolayer coupling in asymmetric bilayers in which each leaflet has an intrinsic tendency to phase-separate.¹⁴ The system we chose, DOPC/cholesterol as our model cytoplasmic leaflet, does not have such a tendency to phase-separate. In this way it provides a model for a cell plasma membrane with a cytoplasmic leaflet having a high fraction of polyunsaturated phospholipids.¹⁶ In a cell plasma membrane, the bilayer midplane separates an exoplasmic leaflet that could have thermodynamically ordered Lo phase domains plus a coexisting Ld phase, from the cytoplasmic leaflet with its Ld phase mixture of polyunsaturated phospholipids that do not have an intrinsic thermodynamic tendency to phase-separate.

Optical microscopy spectroscopic measurements related to leaflet order provide useful information about induced order. Spectra can be obtained from the dye C-Laurdan and then analyzed to find Generalized Polarization, GP. GP results show

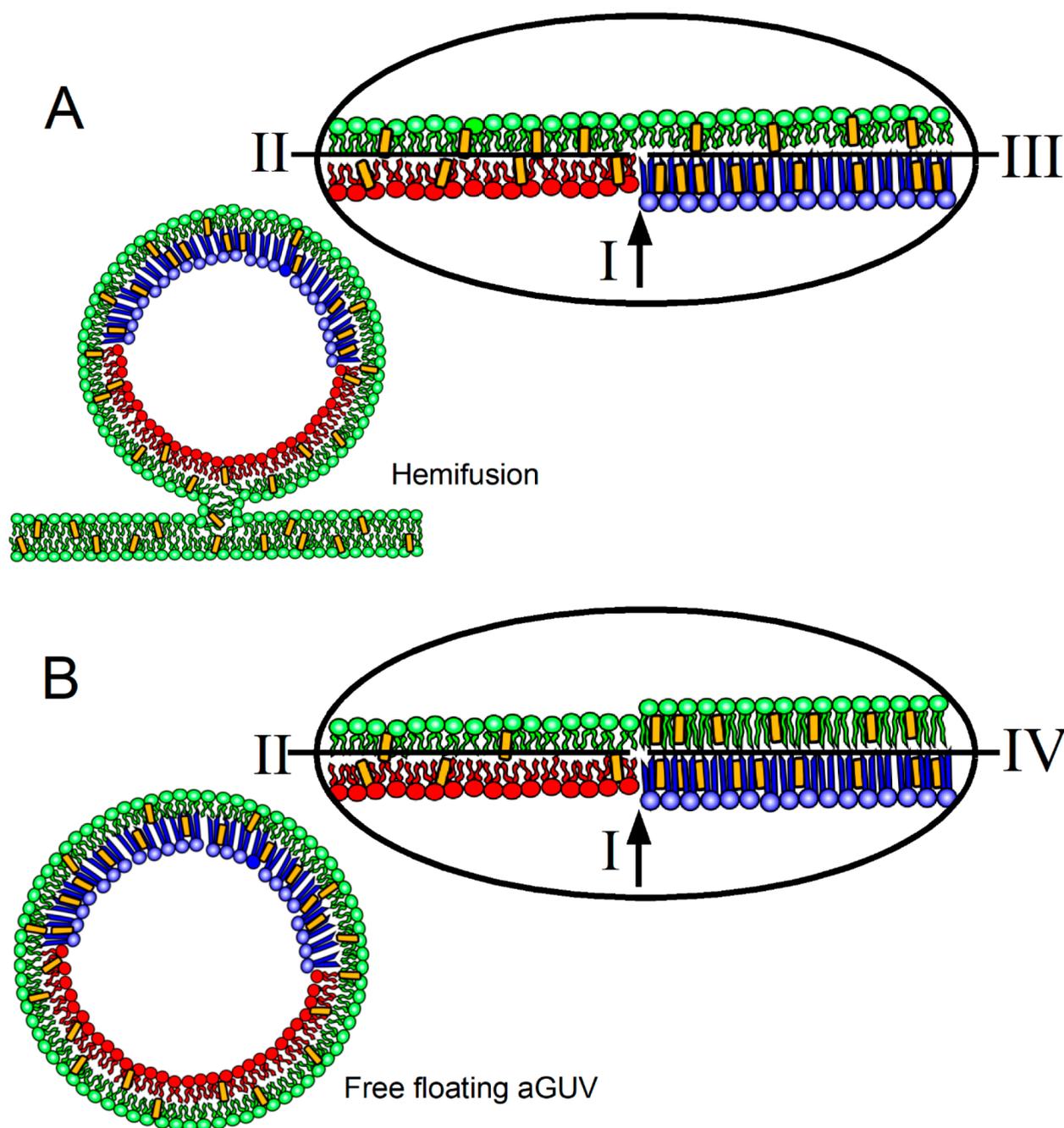


Figure 2. An asymmetric bilayer is formed from a symmetric GUV by replacing the outer leaflet with DOPC/chol from a supported lipid bilayer. The Ld-Lo interface is labeled I and located by a black arrow. Bilayer midplanes II, III, and IV are respectively between Ld and DOPC/chol; between Lo and DOPC/chol; and between Lo and induced order DOPC/chol. Insets show relevant interfaces of the aGUVs. (A) Schematic picture just after completion of hemifusion between an SLB of DOPC/chol = 0.8/0.2 and a GUV of DSPC/DOPC/chol = 0.39/0.39/0.22. During hemifusion, lipids from the SLB replace the GUV outer leaflet, but the inner leaflet of the asymmetric GUV maintains the initial lipid composition of the GUV, with the coexistence of Ld (red) + Lo (blue) phases. Green lipids originated from the SLB of DOPC/chol = 0.8/0.2, with no lipid rearrangements pictured as yet occurring. (B) Sketch of free-floating aGUV after detaching from SLB. Induced ordered domains are represented by green lipids with more ordered acyl chains than in (A). IV shows a midplane interface similar to that in III, but with a lower midplane interfacial tension. Ld/Lo interface I is perpendicular to the bilayer surface, whereas interfaces II, III and IV are parallel.

that the original composition DOPC/chol = 0.8/0.2 changes to a much more positive GP value in the induced order region, but also to an even more negative value in the disordered region across from Ld domains.⁵ This more disordered region shows GP = -0.22, consistent with significant loss of cholesterol from an initial GP value of -0.19 for DOPC/chol = 0.8/0.2 to a value close to the GP of pure DOPC =

-0.23. These GP data imply that the leaflet with induced disordered domains has coexisting domains of especially low cholesterol fraction.⁶ It seems that starting with 0.2 mole fraction of cholesterol in DOPC, the “induced disordered domains” lose a considerable fraction, possibly most of their cholesterol, to either or both the ordered domain in the same leaflet or to the other phase-separated leaflet.

Induced order and induced disorder domains behave like genuine phase-separated domains in the sense that membrane-bound dyes can partition between the induced ordered regions and the surrounding disordered lipid. The partition of a fluorescent lipid between coexisting domains provides a gauge of the difference between regions of the DOPC/chol leaflet that have induced order coexisting with induced disorder.⁵ The partition coefficient of the dye TFPC shows $K_p \sim 5$ between the induced order and induced disordered regions of the DOPC/chol leaflet.⁵ This K_p should be compared with TFPC $K_p = 15$ between symmetric DSPC/DOPC/chol domains of Ld + Lo, favoring Ld.^{5,17}

Mechanism Clues from the Previously Known Penalty for Midplane Phase Mismatch. The induced order behavior that we describe reveals a form of coupling of phase domains across the leaflets. An explanation of such coupling involves the notable chemical simplicity of the interfacial midplane, which is mainly methyl and methylene groups from the lipids in the two apposed leaflets. Without attention here to molecular details, contact of ordered methyl/methylene groups with more disordered methyl/methylenes seems to be disfavored at the midplane interface. It is likely that Lo phase *ordered methyl/methylenes contacting induced ordered methyl/methylenes* across the bilayer results in lower interfacial midplane free energy. This interface is a chemically simple, approximately two-dimensional mixture of methyl/methylene groups from both sides of the bilayer with atom-level structural details not yet known.

Relevant Observations of Symmetric Bilayers. Observations from many researchers starting in 1999 of *symmetric* GUVs having Ld + Lo coexistence show precise registration of each leaflet's phase domains with the apposed leaflet's phase domains, as observed by confocal fluorescence microscopy.^{18–20} The free energy of the midplane, its interfacial tension, seems to be minimized when apposed phase domains are in *contact at their midplane with an identical phase in the other leaflet*. A corollary of the precise registration of like domains across the bilayer is that there must be a free energy penalty when the apposed chain terminal methyl/methylene regions of the other leaflet are not in the same state of order. Because all of the contact of the leaflets is at the bilayer midplane, we identify this interfacial region as the location of any energy penalties when phases in contact are not identical. In particular, given that midplane methyl/methylene is so chemically similar, this is an energy penalty for a lower density of methyl/methylenes being in contact with a higher density. The midplane energetics of *symmetric* bilayers are such that precise phase domain registration of Ld + Lo coexistence in each apposed leaflet of a symmetric GUV is essentially the same as the midplane energetics for two separate, symmetric GUVs, one with a single Lo phase in both leaflets, the other a single Ld phase in both leaflets.

In an attempt to explain how an ordered domain in one leaflet can be “induced” by the other leaflet, we focus on the midplane of the asymmetric bilayer, as originally suggested by Collins.¹³ Induced order in one leaflet must be mediated by the only direct contact that the two leaflets can have, which is contact at and across the bilayer midplane. For the biological case of a cell plasma membrane, the induced order might be one specific way that information can get across the membrane from the exoplasmic leaflet to the cytoplasmic leaflet and vice versa. We propose that induced order is mediated by order/density mismatching at the bilayer midplane, where these

otherwise separate leaflets can interact.^{11,12} We attempt to make a direct connection of the lipid chain ordering with the unfavorable contact of lower with higher density methyl/methylenes at the midplane. One other aspect of lipid packing is the increase in phospholipid chain order as the cholesterol fraction increases. This increased chain order includes the chain terminal methyl/methylene groups. We have previously seen chain ordering caused by cholesterol in earlier studies of the Lo phase, where spontaneous ordering of lipid acyl chains occurs, reducing their cross-sectional area, to accommodate a higher cholesterol fraction, driven by the energy penalty for lipid chain exposure to water, the “Umbrella Effect”.^{21,22}

Asymmetric bilayers are a good model for a cell plasma membrane. The more interesting and complex case than is found for precise domain registration for a symmetric bilayer is when the two bilayer leaflets have different lipid compositions. Preparation of such an experimental asymmetric bilayer that is not tethered to a support could start with an initially symmetric GUV having Ld + Lo phase coexistence. One leaflet of this symmetric bilayer would be replaced using hemifusion with a uniform phase of DOPC/chol.^{5–8} This new, initially uniform DOPC/chol leaflet then undergoes a striking spontaneous rearrangement, with ordered regions of DOPC/chol appearing precisely apposed to Lo phase domains of the other leaflet,^{5–8} and disordered regions appearing precisely across from Ld regions of the apposed leaflet. No high-melting phospholipid is in this DOPC/chol leaflet, yet it gains order. In summary, an initially disordered Ld phase responds to the creation of an unfavorable Ld/Lo midplane contact by becoming more ordered. This induced ordering would lower the interfacial tension in the midplane. Lowering of interfacial tension is well-known to cause a myriad of effects; for example, detergent decreases the size of oil droplets in water. Here we take notice of an interface, the bilayer midplane, where interfacial tension can be important to drive lipid ordering. We do not claim that our case here must involve a high free energy similar to that at a water/oil interface. We do not yet know the magnitude of the midplane interfacial free energies. Instead, we point to the bilayer midplane as a much-neglected bilayer interface. This midplane might have a small or a large unfavorable energy for lower density methyl/methylenes in contact with higher density methyl/methylenes. Measurements of the midplane interfacial energy are needed. Yet, this unfavorable energy at the asymmetric bilayer midplane is sufficient to drive lipid chains to become ordered, as observed by confocal microscopy.

The interfaces that we consider are listed in Figure 2. An asymmetric bilayer is formed from a symmetric GUV of DSPC/DOPC/chol = 0.39/0.39/0.22 by replacing the outer leaflet with DOPC/chol = 0.8/0.2 from an SLB. The Ld/Lo interface is labeled I and located with a vertical black arrow. Bilayer midplanes II, III, and IV are respectively between Ld and DOPC/chol; between Lo and DOPC/chol; and between Lo and induced order DOPC/chol. Figure 2A is a schematic, an idealized picture as if just after completion of hemifusion between an SLB of DOPC/chol and a GUV of DSPC/DOPC/chol. During hemifusion lipids from the SLB replace the GUV outer leaflet. The inner leaflet of the new aGUV maintains the initial lipid composition of the GUV, with its coexisting Ld (red) + Lo (blue) phases. Green lipids originate from the SLB of DOPC/chol = 0.8/0.2. Midplane III would have high interfacial tension because of contact of the ordered Lo with disordered DOPC/chol. Figure 2B shows a free-floating aGUV

after detaching from SLB. An induced order domain is represented by green lipids with more ordered acyl chains than in Figure 2A and having higher cholesterol fraction than in Figure 2A. IV shows a midplane interface similar to that in III, but with a lower midplane interfacial tension where the Lo domain contacts the induced order domain.

Experimental measurements are consistent with the cholesterol fraction increasing in the induced order regions of the DOPC/chol leaflet, while at the same time the cholesterol fraction is *decreasing* in the disordered lipid surrounding the induced order.⁶ These two connected mechanisms, one reducing interfacial tension at the midplane and the other with cholesterol flowing into the induced order region, would occur not in a distinct sequence but instead simultaneously via lipid diffusion during formation of the aGUV.

We emphasize that the DOPC/chol leaflet does not have an intrinsic thermodynamic tendency to phase-separate, and in fact, the mixing of cholesterol with DOPC lipids is known to be favorable. The measured chemical potential profile of cholesterol in DOPC/chol bilayers indicates that the distribution of cholesterol in DOPC bilayers is highly uniform.²³ In the Umbrella Model,^{21,22} the cross-sectional area of the cholesterol hydrophobic moiety is much larger than that of its small hydrophilic hydroxyl headgroup. Cholesterol relies on coverage from neighboring DOPC headgroups to avoid unfavorable exposure of its hydrophobic parts to water. This hydrophobic-driven favorable interaction between cholesterol and PC results in the well-known cholesterol condensing effect and the ordering of PC acyl chains in contact with cholesterol.²¹ If a bilayer leaflet also contains high-melting phospholipids, cholesterol would prefer association with the high-melting phospholipids, in part because there is more space for cholesterol under the headgroups of high-melting, low cross-sectional area phospholipids. Having no high-melting phospholipid, a DOPC/chol symmetric bilayer has no thermodynamic tendency to phase-separate or form domains. We propose a previously unknown function of cholesterol: to form cholesterol-rich domains in a DOPC/chol leaflet and, together with any induced order domains, to lower the midplane interfacial tension caused by the mismatch of order and density between the two leaflets. By forming ordered domains, the Gibbs free energy for the DOPC/chol leaflet would be increased; however the overall Gibbs free energy for the entire bilayer would be reduced because of the lower midplane interfacial tension, shown schematically in Figure 2. Thus, we propose that a part of the driving force for lipid acyl chains to become ordered is the better match at the midplane of Lo domains with induced order domains in the otherwise disordered apposing leaflet. This matching of the order would reduce the unfavorable contact between more and less dense methyl/methylenes at the midplane.

Information Passes through the Bilayer Midplane.

For a symmetric bilayer, the same density of methyl and methylene from each leaflet meets at the midplane. For asymmetric bilayers, we consider these as simply as we can, not speculating about leaflet interpenetration energy or other individual energy factors at the midplane interface; nor do we consider other factors that would influence leaflet phase behaviors, such as possible differences in phospholipid numbers in the two leaflets or the influence of bending modulus.²⁴ In our simple picture, as the new asymmetric bilayer begins to form, transiently unfavorable contacts at the

midplane occur where Lo phase ordered methyl/methylenes meet disordered methyl/methylenes of the new DOPC/chol leaflet. The resulting unfavorable interfacial free energy would be reduced if DOPC/chol were to become more ordered in this region through entropy and enthalpy changes. We observe this induced order by the use of confocal fluorescence microscopy. Figure 2 makes more clear the events that occur in the midplane. We are proposing that the compensating free energy that drives chains to order is the reduction in unfavorable midplane interfacial energy, together with increased cholesterol fraction. A most important point is that the midplane free energy becomes unfavorable when an ordered lipid domain is in contact at the midplane with a disordered lipid domain.

Here, without knowing yet how to measure the midplane interfacial tension, we ascribe aGUV induced order to the lowering of the Gibbs free energy at the bilayer midplane. This is a plausible but not yet proven mechanism to form induced order domains. In our model, the energy penalty at the midplane of an Lo phase contacting an Ld phase would be greater than that for an Lo phase contacting an induced order domain. In contrast, across from an Ld phase, any order mismatching and consequent energy penalty with a different type of Ld phase are likely to be minimal. We are currently working on experiments to measure directly the midplane interfacial tension.

With our attempt to develop an improved model for induced order in asymmetric bilayers, we have not yet answered some important questions about the phenomenology, especially as applied to a cell plasma membrane. We point out that the formation of separated induced order along with disordered domains requires all membrane-bound molecules that can diffuse to partition between these distinct environments. In particular, to address cell plasma membrane behaviors, we would need measurements of lipid partitioning between induced order and induced disorder domains for cytoplasmic leaflet lipids PE, PI, PS, PIP2. We do not yet know even the direction of the partition for any given phospholipid since the induced ordered domain is a new entity, an ordered domain but formed with disordered lipids and enriched in cholesterol. For example, if PI, PS, and PIP2 were to partition the same way between induced order and disordered domains, then highly negatively charged regions would be created in the cytoplasmic leaflet. We emphasize that partition *measurements* are needed. Intuition based on lipid structure must also take into account that highly disordered DOPC is readily induced to become ordered by an apposed ordered phase domain; similar induced ordering can be expected for the PUFA-containing lipids of a cell's cytoplasmic leaflet. In addition, the partitioning of membrane proteins between induced order and surrounding disordered regions should be examined for linkage to proteins that promote the formation of the actin cytoskeleton. The control and connection of induced domains to the cytoskeleton would join the induced order phenomenon with cell shape and motility. Clearly, further investigation is needed in both experiments and computation.

SUMMARY AND OUTLOOK

Confocal fluorescence microscopy imaging of different dyes in each leaflet of asymmetric bilayers is a powerful method to establish the nature of the lipid distributions. In asymmetric lipid bilayers, a gel or Lo phase in one leaflet of the bilayer can induce a superimposed ordered region in the apposed leaflet.

Formation of such an induced order region on its own would be energetically unfavorable because of the decreased entropy and must be balanced by a favorable free energy change. We propose that the favorable free energy change is at the bilayer midplane, which experiences unfavorable free energy, where a gel or Lo phase contacts a disordered phase. By concentration at sites of induced order, cholesterol reorganization is likely to be involved in this phenomenon.

In a cell plasma membrane that has “membrane rafts” in the exoplasmic leaflet and polyunsaturated lipids in the cytoplasmic leaflet, induced order could well occur; therefore, the lipids of the cytoplasmic leaflet should not be assumed to be disordered. Because cholesterol is involved in induced order, future studies would benefit from quantitation of the cholesterol fraction in each type of domain in each bilayer leaflet. Moreover, measurements of bilayer midplane energetics would be of great value to establish factors that affect this aspect of asymmetric biological membranes.

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Notes

The authors declare no competing financial interest.

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