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Cholesterol's balancing act: Defying the status quo

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The structure and organization of biological membranes have fascinated biophysicists and cell biologists for decades. Some key findings that have energized the field include the discovery of domains in lipid mixtures (1) and the subsequent raft hypothesis linking them to cellular phenomena (2), as well as the discovery of compositional asymmetry in cell bilayers (3), which put lipid membranes on the map of energetically regulated physiological players. Almost all current knowledge about lipid and bilayer biophysics is rooted in studies of symmetric model membranes in the form of closed lipid vesicles. The geometric constraints thereof prompt the intuitive assumption that the two leaflets of the bilayer must have the same area and therefore—in symmetric bilayers—the same number of lipids. This implicit assumption unintentionally spilled over to asymmetric membranes, where its validity has been challenged by computer simulations designed to explore the consequences of membrane asymmetry.

The construction of a molecular model of a bilayer requires one to specify how many lipids will comprise each leaflet. It was quickly discovered that perfectly viable bilayers could be produced with different numbers of lipids between leaflets and that the mismatch could have significant consequences for the properties of the resulting membranes (4–7). Concomitantly, experimental observations in model membranes indicated asymmetric membranes are likely to harbor differential packing between their leaflets (8,9). Most recently, lipidomics studies have revealed a dramatic imbalance in the number of lipids in the exoplasmic and cytoplasmic leaflets of red blood cells (10,11). Remarkably, these independent lines of evidence—in silico, in vitro, and in vivo—are converging on a new, mind-bending paradigm for plasma membrane organization: biological membranes are likely asymmetric not only in lipid composition (12) but also in total phospholipid abundance. The latter greatly challenges our intuition. How can a membrane have many more phospholipids in one leaflet than the other while preserving its integrity and successfully shielding lipid hydrocarbon chains from water?

In this issue of *Biophysical Journal*, Varma and Deserno show us that physical intuition and counter-intuitive observations can be reconciled by biomembrane's oddball, cholesterol (13). Unlike phospholipids, cholesterol can move rapidly between leaflets to satisfy both its own needs and those of the bilayer. For example, a compositionally symmetric phospholipid membrane can exist in a meta-stable state with different numbers of lipids in its two leaflets because lipid redistribution, involving polar headgroup translocation through the bilayer's hydrophobic core, is too costly. Instead, the lipids in the two leaflets can compress and expand, respectively, to equalize total leaflet areas at the (relatively lower) cost of unfavorable *differential stress*—that is, tensile and compressive forces that take the lipids away from their preferred packing. In this case, cholesterol could ease these stresses by simply flipping to "fill the gaps" created by mismatched lipid numbers. We can also consider a different scenario in which the two leaflets have the same number of lipids but a pronounced compositional asymmetry, such that one leaflet has mostly saturated chains and the other is mostly unsaturated. Here, cholesterol will migrate toward the saturated half (with which it forms energetically more favorable interactions) even if that makes the lipids unhappy overall and creates differential stress. Using well-defined physical principles and some mathematical wizardry, Varma and Deserno describe these effects theoretically and test their derivations with molecular dynamics simulations. The simulations show that entropy is also important in this tug of war, by favoring a symmetric interleaflet distribution and preventing cholesterol (at up to 20 mol %) from completely eliminating differential stress even in the absence of an enthalpic bias for either side. While these peculiarities of cholesterol's behavior have been previously commented on (7,14), the authors here set out to answer a specific question: how do the various forces and preferences ultimately determine cholesterol's interleaflet distribution in a compositionally asymmetric membrane with an imbalanced number of

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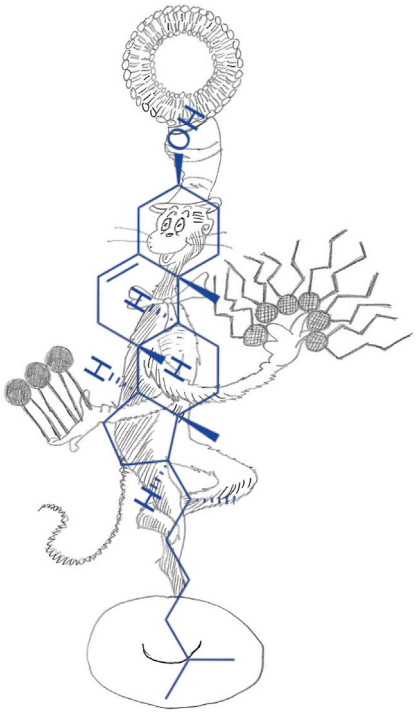

FIGURE 1 Cholesterol's interleaflet distribution in compositionally asymmetric membranes. To see this figure in color, go online.

lipids (Fig. 1)? Unsurprisingly, the answer turns out to be quite complex, but the authors translate their careful calculations into qualitative insights accessible to the general reader and especially to experimentalists seeking to use the results to interpret experiments or design new ones.

One key takeaway is that if only leaflet area balance and cholesterol preferential partitioning are taken into account, number asymmetry and differential stress go hand in hand. As a result, cholesterol's interleaflet distribution is highly correlated with both, and predictions from the theory can be used to probe the effects of its (re) distribution in vitro. The authors specifically mention the extraction of cholesterol from lipid bilayers (e.g., using β-cyclodextrin) and how, depending on the dominating force determining cholesterol's interleaflet residence, this perturbation may increase or decrease the differential stress in the membrane. For example, if cholesterol was helping alleviate the stress due to lipid imbalance by filling the gaps, its extraction would increase the differential stress in the leaflets; conversely, if cholesterol was acting upon its preferences for certain lipids thus causing the stress to begin with, its extraction would decrease the unfavorable tensile and compressive forces felt by the lipids. Thus, an important question remains regarding the specific bilayer response expected in each case. The authors acknowledge that if the cholesterol-containing bilayer exists in a stable condition in the presence of differential stress (due to balancing of the bending torque), a change in stress in combination with additional factors not accounted for by the theory may upset this balance and lead to identical morphological responses regardless of cholesterol's initial intent. It seems that one possible solution to this problem may be to compare the effects of cholesterol extraction with those of phospholipid extraction. The latter would counterbalance any differential stress caused by cholesterol preferential partitioning or, conversely, exacerbate the stress being alleviated by cholesterol. The bilayers would be more tolerant to the perturbation in one case and not so much in the other, and membrane integrity (assayed by permeability or light scattering) may help distinguish between the two scenarios.

A major outcome of the study is its direct applicability to the lipid and cholesterol organization of living membranes. For example, a detailed report of the leaflet lipid compositions of red blood cell plasma membranes was recently published, highlighting interleaflet asymmetries in both lipid type and saturation (15). An ongoing extension of that work also suggests a rather large difference in the total phospholipid abundances between the two red blood cell leaflets (10,11). Preliminary results from lipidomics data, corroborated by accompanying experiments in silico and in vitro, point to an *overpopulated* cytoplasmic leaflet composed primarily of bulky polyunsaturated lipids and an *underpopulated* exoplasmic leaflet containing mostly tightly packing sphingomyelin lipids. A question arises naturally as to how such a large imbalance may be possible: both preferential interactions and number asymmetry would pull cholesterol toward the exoplasmic leaflet in this case, but is such an interleaflet lipid and cholesterol distribution physically realistic? According to the theory presented by Varma and Deserno, it is indeed within the realm of possibilities. The authors find that if the cytoplasmic leaflet has as much as 70% more phospholipids than the exoplasmic one, the large majority of cholesterol would be located on the exoplasmic side, effectively comprising about 60 mol % of the leaflet's molecules. A similarly large imbalance was suggested by measurements of cholesterol's distribution in cells using orthogonal cholesterol biosensors (16). Under these conditions, the membrane would be storing a sizable differential stress, but the theory suggests that bilayers can adequately harbor these big phospholipid and cholesterol asymmetries. The resulting picture of the cell plasma membrane, having fewer phospholipids and more cholesterol in its exoplasmic leaflet, defies the status quo view of its organization and opens many avenues for exploration and speculation.

This new study thus shows how highly coarse-grained models built on a few reasonable assumptions can be scientifically illuminating and establish a basis for future hypotheses and experiments. The relative simplicity of the assumptions, however, inevitably comes at a cost, and the authors very openly and critically discuss the shortcomings of their framework and the limitations to its application. For example, due to practical reasons, their coarse-grained simulations cannot accommodate cholesterol at higher than 20 mol % of all membrane lipids, preventing the authors from uncovering potentially novel effects at higher cholesterol concentrations. Furthermore, as mentioned above, the model focuses solely on the interplay between number asymmetry (or differential stress) and cholesterol's preferential partitioning, thus ignoring contributions from lipid spontaneous curvature or the effect of cholesterol

on the bending energies in the membrane (17). The theory also ignores the condensing effect of cholesterol on lipid packing, which, while extensively documented experimentally, remains challenging to incorporate into generalizable models.

Despite these limitations, the derived mathematical framework presents an excellent springboard for future studies and helps transform a once counterintuitive idea into a tangible and testable hypothesis. While establishing and maintaining asymmetric bilayers requires significant energy input in vivo, cholesterol's flow between leaflets is determined indirectly by its interaction preferences and the resulting forces within the membrane. Cells can thus control cholesterol's leaflet concentrations via fine-tuning lipid compositions and abundances, and we can make inferences about its ultimate leaflet residence both at steady state and upon different perturbations with models like those developed in Varma Deserno (13). The latter constitutes a critical step toward describing and understanding the organization and functionality of complex living membranes.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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