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Biomembranes balance many types of leaflet asymmetries

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Abstract

Many biological membranes host different lipid species in their two leaflets. Since their spontaneous curvatures are typically not the same, this compositional asymmetry generally entails bending torques, which can be counteracted by differential stress—the difference between the two leaflet tensions. This stress, in turn, can affect elastic parameters or phase behavior of the membrane or each individual leaflet, or push easily flippable species, especially cholesterol, from the compressed leaflet into the tense leaflet. In short, breaking the symmetry of a single observable (to wit: composition), essentially breaks all other symmetries as well, with many potentially interesting consequences. This brief report examines the elastic aspects of this interplay, focusing on some elementary conditions of mechanical and thermodynamic equilibrium, but also shows how this poses novel questions that we are only beginning to appreciate.

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Membrane asymmetry, Differential stress, Curvature elasticity, Cholesterol distribution, Phase behavior.

Introduction

In the early 1970s, biologists discovered that biomembranes have leaflet-specific compositions: lipids such as sphingomyelin and phosphatidylcholine were predominantly found in the outer (exoplasmic) leaflet, while phosphatidylethanolamine or the negatively charged phosphatidylserine were mostly in the inner (cytosolic) one. Modern investigations have added substantial detail to this early picture of membrane asymmetry, resulting in fine-grained lipidomes resolved not just by headgroup but also chain lengths and saturation [1].

Since lipids can transition ("flip-flop") between leaflets on time scales ranging from hours to days [2], asymmetry must be actively maintained as a non-equilibrium steady state by a variety of energy consuming processes that collectively enable lipid sorting—a prominent one being lipid transport proteins now known as "flippases" [3–5]. These lipid pumps appear to be surprisingly sluggish, having typical rates on the order of 10 lipids/min [6]. However, this still exceeds the spontaneous flip-flop rate of single lipids by about four orders of magnitude and so a crude balance of rate and abundance indicates that lipids can outnumber their flippases by maybe 10^4:1 while maintaining a sizable asymmetry.

Making asymmetric membranes

A bioinformatics analysis of transmembrane proteins anchored by a single-pass α-helix strongly suggests that plasma membrane asymmetry is evolutionarily conserved across all eukarya [1]. While it is not difficult to post-dict plausible biological functions, it would be more gratifying to first understand what distinct properties asymmetry enables and then identify how these are exploited. This path has long been thwarted by our inability to reproducibly synthesize model membranes of controllable asymmetry, but this situation has profoundly changed. A recent compendium by Krompers and Heerklotz [7] examines 72 published protocols in detail, sorts them into 4 main categories, and examines their individual merits. Without doubt, the current renaissance of membrane asymmetry has been ushered in by these experimental breakthroughs.

Simulating asymmetric membranes seems straightforward by comparison: just set up the desired leaflet-specific lipid compositions and press "run". However, the broken symmetry demands a far-reaching new decision: *how many lipids should each leaflet hold*? Park *et al.* [8] reviewed the physical ramifications of four subtly different choices, especially with regard to conceivably unintended torque-derived stresses that can be relaxed via so-called $P2_1$ boundary conditions. In a follow-up

study, they use this approach to reliably measure the spontaneous curvature of embedded peptides [9].

Of course, a stress-free state might not actually be the experimentally relevant one—to decide this one would need actual data, which are exceptionally difficult to get. Moreover, the relaxed state need not be flat either. This causes problems with standard periodic boundary conditions, but there are simple yet effective ways to relax these constraints [10]. Furthermore, studying the consequences of asymmetry at scales larger than, say, 100 nm typically requires coarse-grained models, whose often unphysically high flip-flop rates spoil asymmetry and thus require suitable countermeasures [11].

What does asymmetry do to membranes?

Experiments on compositionally asymmetric model membranes have revealed a number of surprises. A widely observed phenomenon is that these systems tend to be elastically stiffer than their symmetric counterparts. An increase of the bending modulus between 50% [12] and 150% [13] has been observed for POPC/DOPC systems, as well as in the DMPC/DOPC case (near the gel transition of DMPC) [14] and in asymmetric systems involving the aminophospholipids POPE and POPS [15]. A slight increase in the area stretching modulus was also observed in POPC vesicles (at 24 °C) that had 20% of their lipids on one side replaced by POPA—but only if that replacement was done on the inner side [16]. This is extraordinary given that the measurement was done with GUVs of diameter 10 μm or larger.

Another remarkable finding pertains to the main phase transition in asymmetric vesicles. Eicher *et al.* [17] have examined asymmetric large unilamellar vesicles (radius $R_0 \approx 60$ nm) with POPE/POPC leaflets and shown that the two leaflets cooperatively gel at a single temperature ($\approx 16\,^{\circ}$C) if POPE resides on the inside, but this transition is spread over a range between 4 °C and 20 °C, with notable peaks at the endpoints, if POPE resides on the outside.

Other remarkable properties are seen in the phase behavior of mixtures (see Section "Cholesterol"). The common challenge is that asymmetry invariably modifies the two leaflets differently, and it is frequently unclear how to experimentally resolve this.

Types of asymmetry

Once the up-down symmetry of a bilayer is broken in one particular way (say, lipid type), there is no reason why any other observable definable on the leaflet level should remain symmetric. Even a difference in the embedding medium on both sides suffices to trigger membrane asymmetry, for instance by developing curvature via unbalanced depletion interactions [18] or a dipole field via charge regulation in the presence of a pH difference across the membrane [19].

The main focus of the present paper is to examine *elastic* asymmetries, specifically those that result in a difference between the tensions Σ_+ and Σ_- of the upper and lower leaflet. If these are not the same, it is convenient to define the symmetrized and antisymmetrized values

$$\text{total tension}: \qquad \Sigma = \Sigma_+ + \Sigma_-, \tag{1a}$$

$$\text{differential stress}: \qquad \Delta\Sigma = \Sigma_+ - \Sigma_-, \tag{1b}$$

such that $\Sigma_\pm = \frac{1}{2}(\Sigma \pm \Delta\Sigma)$, because (*i*) the bilayer response is often determined by $\Delta\Sigma$ and (*ii*) we will find that frequently $|\Delta\Sigma| \gg |\Sigma|$.

How differential stress arises and why it matters

Membranes curve in the presence of bending torques [20,21]. The two most obvious *internal* sources are the existence of (1) a spontaneous bilayer curvature $K_{0\mathrm{b}}$ and (2) differential stress $\Delta\Sigma$. Recently, Hossein and Deserno [22] have argued that since bending-induced relative leaflet stretching is to lowest order proportional to the curvature, one may write the combined energy as a sum of a local and a global bending energy density,

$$e(K, \overline{K}) = \frac{1}{2}\kappa(K - K_{0\mathrm{b}})^2 + \frac{1}{2}\kappa_{\mathrm{nl}}(\overline{K} - K_{0\mathrm{s}})^2, \tag{2}$$

where K is the local curvature, $\overline{K}$ is the curvature averaged over the whole membrane, κ and $\kappa_{\mathrm{nl}} = K_A z_0^2$ are local and non-local bending moduli, respectively, K_A is the bilayer area modulus, z_0 is the distance of a leaflet's neutral surface from the bilayer midplane, and $K_{0\mathrm{s}}$ is the curvature at which the bending-induced area strain vanishes. If we restrict to constant mean curvature surfaces (*e.g.* planes, spheres, and cylinders), we need not distinguish between K and $\overline{K}$. In that case, the torque due to Eqn. (2) is

$$\mathcal{T} = \frac{\partial e}{\partial K} = \kappa(K - K_{0\mathrm{b}}) + \kappa_{\mathrm{nl}}(K - K_{0\mathrm{s}}), \tag{3}$$

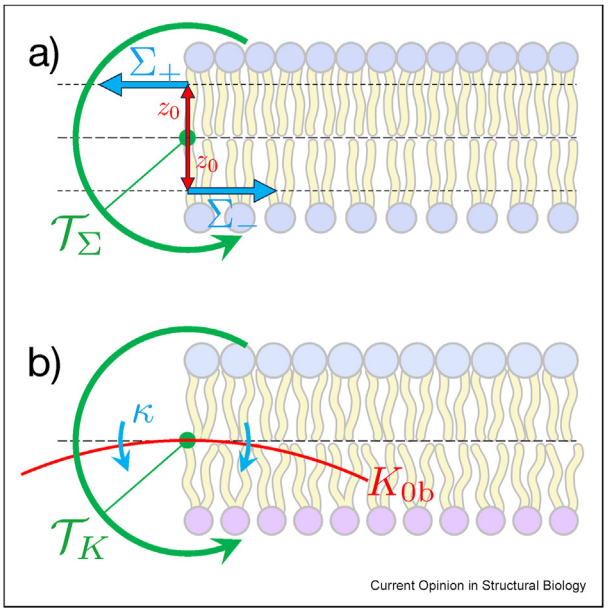
whereby the first term, $\mathcal{T}_K$, arises due to the curvature elasticity of the membrane, while the second term, $\mathcal{T}_\Sigma$, arises from the differential stress. Figure 1 illustrates these two different mechanisms.

While the full torque clearly vanishes for the equilibrium curvature,

$$K_0^* = \frac{\kappa K_{0\mathrm{b}} + \kappa_{\mathrm{nl}} K_{0\mathrm{s}}}{\kappa + \kappa_{\mathrm{nl}}}, \tag{4}$$

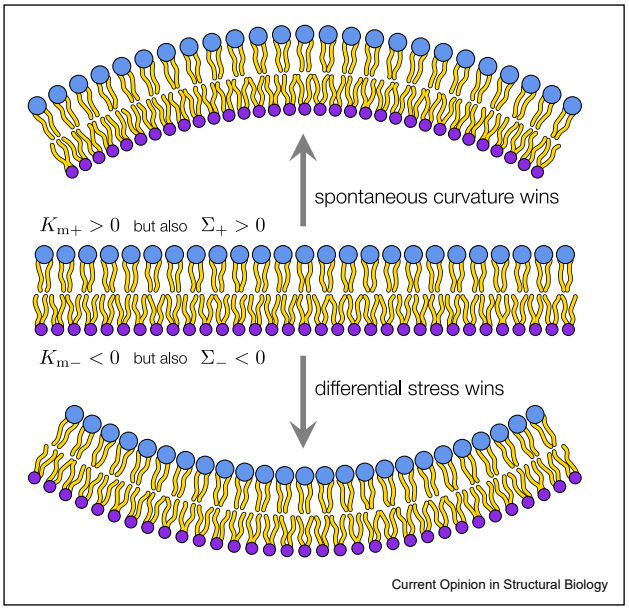
the *differential stress* does not [22]:

Figure 1



Two ways by which a bending torque arises in a membrane. **(a)** Differential stress creates torque via a force times lever arm mechanism: $\mathcal{T}_\Sigma = \Sigma_+ z_0 - \Sigma_- z_0 = \Delta\Sigma\, z_0$. **(b)** Spontaneous curvature creates a torque via a deviation from the preferred curvature times bending modulus mechanism: $\mathcal{T}_K = \kappa(K - K_{0b})$.

Figure 2



An asymmetric membrane of given composition will generally have internal torques due to both spontaneous curvature and differential stress. If these oppose one another, then the equilibrium shape of the membrane depends on which one is stronger. If they balance, the membrane is flat, yet differentially stressed. Observe that the three pictured configurations all have the same compositional asymmetry, but they differ in both shape and internal stresses. To fully specify the thermodynamic state, it hence does not suffice to merely know the leaflet compositions.

$$\Delta\Sigma|_{\mathcal{T}=0} = \frac{\kappa}{z_0}(K_{0b} - K_0^*). \tag{5}$$

Demanding a spontaneously flat membrane, $K_0^* = 0$, implies $\kappa_{nl}K_{0s} = -\kappa K_{0b}$ (from Eqn. (4)), or equivalently $\Delta\Sigma = \kappa K_{0b}/z_0$ (from Eqn. (5)). Using typical values for bending rigidities and spontaneous curatures of common lipid-asymmetric systems [23], as well as $z_0 \approx 1$ nm, reveals that the differential stress can easily be several mN/m [22], *far* bigger than typical cellular membrane tensions [24]. Since GUVs are essentially flat, this should be the differential stress they are under if asymmetric; without it, the bending torque due to K_{0b} would immediately tubulate them. Figure 2 illustrates the possible outcomes when $\mathcal{T}_K$ and $\mathcal{T}_\Sigma$ oppose one another, showing that there are many shapes and states of internal stress that are compatible with a given lipidomic asymmetry.

Differential stress has many physical consequences. It increases the rate at which lipids flip out of the compressed leaflet, and at larger values may trigger transient instabilities like micellar buds [25]. Its torque can affect the function of transmembrane proteins if their operational states differ in relative leaflet footprint, a possibility supported by recent work on the bacterial protein OmpLA [26]. Coarse-grained simulations even suggest that these stresses, rather than compositional differences, underlie the experimentally observed elastic stiffening [22,27]. This cannot be the full story, though, because stiffening observed in Ref. [15] does not appear to correlate well with estimated differential stress.

If we consider the much smaller POPE/POPC LUVs from Ref. [17] and assume they have formed such that their torque $\mathcal{T}$ vanishes (*i.e.* $K_0^* = 2/R_0$), then the differential stress (always taken with respect to the POPE leaflet) is $\Delta\Sigma_\pm = \kappa z_0^{-1}(K_{0b} \mp 2/R_0)$, where the "+" case has POPE on the outside. Plugging in $K_{0b} \approx -0.09\,\mathrm{nm}^{-1}$, $\kappa z_0^{-1} \approx 125$ pN (both evaluated with data from Ref. [23]), and $R_0 \approx 60$ nm reveals that these stresses are enormous: $\Delta\Sigma_+ \approx -15.5$ mN/m and $\Delta\Sigma_- \approx -7.11$ mN/m (*i.e.*, always compressive in the POPE leaflet); moreover, even their difference is huge: $\Delta\Delta\Sigma = |\Delta\Sigma_+ - \Delta\Sigma_-| \approx 8.4\,\mathrm{mN/m}$, which might explain why POPE orientation (outside *vs.* inside) matters. More generally, that difference is given by $\Delta\Delta\Sigma = 4\kappa/z_0R_0$, independent of differential stress but strongly dependent on vesicle size. For GUVs with a diameter of 10 μm we still have $\Delta\Sigma_\pm \approx 11$ mN/m, but

now $\Delta\Delta\Sigma \approx 0.1$ mN/m, rendering the asymmetry orientation less relevant.

By Le Chatelier's principle, such huge lateral stresses should indeed affect the gel transition in each leaflet. This has recently been described by a model analogous to two coupled van der Waals gases [28,29]. The sharp transition was found to be replaced by a coexistence range with a width $\Delta T \sim 0.5$ Km/mN $\times\Delta\Sigma$ (which is somewhere between 3 K and 8 K for the LUVs in Ref. [17]). The same reasoning applied to the equally area-sensitive l_o/l_d transition suggest that its much smaller latent heat would render the transition temperature (via the Clausius-Clapeyron equation) much more stress sensitive [28]. However, in this case, forced translocation of cholesterol becomes an important consideration, as we will discuss next.

Cholesterol

At 40 mol%, cholesterol is the single most abundant molecular species in animal plasma membranes, but its transleaflet distribution has long been a source of debate [30]. While two ABC transporters, ABCA1 and ABCG1, have recently been implicated in significant (12:1) cholesterol sorting into the outer leaflet [31], transport alone can barely explain this, as revisiting the flippase estimate from the introduction shows: cholesterol's flip-flop rate is at least six orders of magnitude faster than that of ordinary phospholipids, so putative cholesterol flippases would—for instance—have to be 100 times more abundant and 10 000 times more efficient than conventional flippases. As Steck and Lange point out, they would also completely exhaust the cells' energy budget [30]. This suggests that sorting is heavily assisted by equilibrium thermodynamics.

Using MARTINI-level simulations, it was shown that cholesterol can reduce differential stress by escaping from the compressed leaflet [32], while its well-known preference for more saturated lipids [33] can pull it into the more ordered leaflet of asymmetric bilayers [34,35]. More generally, the thermodynamic observable that cholesterol will seek to equilibrate between the leaflets is its chemical potential, which in turn depends on variables such as differential stress, solvation free energies, and partitioning entropy. A recent idealized model accounting for these three terms shows that (at a given phospholipid asymmetry) neither of the observables take their optimal value in equilibrium [36]: in general, differential stress is not relaxed, partitioning is weaker than expected from non-ideal mixing, and entropy does not achieve an even distribution; instead, compensatory effects balance these observables, while detuning them triggers a linear response of the others (with predictable "lever arms"). These results are replicated in coarse-grained simulations that follow the simple theoretical model surprisingly well.

Nevertheless, the real-world situation is significantly more complex. To start, the stress-free leaflet area does not arise additively from its components. This is especially true for cholesterol, whose condensing effect may even give it a negative differential area [39]. Similar non-additivities plague basic observables such as moduli and spontaneous curvature [40], which in turn affects cholesterol partitioning [41]. Better theoretical models have to account for all these effects, which appear challenging given that cholesterol's chemical potential in binary and ternary lipid mixtures was recently measured to be very non-ideal [42]. And yet, such studies could deeply inform our understanding of cellular membranes' molecular housekeeping. For instance, Girard and Bereau have suggested that if partitioning thermodynamics is included as a factor in lipid homeostatis (say, in the Lands cycle), complex lipidomes may be maintained while regulating only a small subset of species [43,44].

Conclusion and outlook

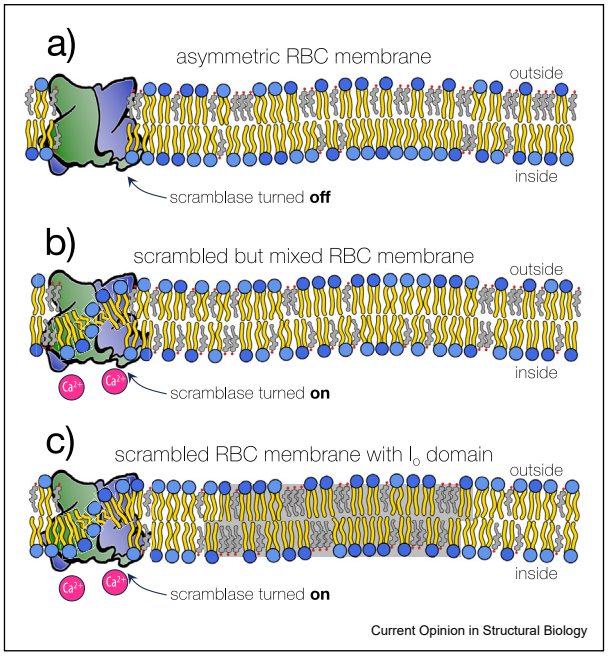
Bilayer asymmetry strongly affects membrane shape, deformation energetics, stresses on transmembrane proteins, cholesterol distribution, the temperature of phase transitions, and overall membrane phase behavior. It is hard to see how this would not impact a vast array of biological processes, and we may well be at the beginning of an exciting period where unearthing the underlying mechanistic connections affords a fresh look at—maybe even new explanations for—well-catalogued biomembrane functions.

A striking example is the enduring question of membrane rafts, for which the coexistence between liquid ordered and liquid disordered (l_o/l_d) phases observed in mixtures of saturated lipids, unsaturated lipids, and cholesterol (in roughly equal proportions) is a widely studied simplified model [45]. Evidently, phase coexistence is heavily dependent on the mixing ratios, and if preferential partitioning and differential stress affect the distribution of cholesterol, leaflet compositions may deviate strongly from the bilayer average. For instance, recent work on red blood cells points to a >50% overabundance of phospholipids on the interior leaflet, which would drive most cholesterol into the outer (and additionally more saturated) one [37]. This conclusion is supported by theory and simulation [36], except that the predicted sign of the differential stress differs between these two studies. At any rate, *neither* of the leaflets might have a composition in the coexistence regions (or close to criticality), suggesting that domains only form upon (full or at least partial) leaflet scrambling [46]. This could equip cells with a way to rapidly create signaling platforms by transiently and locally turning on calcium-activated scramblases [38,47]. Once the leaflet compositions have been sufficiently mixed, one leaflet, or both of them, may enter a phase state whose

composition is conducive to demixing, or at least critical fluctuations. The three stages are illustrated in Figure 3. Experimentally, symmetric vesicles comprising the lipids extracted from the plasma membranes of RBL-2H3 cells have been shown to phase separate up to 55°C, while plasma membrane vesicles (PMVs) shed from those same cells only do so up to around physiological temperature [46]. These PMVs—which require active scramblases to form—hence retain some of their asymmetry. This might hence also hold for a plasma membrane in which scramblases are transiently turned on.

In the asymmetric case, one can arrange for a situation in which only one of the two leaflets has a composition that can phase separate into domains. It has been shown that in such a case the domains on one side can imprint (some of) their properties to the other. For instance, l_o domains can locally order a membrane phase that would not otherwise exhibit membrane order, as was first shown in supported bilayers [48] and free-standing (Montal-Mueller) membranes [49], followed by experiments in vesicles [50,51]. Conversely, l_d domains can locally suppress the appearance of ordered domains in leaflets that would otherwise be prone to exhibit them [49,52]. It has also been shown that lipid tail interdigitation may play an important role in this leaflet coupling [53]: it typically leads to a disordering of the side that is being invaded, but very long chains in strongly asymmetric lipids (such as milk sphingomyelin) penetrating into a saturated phase can instead increase order.

Figure 3



Plasma membrane scrambling could trigger domain formation: **(a)** An asymmetric membrane similar to the red blood cell membranes discussed in Refs. [36,37], *i. e.* with a sizable phospholipid imbalance in the cytosolic (lower) leaflet and a resulting large cholesterol excess in the exoplasmic (upper) one. Neither of the leaflets have a composition that is conducive to l_o/l_d phase separation (the lower is too disordered, the upper contains too much cholesterol). **(b)** After sufficient scrambling, *e. g.* via the calcium-dependent scramblase TMEM16F [38], the compositions may assimilate enough to trigger l_o/l_d phase separation in one leaflet (or both of them), or at least reach the vicinity of the l_o/l_d critical point to enable transient critical fluctuations. **(c)** As a consequence, raft-like domains (or fluctuations) can form, which here is shown as a nanoscopic-ordered region registered across both leaflets.

Declaration of competing interest

The author declares no conflict of interests.

Data availability

No data was used for the research described in the article.

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