Trends in
Biochemical Sciences

CellPress
OPEN ACCESS

Review

Exploring membrane asymmetry and its effects on membrane proteins

Georg Pabst [1,2,3,*] and Sandro Keller [1,2,3]

Plasma membranes utilize free energy to maintain highly asymmetric, non-equilibrium distributions of lipids and proteins between their two leaflets. In this review we discuss recent progress in quantitative research enabled by using compositionally controlled asymmetric model membranes. Both experimental and computational studies have shed light on the nuanced mechanisms that govern the structural and dynamic coupling between compositionally distinct bilayer leaflets. This coupling can increase the membrane bending rigidity and induce order – or lipid domains – across the membrane. Furthermore, emerging evidence indicates that integral membrane proteins not only respond to asymmetric lipid distributions but also exhibit intriguing asymmetric properties themselves. We propose strategies to advance experimental research, aiming for a deeper, quantitative understanding of membrane asymmetry, which carries profound implications for cellular physiology.

Highlights

All plasma membranes exhibit asymmetry, both in lipid distribution and protein topology. Cells invest significant free energy to uphold this asymmetry.

Despite its critical physiological role, our quantitative understanding of membrane asymmetry is limited, due primarily to insufficient *in vitro* methods for creating asymmetric model membranes.

New experimental protocols now allow for the preparation of vesicles with tailored, asymmetric lipid distributions.

Leaflets with distinct lipid compositions can affect each other's properties, such as lateral diffusion, hydrocarbon chain ordering, and bending elasticity.

Integral membrane proteins not only adapt to lipid asymmetry but also exhibit asymmetric topological orientations, which in turn can depend on lipid composition.

Asymmetry: a universal but expensive hallmark of plasma membranes

Despite more than half a century having passed since the discovery of asymmetric membranes in red blood cells [1,2], and later in other mammalian cells [3–5] and in prokaryotes [6,7], quantitative insights are still scarce. With advancing technologies, we now find ourselves in the infancy of a deeper exploration of asymmetric membranes and their interplay with membrane proteins. Thus, a 'paradigm shift' in our view of plasma membrane functionality seems certain as we approach a more profound understanding of membrane asymmetry.

When defining the state of a plasma membrane, it is clear that maintaining an asymmetric (that is, non-equilibrium) distribution of lipids consumes substantial amounts of free energy. This free energy is expended through the delicate interplay of various integral membrane proteins involved in lipid translocation, including **flippases** (see Glossary), **floppases**, and **scramblases** [8]. Furthermore, membrane asymmetry appears to be intimately linked to the biogenesis of membrane proteins and cellular membranes themselves (Box 1): along the secretory pathway, the distributions of both membrane-protein shapes and lipids shift from symmetric to asymmetric [5].

Why do cells make such concerted efforts to maintain a highly asymmetric distribution of lipids in their plasma membranes? Although evidence suggests that membrane asymmetry might help prevent problems such as blood coagulation and aid in properly orienting membrane proteins [9,10], our understanding remains rather circumstantial. A deeper, more quantitative understanding of asymmetry promises key insights into a plethora of physiological processes occurring in or close to plasma membranes. However, such a quantitative understanding has been impeded for many years because it is challenging to construct asymmetric membranes with precisely defined lipid compositions. While asymmetric free-standing or solid-supported lipid bilayers have been known for some time [4,11], these model membranes tend to lose asymmetry quickly, and they can be studied using only a limited number of experimental techniques. Nevertheless,

[1]Biophysics, Institute of Molecular Bioscience (IMB), NAWI Graz, University of Graz, Graz 8010, Austria
[2]BioTechMed-Graz, Graz, Austria
[3]Field of Excellence BioHealth, University of Graz, Graz, Austria

*Correspondence:
georg.pabst@uni-graz.at (G. Pabst).

Box 1. Biogenesis of asymmetry

Membranes commence as rather symmetric lipid bilayers within the endoplasmic reticulum (ER). However, membrane asymmetry emerges through the asymmetric insertion of proteins and continuously increases as lipids and proteins are trafficked from the ER towards the plasma membrane. This increase in asymmetry is evident in both the lipid distribution between the two leaflets and the topological orientation of the membrane proteins embedded within [56].

From a functional perspective, the differences in asymmetry between the ER membrane and the plasma membrane are easy to rationalize, at least at the qualitative level. On the one hand, a major function of the ER membrane is to facilitate the synthesis and bilayer insertion of lipids and proteins. The ability to execute these functions is attributed to the inherent softness and flexibility of the ER membrane, properties arising from its symmetric lipid composition, high unsaturated phospholipid content, and low cholesterol content.

Nevertheless, the primary roles of the plasma membrane are to act as a selective permeability barrier and to enable transmembrane signaling and selective transport. In a recent proposition by Schütz and Pabst [57], it has been suggested that, to a first approximation, each leaflet of the plasma membrane is responsible for one of these core functions. Specifically, the exoplasmic leaflet, enriched in saturated hydrocarbon chains, predominantly manages the membrane's barrier function. By contrast, the cytoplasmic leaflet, with a high content in unsaturated hydrocarbon chains, serves as a fluid platform, allowing the rapid two-dimensional diffusion of peripheral membrane proteins that are part of signaling complexes.

recent advances in producing asymmetric lipid vesicles have paved the way for pursuing these objectives (Box 2).

In this context, our goal in this review is to provide a critical appraisal of the knowledge gained from asymmetric model membranes and to draw out their implications for future research. First, we delve into the lipid architecture of red blood cells, whose asymmetric membrane is representative of many mammalian plasma membranes. From there, we explore the complexities of asymmetric membranes and how the composition of one leaflet can affect the properties of the other, sometimes in predictable ways and at other times in surprising ways. We then shift our focus to integral membrane proteins, highlighting a few examples that showcase the link between the distinct properties of asymmetric membranes and protein functions. Finally, we discuss potential future avenues of research on membrane asymmetry, emphasizing improved protocols for the preparation of well-defined asymmetric lipid vesicles that host membrane proteins with controlled transmembrane orientations. Understanding the principles underlying membrane asymmetry holds significant promise for various applications, including personalized medical treatments. We thus hope to encourage active engagement in this rapidly evolving field.

The many facets of membrane asymmetry in erythrocytes

Membrane asymmetry manifests in diverse ways, ranging from the intrinsically asymmetric distribution of lipids, through the asymmetric insertion of membrane-interacting compounds, to the asymmetric topological orientation of integral membrane proteins. In terms of lipid distribution, we can categorize membrane asymmetry into two primary types: headgroup asymmetry and hydrocarbon-chain asymmetry. To illustrate this, we focus in the following on data reported in great detail for erythrocytes (Figure 1).

Headgroup asymmetry

The exoplasmic leaflet of erythrocyte membranes is composed predominantly of choline phospholipids such as phosphatidylcholine (PC) and sphingomyelin (SM). By contrast, the inner leaflet is enriched in aminophospholipids, including phosphatidylserine (PS) and phosphatidylethanolamine (PE), but also features notable fractions of PC and PE plasmalogens (PEp), as well as phosphatidylinositol (PI) [5] (Figure 1). This headgroup asymmetry imparts distinct characteristics to the two leaflets.

Glossary

Compositional asymmetry: the difference in lipid composition between the two leaflets of a bilayer membrane.
Flip–flop: passive translocation (diffusion) of a lipid from one leaflet into the other.
Flippase: an integral membrane protein that catalyzes the ATP-dependent translocation of lipids from the exoplasmic into the cytoplasmic leaflet.
Floppase: an integral membrane protein that catalyzes the ATP-dependent translocation of lipids from the cytoplasmic into the exoplasmic leaflet.
Interdigitation: the partial penetration of hydrocarbon chains from one leaflet into the opposing leaflet.
Interleaflet coupling: interaction ('crosstalk') between the two leaflets that goes beyond their mere physical proximity. This can be due to any physical mechanism that changes the properties of a given leaflet in response to the properties of the opposing leaflet.
Intrinsic lipid curvature: the preferred shape of a given lipid molecule, determined by the difference in projected area between its headgroup and its hydrocarbon chain.
Lateral pressure profile: depth-dependent variation of the lateral pressure within a lipid-bilayer membrane. Net repulsive interactions in the headgroup regime and membrane interior are balanced by net attractive interactions at the polar–nonpolar interface.
Number asymmetry: the difference in the number of molecules of a particular lipid between the two leaflets of a bilayer membrane.
Polyunsaturated fatty acids (PUFAs): hydrocarbons containing two or more double bonds.
Positive-inside rule: a fundamental principle governing membrane-protein topology which states that, if the loops connecting the transmembrane regions of an integral membrane protein are rich in cationic amino acid residues (specifically, lysine and arginine), these loops are typically located on the cytoplasmic side of the plasma membrane ('inside').
Proteoliposome: a vesicle (liposome) composed of a lipid bilayer with embedded membrane proteins. Proteoliposomes are commonly utilized as model systems to study membrane proteins in a lipid bilayer environment under controlled *in vitro* conditions.

Box 2. Ways to prepare compositionally asymmetric lipid vesicles

Layer-by-layer assembly

Layer-by-layer assembly combines 'oil' and 'water' phases to create lipid bilayers (Figure I). Initially, lipid monolayers form in the oil phase as inverted micelles (water-in-oil topology). Outer-leaflet lipids are then introduced by passing these inverted micelles through a lipid monolayer at the interface with the water phase. This method is often used for generating asymmetric giant unilamellar vesicles (GUVs), with various adaptations [58–60].

Outer-leaflet modification

This technique alters the outer-leaflet lipid composition of pre-existing vesicles of various sizes, achieved by catalyzed lipid exchange or enzymatic modification (Figure I). The most widely used and versatile method for lipid exchange employs cyclodextrin [61]. Alternatively, transfer proteins have been utilized for similar purposes [62]. By contrast, enzymatic procedures chemically modify lipid headgroups. Examples include the use of phosphatidylserine decarboxylase [63,64] to convert PS to PE lipids, or phospholipase D to convert PC into PE or PS [65]. A related technique applies Ca^{2+}-induced hemifusion of GUVs, where GUVs partially fuse with a solid support coated with a single bilayer of a different lipid composition [9]. In this docked state, outer-leaflet lipids can exchange with those of the solid support. Hemifusion can be reversed by chelating Ca^{2+} [66].

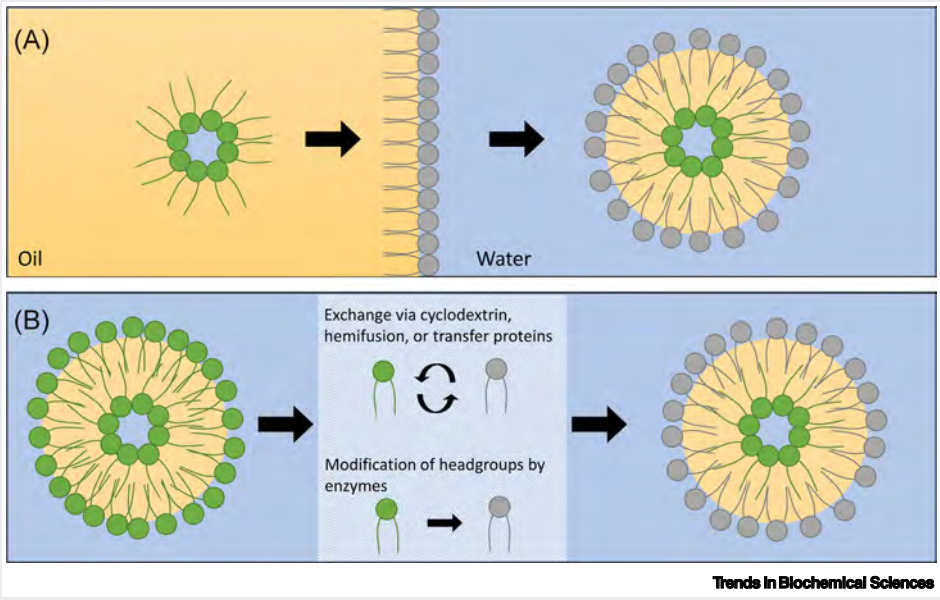

Figure I. Scheme of generating asymmetric lipid vesicles via layer-by-layer assembly (A) or outer-leaflet modification (B). For details, see [67].

Scramblase: an integral membrane protein that catalyzes the spontaneous (free-energy-independent) bidirectional translocation of lipids between the two leaflets.
Topology: the orientation of an integral membrane protein relative to the lipid bilayer. In a broader context, topology can also describe the number and relative orientation of the protein's transmembrane (TM) segments.

First, both PS and PI are anionic, which contributes to a charge imbalance between the two leaflets. Second, PS, PE, and PEp can form intermolecular hydrogen bonds [12]. By contrast, the only hydrogen-bond former among the prevailing outer-leaflet lipids is the amino-group-carrying SM. Both headgroup charge and hydrogen-bonding capacity influence lipid packing and dynamics, albeit with different implications. While charge–charge interactions are responsible for isotropic, long-range effects, hydrogen bonding is strongly directional and operates over shorter distances, thus conferring greater specificity. Despite their short-range nature, hydrogen bonds have been shown to be the primary drivers of PS packing, even in the absence of additional salt, when charge–charge interactions are strongest [13].

Hydrocarbon-chain asymmetry

While headgroup asymmetry plays a significant role in membrane asymmetry, hydrocarbon-chain asymmetry is also crucial to understand. The inner leaflet of mammalian membranes tends to be rich in **polyunsaturated fatty acids (PUFAs)**, whereas the outer leaflet contains predominantly

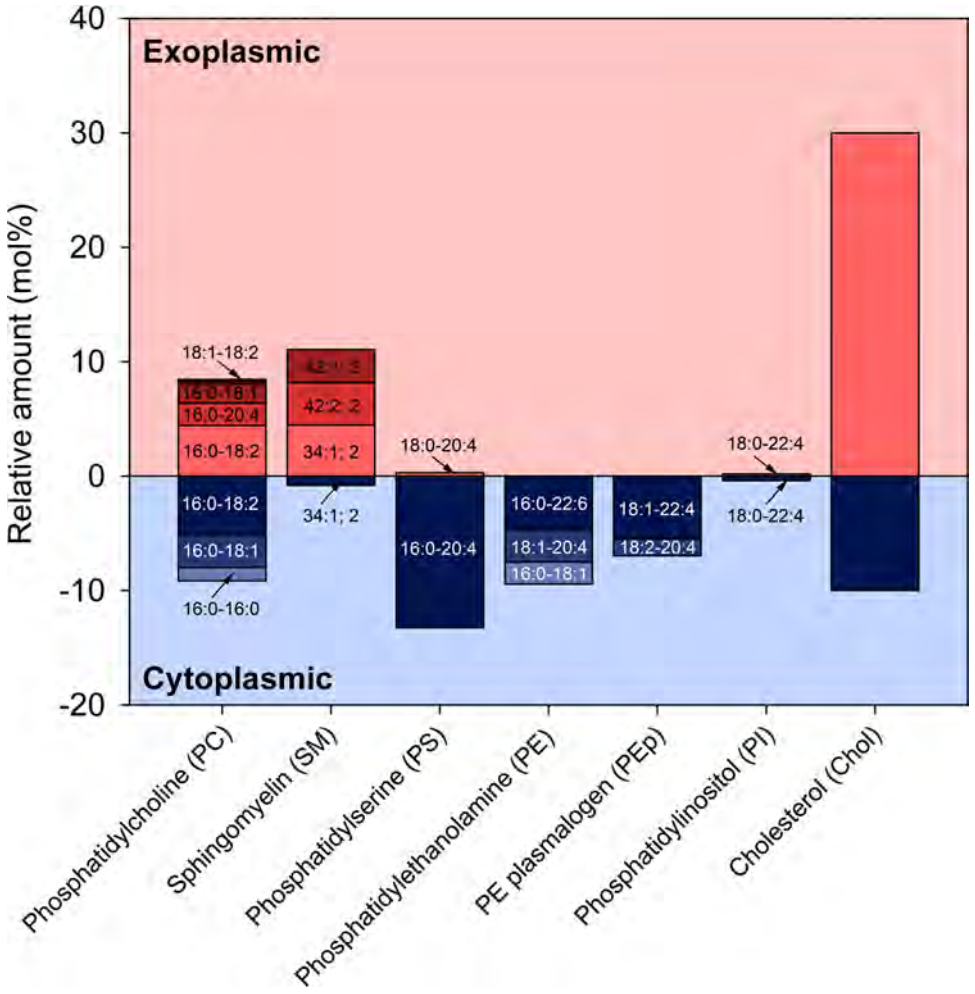

Figure 1. Distribution of major lipid species in human erythrocytes, considering an imbalance of exoplasmic to cytoplasmic phospholipids of 1:2 [5,20]. The total amount of a lipid with a given headgroup is given by adding up its amounts in the exoplasmic and cytoplasmic leaflets. The hydrocarbon-chain data shown have been reduced to the most abundant values, as reported in [5]. Hydrocarbon nomenclature for glycerophospholipids: (number of carbon atoms): (number of double bonds) for *sn*-1 chain and (number of carbon atoms):(number of double bonds) for *sn*-2 chain; for sphingolipids: (number of carbon atoms):(number of double bonds); number of hydroxyl groups.

lipids with saturated hydrocarbon chains (Figure 1) [5]. A notable exception to this rule is PC: while the inner leaflet contains a small fraction (~1.2 mol%) of disaturated PC (palmitic acid, 16:0), all *sn*-2 chains of outer-leaflet PC are unsaturated. As with the headgroups, the hydrocarbon chains also shape the properties of lipid membranes through the diverse conformations that they can adopt, thereby modulating lipid packing. Of particular interest in this regard is the question of whether the properties of one leaflet can influence those of the other. Such **interleaflet coupling** might facilitate key cellular processes such as transmembrane signal transduction beyond the well-studied role of membrane proteins (Figure 2).

Experimental models of asymmetric membranes

Although all-atom molecular dynamics (MD) simulations are capable of studying bilayer membranes made from multicomponent lipid mixtures like the one represented in Figure 1 [5], such

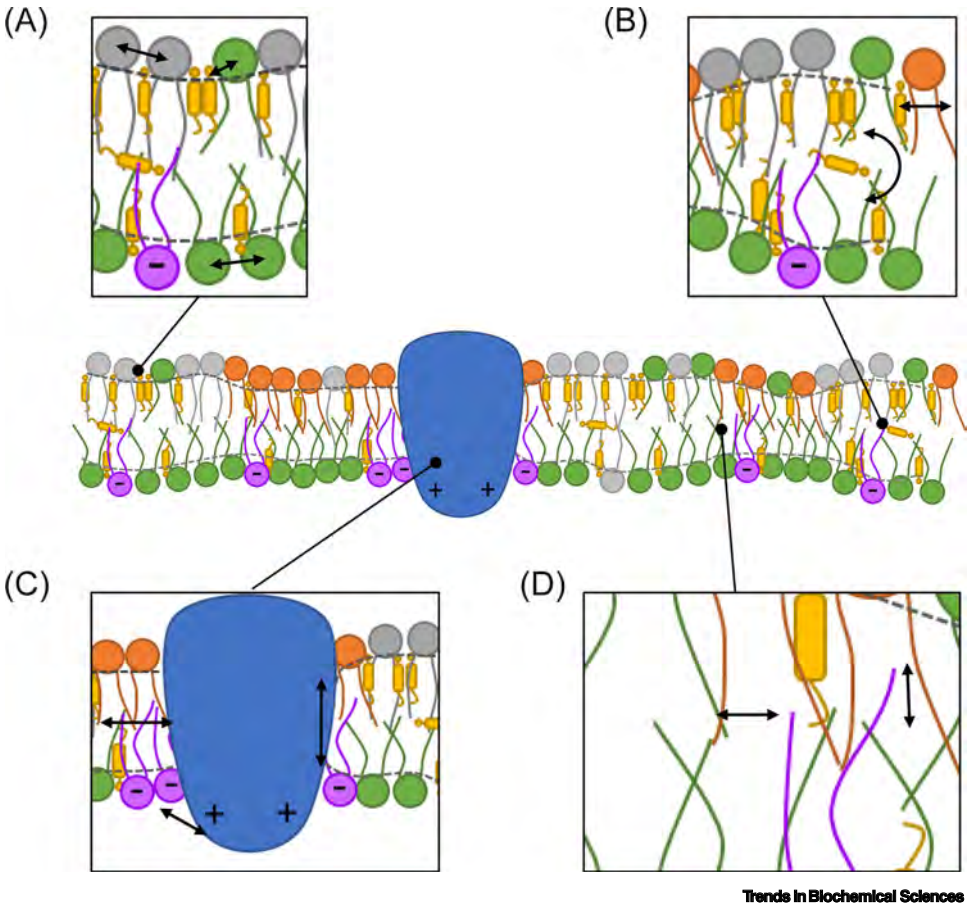

Figure 2. Schematic overview of coupling interactions in asymmetric membranes. (A) Headgroup interactions, including electrostatic interactions and hydrogen bonding. (B) Transversal diffusion ('flip–flop') of cholesterol, driven by number asymmetry and preferential interactions with saturated hydrocarbon chains. (C) Connection between protein folding or function and membrane asymmetry resulting from charge–charge interactions ('positive-inside rule') [47] and elastic coupling to the lipid membrane, including factors such as hydrophobic matching and the lateral pressure profile across the lipid bilayer. (D) Lateral tension in the lipid bilayer's midplane, influenced by splaying and interdigitation of the hydrocarbon chains.

meticulously controlled complex lipid asymmetries are not yet attainable in experiments. Therefore, most recent experimental efforts focus on simple asymmetric lipid vesicles consisting of only two types of lipids. Even so, comparing results across different laboratories or different batches of membrane preparations presents challenges. One reason is the difficulty in controlling membrane composition. For example, outer leaflets might unintentionally contain traces of inner leaflet lipids, or both leaflets could contain remnants from the preparation process (e.g., oil, cyclodextrin, etc.). Thus, it is essential to account for all potential changes to each leaflet's lipid composition, including passive lipid **flip–flop**, which occurs over days to weeks [14].

Evidence of interleaflet coupling

Asymmetric lipid vesicles have been central to studies showcasing the coupling between the two leaflets. For instance, Chiantia and London, using fluorescence correlation spectroscopy (FCS) with uniquely labeled lipids in each leaflet, demonstrated that the presence of SM in the outer leaflet of asymmetric membranes can influence the lateral diffusion of di-unsaturated or mono-unsaturated PC in the inner leaflet [15]. The magnitude of this effect was found to depend on

the extent of hydrocarbon-chain **interdigitation** in mixed-chain lipids. Yet, using fluorescence lifetime imaging (FLIM), the authors found no discernible impact of this interdigitation on structural lipid ordering. This observation underscores the intricate nature of membrane asymmetry and suggests that dynamic interleaflet coupling need not always result in noticeable structural changes.

A particularly noteworthy observation in this context was made using asymmetric lipid vesicles composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), analyzed using differential scanning calorimetry (DSC) [16]. A coupled gel-to-fluid melting transition was observed only when POPE was predominantly present in the inner leaflet and POPC in the outer one, but not in the case of reversed asymmetry. This curious observation was attributed to the different **intrinsic lipid curvatures** of POPC and POPE, which makes POPE favor the inner leaflet's slightly more curved structure. In addition, a potential role for hydrogen bonding between PE lipids in these observations should not be overlooked: while the ethanolamine moieties of neighboring PE headgroups are 'pulled together' by intermolecular hydrogen bonds, such bonds cannot form between the phosphocholine (i.e., trimethylated ethanolamine) moieties of PC headgroups (Figure 2A).

Significant coupling between the inner and outer leaflets – even above the gel-to-fluid phase transition – has recently been confirmed through the analysis of individual leaflet structures using small-angle X-ray and neutron scattering [17]. The inner leaflet of the lipid vesicles employed in this study consisted of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), while the outer leaflet featured either PCs carrying two hydrocarbon chains of different lengths or milk sphingomyelin, a natural mixture rich in lipid species having two chains that differ greatly in their lengths. Consequently, the different-length hydrocarbon chains in the outer leaflet could interdigitate to various extents into the inner leaflet. Milk sphingomyelin displayed the most extensive interdigitation among all lipids studied, leading to lateral condensation of the inner DPPC leaflet. By contrast, all PCs carrying hydrocarbon chains with a less pronounced length mismatch caused lateral expansion of the inner DPPC leaflet. This behavior illustrates the delicate interplay between entropic repulsion, which arises from fluctuating hydrocarbon termini, and van der Waals attraction among the hydrocarbon chains, which is modulated by the extent of interdigitation.

Impact of cholesterol on membrane asymmetry

Cholesterol is the most abundant lipid in mammalian plasma membranes. However, the question of whether cholesterol partitions primarily into the cytoplasmic or into the exoplasmic leaflet of plasma membranes still is highly controversial [18,19]. Recent lipidomics data on human erythrocytes suggest that the cytoplasmic leaflet contains more than twice as many phospholipid molecules as the exoplasmic leaflet [20]. Compensating for this imbalance in the number of phospholipids (**number asymmetry**) implies that cholesterol should reside primarily in the exoplasmic leaflet (Figure 1). While such detailed information is currently unavailable for other mammalian membranes, studies have demonstrated variations both in overall cholesterol concentrations and in leaflet compositions among different species, cell types, and even throughout the life cycle of erythrocytes [4,18,21].

Turning our attention to number asymmetry in plasma membranes, two additional factors influencing the distribution of cholesterol need to be taken into consideration (Figure 2B). First, cholesterol molecules move between the two leaflets much faster (about ten orders of magnitude faster) than do phospholipids [14,22]. Second, cholesterol has a higher affinity for saturated lipids than for unsaturated ones [23]. In a recent theoretical study, Varma and Deserno quantified the effects of lateral stress differences between the two leaflets ('differential stress') that originate

either from number asymmetry or from preferential phospholipid–cholesterol interactions [24]. These authors found that number asymmetry tends to move cholesterol from the overcrowded leaflet (compressive stress) to the undercrowded leaflet (tensile stress), such as to relax differential stress. By contrast, differential stress can also be induced by cholesterol because of its interaction bias favoring the leaflet containing more saturated phospholipids, even if the total number of phospholipids (saturated and unsaturated) is the same in both leaflets. Consequently, the distribution of cholesterol between the two leaflets can become asymmetric for two different reasons: either to equalize area differences between the two leaflets, resulting in 'number asymmetry', or in response to cholesterol's affinity for saturated lipids. It should be noted, however, that these theoretical considerations were limited to a relatively low cholesterol content of 20 mol%.

At higher cholesterol contents in the bilayer, additional effects come into play, such as its ability to induce fluid–fluid domain coexistence. Recent fluorescence microscopy experiments have revealed that highly ordered lipid domains in one leaflet can induce regions of increased order in the opposing leaflet, whose lipids would otherwise mix homogeneously with cholesterol [25]. In line with an earlier report [26], these observations suggest that cholesterol diffuses not only from the originally ordered domains across the bilayer but also laterally into regions of induced order in the opposing leaflet. Recently, it has been speculated that such behavior might stem from increased interfacial tension in the lipid bilayer's midplane [27] (Figure 2D). This view can be easily accommodated within the interdigitation-mediated coupling mechanism discussed above [17]. Finally, the enrichment of cholesterol in the exoplasmic leaflet has an additional, previously unnoticed implication for the overall balance of phospholipids: the number of PC lipids in each leaflet is about equal (Figure 1), contrary to textbook wisdom claiming that PC is more abundant in the outer than in the inner leaflet of mammalian plasma membranes [28].

In addition to the structural coupling between their two leaflets, asymmetric membranes have also been shown to be less elastic than symmetric ones. Several research groups have independently observed that cholesterol-free asymmetric membranes display bending rigidities up to twice as high as those of symmetric membranes formed from either of their cognate leaflets' lipid compositions [29–32]. This phenomenon cannot be explained solely by factors such as differences in spontaneous curvature or area requirements between the two leaflets [33]. Rather, these findings strongly suggest that intraleaflet lipid–lipid interactions, such as charge–charge interactions and hydrogen bonding, must also be considered to fully understand the peculiar properties of asymmetric membranes.

Induced membrane asymmetry and membrane permeation

Even in initially symmetric lipid vesicles, asymmetry can be introduced by adding compounds that self-insert into the lipid bilayer but do not rapidly translocate ('flip–flop') across it. A comprehensive calorimetric study by Heerklotz found that the asymmetry stress induced by the partitioning of alkyl maltoside detergents into the outer leaflet of POPC vesicles is enthalpic in origin [34]. Furthermore, this induced asymmetry stress causes membrane permeation at detergent concentrations much lower than those needed for membrane solubilization. In subsequent work, the same researcher and co-workers demonstrated that the gradual build-up of asymmetry stress can have vastly different consequences, depending on the membrane-interacting compound. On the one hand, asymmetry stress can eventually enable membrane permeation, termed 'cracking in', as observed for the antimicrobial lipopeptide surfactin [35]. On the other hand, asymmetry stress might lead to a condition described as 'staying out', where further membrane insertion is prevented, as these authors found for lysolipids [36]. It is crucial to realize that, even in this seemingly unremarkable scenario, the inner bilayer leaflet must undergo significant structural and dynamic adjustments by laterally expanding and thinning to accommodate

the outer leaflet's area expansion caused by asymmetric membrane insertion. As a result, the asymmetric inclusion of compounds such as lysolipids, amphipathic peptides, and peripheral membrane proteins into the outer leaflet of a plasma membrane may serve as a unique mechanism for conveying information to a cell's interior through changes in the inner leaflet's physical properties.

Effects of membrane asymmetry on proteins

Not only is the asymmetric distribution of lipids between the two leaflets of mammalian plasma membranes interesting in its own right, it also has important implications for the working of membrane proteins. These proteins are essential because they mediate transmembrane signaling and solute transport, thus forming indispensable players in cellular signaling and metabolism. In this discussion we concentrate on membrane proteins and their interplay with collective membrane properties, including the **lateral pressure profile**, membrane thickness, domain formation, and overall lipid composition. We will not consider specific protein–lipid interactions with, for example, cholesterol or 1,2-diacyl-*sn*-glycero-3-phospho-(1-D-*myo*-inositol 4,5-bisphosphate) (PIP_2).

Bilayer partitioning and domain formation

Lin and London [37] have demonstrated that **compositional asymmetry** can significantly modulate the behavior of membrane-inserting proteins. Specifically, the conformation of the pore-forming cholesterol-dependent cytolysin, perfringolysin O (PFO), in asymmetric lipid vesicles diverged from that in symmetric vesicles. Interestingly, when PC was present in the outer leaflet, a higher cholesterol concentration was needed to induce partitioning of PFO from the aqueous phase into the lipid bilayer. By contrast, the presence of PS and PE in the inner leaflet of asymmetric vesicles stabilized a deeply inserted conformation of PFO that did not form pores, despite containing transmembrane segments. The authors speculated that this conformation might represent a crucial intermediate in PFO's pore-formation process. Similarly, Scott *et al*. [38] reported that PS asymmetry promotes the protonation of titratable amino acid side chains of 'pH low insertion peptide' (pHLIP), thereby facilitating its membrane insertion.

Membrane asymmetry not only plays a pivotal role in the water–membrane partitioning of self-inserting proteins, but also exerts control over the sorting of membrane proteins into distinct domains within the lipid bilayer. For example, after reconstitution into simple, symmetric membranes, the nicotinic acetylcholine receptor (AChR) did not preferentially partition into liquid-ordered (Lo) domains [39]. By stark contrast, introducing membrane asymmetry by adding SM to the outer leaflet of initially symmetric lipid bilayers caused AChR to partition into Lo domains, as shown by Förster resonance energy transfer (FRET) measurements [40].

Mechanoresponse of membrane transport

Over two decades ago, Perozo *et al.* [41] posited a pivotal role for membrane asymmetry in triggering the gating of the mechanosensitive channel, MscL. Mechanosensitive channels facilitate the transfer of ions and other solutes across membranes in response to alterations in bilayer tension: a crucial function in physiological processes including touch, hearing, and proprioception in mammalian cells, additionally turgor control in plant cells, and osmoregulation in bacteria. The opening of MscL channels had previously been hypothesized to be prompted by membrane deformations that result in notable changes in the bilayer's lateral pressure profile.

To mimic the process of mechanically triggered channel opening, the researchers constructed vesicles from various phospholipids, such as PC and PE [41]. They then induced asymmetry stress – as discussed in the previous section – by adding lysophosphatidylcholine, which partitioned into the outer membrane leaflet of the lipid vesicles. This way the authors could indeed trigger a mechanoresponse, that is, the opening of MscL channels.

Mechanoresponse of enzymatic activity

We have recently discovered that the activity of the bacterial membrane enzyme outer membrane phospholipase A (OmpLA) is modulated by membrane asymmetry [42]. Specifically, the phospholipid hydrolysis rate of OmpLA in asymmetric membranes was found to be slower than in symmetric membranes (Figure 3A). Of note, this rate diminished further as membrane asymmetry was systematically increased in lipid vesicles following careful control of asymmetric phospholipid distributions.

These observations were rationalized through a simple allosteric model. According to this model, augmenting the compositional asymmetry – and, thereby, the differential stress – between the two bilayer leaflets increases the free-energy gap between OmpLA's inactive and active states. Consequently, transitioning from the inactive to the active state of OmpLA necessitates performing more work against lateral-pressure differences in asymmetric than in symmetric membranes. This study thus offered a quantitative understanding of how membrane asymmetry might impact the activity of an integral membrane enzyme that naturally resides in highly asymmetric membranes. From a functional perspective, it appears reasonable that a phospholipase such as

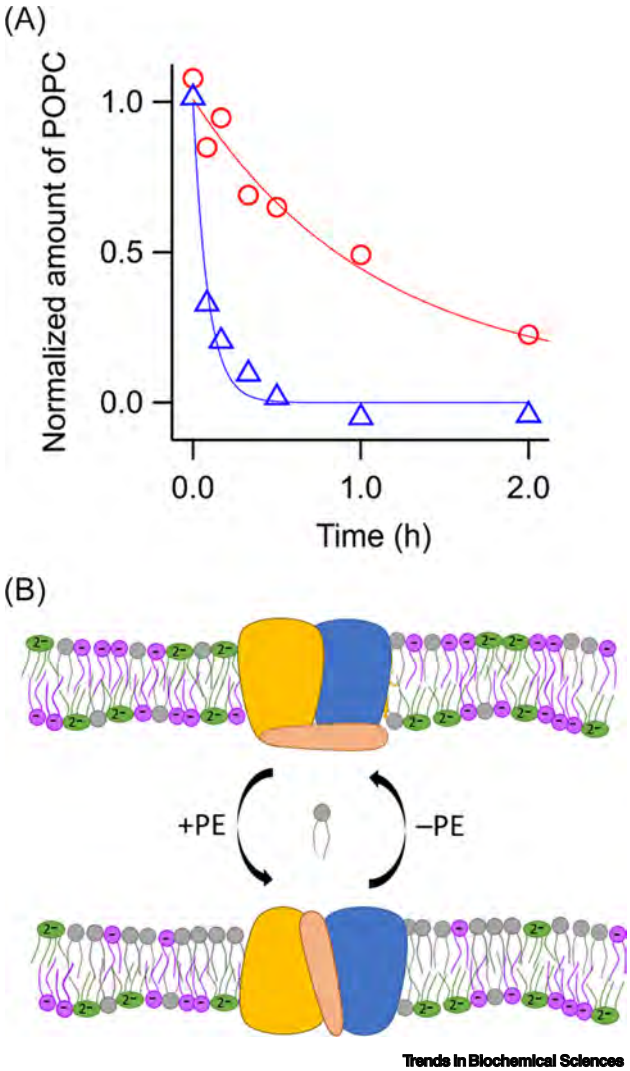

Figure 3. Effects of asymmetric lipid distributions on integral membrane proteins. (A) Normalized relative change in the amount of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) left in symmetric and asymmetric proteoliposomes after triggering the phospholipase activity of the integral outer membrane phospholipase A (OmpLA). Shown are experimental data (symbols) and mono-exponential fits (lines). Symmetric bilayers were made from POPC only, whereas asymmetric bilayers were made from POPC in the inner leaflet and predominantly 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), (~88%) in the outer leaflet. POPC was quantified using high-performance thin-layer chromatography (HPTLC). (B) Schematic showing the reversible topology switching of the N-terminal domain of LacY in response to adding or removing phosphatidylethanolamine (PE).

OmpLA should remain inactive under normal, asymmetric conditions, but become activated when membrane asymmetry is reduced or lost due to membrane perturbation.

Membrane-protein topology

Membrane proteins not only react to changes in asymmetric lipid distributions but also exhibit intriguing asymmetric properties themselves. Dowhan and co-workers discovered that the N-terminal six-helix bundle of lactose permease, LacY, can switch its topological orientation within the bilayer in response to changes in lipid composition [43–45]. Specifically, these authors showed that the topological orientation of this rather large helical bundle can be reversibly changed through the addition or removal of PE (Figure 3B), both in asymmetric vesicles [43] and in live bacteria [44,45]. These surprising findings challenged the expectation that the asymmetric topological orientation of a membrane protein would remain static once the protein is embedded within a lipid-bilayer membrane. Instead, this study unveiled that the membrane's lipid composition exercises a direct influence on the asymmetric **topology** of LacY, allowing for dynamic, thermodynamically controlled adjustments even post membrane insertion in response to changes in lipid composition.

Concluding remarks

The study of asymmetric model membranes has revealed fascinating insights into structural and dynamic properties of lipids and proteins. However, the dynamic coupling between the two leaflets of lipid-bilayer membranes emerges from multiple effects, as depicted in Figure 2. In addition to compositional differences between the two leaflets, differences in the overall number of lipids also affect membrane properties. However, the reported effects of membrane asymmetry on membrane proteins drive research on asymmetric model systems (see Outstanding questions). Achieving success requires not only meticulous and persistent efforts but also the courage to explore uncharted territories. Thus, we must strive to refine the preparation of asymmetric lipid vesicles, ensuring well-defined and controllable lipid compositions in both leaflets. This aspect is crucial for the quantitative analysis of experimental data obtained by using asymmetric membranes. The significance of tightly controlling asymmetry in model membranes is amplified when we consider the various mechanisms of interleaflet coupling, especially when striving to enhance the compositional complexity of these membranes.

At present, cyclodextrin-mediated lipid exchange is the most widely used method for generating asymmetric lipid vesicles, but currently available protocols offer only limited control over the distribution of charged lipids between the two leaflets; exceptions are mildly charged membranes [46]. To address this challenge, an in-depth exploration of the thermodynamics and kinetics of cyclodextrin interactions with different lipids, and potentially the development of compounds with superior lipid-exchange properties, holds the potential to refine and optimize this situation.

Simultaneously, the reconstitution of membrane proteins with controlled orientation within asymmetric membranes needs to be addressed with high priority. First, the uneven amino acid distribution in transmembrane domains along the bilayer's normal axis leads to orientation-specific responses [47,48]. Second, the majority of membrane proteins have a specific functional orientation, such as transport or signal transduction, which necessitates asymmetric reconstitution for detailed analysis. This multifaceted challenge begins with protein production, extraction, and purification. Recent advancements in lipid nanodisc technology hold the promise of significantly enhancing this workflow [49]. Orienting proteins within asymmetric lipid vesicles can be facilitated by leveraging the **positive-inside rule** [47] (Figure 2C). According to this rule, the higher content of anionic lipids in the inner leaflet of mammalian plasma membranes correlates with – and possibly explains – the enrichment of cationic amino acid residues on the cytosolic

Outstanding questions

What factors govern the lipid imbalance in mammalian plasma membranes? With the cytoplasmic leaflet containing roughly twice as many phospholipids as the exoplasmic leaflet, questions arise as to the interplay among flippases, floppases, and scramblases, and the role of cholesterol. What mechanisms are responsible for this asymmetry of phospholipid distribution? Is the asymmetric distribution of cholesterol regulated similarly, or is it solely a consequence of cholesterol's rapid flip–flop in response to phospholipid asymmetry?

How is lipid asymmetry related to ion asymmetry? Plasma membranes create boundaries between aqueous compartments with enormous differences in ion concentrations. The prevalent distributions of inorganic ions hint at critical interactions of PC and SM with Na^+ (and potentially Ca^{2+}) in the exoplasmic leaflet and PE, PS, and PI with K^+ in the cytoplasmic leaflet. How does membrane asymmetry influence these physiologically vital interactions?

Can proteins sense and harness membrane asymmetry? Each leaflet of the plasma membrane has a distinct lipid composition, offering multiple ways for lipids to regulate the functions of both integral and peripheral membrane proteins. How does membrane asymmetry contribute to essential physiological activities such as transmembrane signaling and transport facilitated by integral membrane proteins? Is it possible that asymmetry stress serves as a direct means of transmitting signals across the plasma membrane?

How can we develop more accurate models of asymmetric membranes? Recently there have been significant advances in producing *in vitro* models with compositionally asymmetric lipid bilayers, both with and without proteins. How can we further enhance lipid diversity in these models without compromising the control over composition and asymmetry? Can we devise techniques that allow for the controlled asymmetric incorporation of various types of membrane proteins into the same **proteoliposome**?

side of integral membrane proteins. For example, Manin *et al*. have recently demonstrated that membrane insertion and folding of two bacterial outer membrane proteins into asymmetric lipid vesicles is facilitated if the inner leaflet is enriched in phosphatidylglycerol [50]. Although the authors did not demonstrate the orientation of the studied proteins, their results suggest an alignment of oppositely charged membrane leaflet and protein moieties.

As an alternative method for achieving asymmetric reconstitution of membrane proteins, one could explore the use of water-soluble proteins as fusion tags that cannot traverse membranes but can be removed after asymmetric reconstitution [51]. In the same vein, it is possible to employ polyhistidine affinity tags that bind to immobilized metal matrices to orient membrane proteins [52]. By contrast, a simple but potentially effective way for some membrane proteins might be to selectively target that protein subpopulation that has its ligand-binding site in the outer vesicle leaflet and, thus, accessible to the outside. In this case, protein reconstitution need not be asymmetric, and the other subpopulation having the 'wrong' orientation would simply be ignored, as it does not affect the activity of the target population.

Finally, the use of live cells, coupled with the judicious manipulation of the lipid composition of their outer leaflet [53,54], would circumvent issues related to protein orientation. Nonetheless, the complexity of live cells poses challenges for obtaining quantitative insights. A preferred approach could thus involve a dual strategy, combining membrane-mimetic systems with live cells for a holistic exploration of the effects of membrane asymmetry on membrane proteins.

The path ahead, with twists and setbacks, may feel like navigating a 'long and winding road' [55]. Nevertheless, the process of meticulously assembling asymmetric bilayers as more realistic models of plasma membranes will unveil the reasons why nature invests considerable amounts of free energy in creating and upholding membrane asymmetry. This knowledge is crucial for comprehending lipid maps obtained from both healthy and diseased tissues, and hence holds significant promise for advancing personalized medical treatments. Given these prospects, we firmly believe that this journey is essential and promising.

Acknowledgments

We thank our (former) graduate students and postdocs Barbara Eicher, Moritz P.K. Frewein, Drew Marquardt, Paulina Piller, and Enrico F. Semeraro for their invaluable contributions to membrane-asymmetry research, and Milka Doktorova, Frederick Heberle, Heiko Heerklotz, and Erwin London for numerous enlightening discussions. This research was funded in whole, or in part, by the Austrian Science Funds (FWF) [10.55776/P32514, 10.55776/P27083, 10.55776/I5359], as well as by the Institut Laue–Langevin (ILL) graduate school. For the purpose of open access, the author has applied a CC BY public copyright license to any Author Accepted Manuscript version arising from this submission.

Declaration of interests

No interests are declared.

References

1. Bretscher, M.S. (1972) Asymmetrical lipid bilayer structure for biological membranes. *Nat. New Biol.* 236, 11–12
2. Verkleij, A.J. *et al.* (1973) The asymmetric distribution of phospholipids in the human red cell membrane. A combined study using phospholipases and freeze-etch electron microscopy. *Biochim. Biophys. Acta* 323, 178–193
3. Devaux, P.F. (1991) Static and dynamic lipid asymmetry in cell membranes. *Biochemistry* 30, 1163–1173
4. Kiessling, V. *et al.* (2009) Domain coupling in asymmetric lipid bilayers. *Biochim. Biophys. Acta* 1788, 64–71
5. Lorent, J.H. *et al.* (2020) The mammalian plasma membrane is defined by transmembrane asymmetries in lipid unsaturation, leaflet packing, and protein shape. *Nat. Chem. Biol.* 16, 644–653
6. Rothman, J.E. and Kennedy, E.P. (1977) Asymmetrical distribution of phospholipids in the membrane of *Bacillus megaterium*. *J. Mol. Biol.* 110, 603–618
7. Bogdanov, M. *et al.* (2020) Phospholipid distribution in the cytoplasmic membrane of Gram-negative bacteria is highly asymmetric, dynamic, and cell shape-dependent. *Sci. Adv.* 6, aaz6333
8. Shukla, S. and Baumgart, T. (2021) Enzymatic trans-bilayer lipid transport: mechanisms, efficiencies, slippage, and membrane curvature. *Biochim. Biophys. Acta Biomembr.* 1863, 183534

9. St. Clair, J.R. *et al.* (2017) Preparation and physical properties of asymmetric model membrane vesicles. In *The biophysics of cell membranes. Biological consequences* (Epand, R.M. and Ruysschaert, J.-M., eds), pp. 1–27, Springer
10. Clarke, R.J. *et al.* (2020) Physiological roles of transverse lipid asymmetry of animal membranes. *Biochim. Biophys. Acta* 1862, 183382
11. Neher, E. (1974) Asymmetric membranes resulting from the fusion of two black lipid bilayers. *Biochim. Biophys. Acta Biomembr.* 373, 327–336
12. Marsh, D. (2013) *Handbook of Lipid Bilayers* (2nd edn), CRC Press
13. Petrache, H.I. *et al.* (2004) Structure and fluctuations of charged phosphatidylserine bilayers in the absence of salt. *Biophys. J.* 86, 1574–1586
14. Marquardt, D. *et al.* (2017) 1H NMR shows slow phospholipid flip-flop in gel and fluid bilayers. *Langmuir* 33, 3731–3741
15. Chiantia, S. and London, E. (2012) Acyl chain length and saturation modulate interleaflet coupling in asymmetric bilayers: effects on dynamics and structural order. *Biophys. J.* 103, 2311–2319
16. Eicher, B. *et al.* (2018) Intrinsic curvature-mediated transbilayer coupling in asymmetric lipid vesicles. *Biophys. J.* 114, 146–157
17. Frewein, M.P.K. *et al.* (2022) Interdigitation-induced order and disorder in asymmetric membranes. *J. Membr. Biol.* 255, 407–421
18. Liu, S.-L. *et al.* (2017) Orthogonal lipid sensors identify transbilayer asymmetry of plasma membrane cholesterol. *Nat. Chem. Biol.* 13, 268–274
19. Courtney, K.C. *et al.* (2018) Comment on 'Orthogonal lipid sensors identify transbilayer asymmetry of plasma membrane cholesterol'. *eLife* 7, e38493
20. Doktorova, M. *et al.* (2023) Cell membranes sustain phospholipid imbalance via cholesterol asymmetry. *bioRxiv*, Published online July 31, 2023. https://doi.org/10.1101/2023.07.30.551157
21. Ivanov, I.T. (2007) Allometric dependence of the life span of mammal erythrocytes on thermal stability and sphingomyelin content of plasma membranes. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 147, 876–884
22. Gu, R.-X. *et al.* (2019) Cholesterol flip-flop in heterogeneous membranes. *J. Chem. Theory Comput.* 15, 2064–2070
23. Iaea, D.B. and Maxfield, F.R. (2015) Cholesterol trafficking and distribution. *Essays Biochem.* 57, 43–55
24. Varma, M. and Deserno, M. (2022) Distribution of cholesterol in asymmetric membranes driven by composition and differential stress. *Biophys. J.* 121, 4001–4018
25. Enoki, T.A. and Feigenson, G.W. (2022) Improving our picture of the plasma membrane: rafts induce ordered domains in a simplified model cytoplasmic leaflet. *Biochim. Biophys. Acta* 1864, 183995
26. Lin, Q. and London, E. (2015) Ordered raft domains induced by outer leaflet sphingomyelin in cholesterol-rich asymmetric vesicles. *Biophys. J.* 108, 2212–2222
27. Feigenson, G.W. *et al.* (2023) An unexpected driving force for lipid order appears in asymmetric lipid bilayers. *J. Am. Chem. Soc.* 45, 21717–21722
28. Buehler, L. (2015) *Cell Membranes*, Taylor & Francis Group
29. Elani, Y. *et al.* (2015) Measurements of the effect of membrane asymmetry on the mechanical properties of lipid bilayers. *Chem. Commun.* 51, 6976–6979
30. Lu, L. *et al.* (2016) Membrane mechanical properties of synthetic asymmetric phospholipid vesicles. *Soft Matter* 12, 7521–7528
31. Rickeard, B.W. *et al.* (2020) Transverse lipid organization dictates bending fluctuations in model plasma membranes. *Nanoscale* 12, 1438–1447
32. Frewein, M.P.K. *et al.* (2023) Distributing aminophospholipids asymmetrically across leaflets causes anomalous membrane stiffening. *Biophys. J.* 122, 2445–2455
33. Hossein, A. and Deserno, M. (2020) Spontaneous curvature, differential stress, and bending modulus of asymmetric lipid membranes. *Biophys. J.* 118, 624–642
34. Heerklotz, H. (2001) Membrane stress and permeabilization induced by asymmetric incorporation of compounds. *Biophys. J.* 81, 184–195
35. Fan, H.Y. *et al.* (2014) Utilizing zeta potential measurements to study the effective charge, membrane partitioning, and membrane permeation of the lipopeptide surfactin. *Biochim. Biophys. Acta* 1838, 2306–2312
36. Fan, H.Y. *et al.* (2016) 'Staying out' rather than 'cracking in': asymmetric membrane insertion of 12:0 lysophosphocholine. *Langmuir* 32, 11655–11663
37. Lin, Q. and London, E. (2014) The influence of natural lipid asymmetry upon the conformation of a membrane-inserted protein (perfringolysin O). *J. Biol. Chem.* 289, 5467–5478
38. Scott, H.L. *et al.* (2019) Phosphatidylserine asymmetry promotes the membrane insertion of a transmembrane helix. *Biophys. J.* 116, 1495–1506
39. Bermúdez, V. *et al.* (2010) Partition profile of the nicotinic acetylcholine receptor in lipid domains upon reconstitution. *J. Lipid Res.* 51, 2629–2641
40. Perillo, V.L. *et al.* (2015) Transbilayer asymmetry and sphingomyelin composition modulate the preferential membrane partitioning of the nicotinic acetylcholine receptor in Lo domains. *Arch. Biochem. Biophys.* 591, 76–86
41. Perozo, E. *et al.* (2002) Physical principles underlying the transduction of bilayer deformation forces during mechanosensitive channel gating. *Nat. Struct. Biol.* 9, 696–703
42. Piller, P. *et al.* (2023) Allosteric modulation of integral protein activity by differential stress in asymmetric membranes. *PNAS Nexus* 2, pgad126
43. Vitrac, H. *et al.* (2015) Dynamic membrane protein topological switching upon changes in phospholipid environment. *Proc. Natl. Acad. Sci. U. S. A.* 112, 13874–13879
44. Bogdanov, M. and Dowhan, W. (2012) Lipid-dependent generation of dual topology for a membrane protein. *J. Biol. Chem.* 287, 37939–37948
45. Bogdanov, M. *et al.* (2002) A polytopic membrane protein displays a reversible topology dependent on membrane lipid composition. *EMBO J.* 21, 2107–2116
46. Markones, M. *et al.* (2018) Engineering asymmetric lipid vesicles: accurate and convenient control of the outer leaflet lipid composition. *Langmuir* 34, 1999–2005
47. von Heijne, G. (2006) Membrane-protein topology. *Nat. Rev. Mol. Cell Biol.* 7, 909–918
48. Sharpe, H.J. *et al.* (2010) A comprehensive comparison of transmembrane domains reveals organelle-specific properties. *Cell* 142, 158–169
49. Glueck, D. *et al.* (2022) Electroneutral polymer nanodiscs enable interference-free probing of membrane proteins in a lipid-bilayer environment. *Small* 18, e2202492
50. Machin, J.M. *et al.* (2023) Protein–lipid charge interactions control the folding of outer membrane proteins into asymmetric membranes. *Nat. Chem.* 15, 1754–1764
51. Shih, Y.-P. *et al.* (2005) Self-cleavage of fusion protein *in vivo* using TEV protease to yield native protein. *Protein Sci.* 14, 936–941
52. Bornhorst, J.A. and Falke, J.J. (1973) Purification of proteins using polyhistidine affinity tags. In *Methods in Enzymology: Part D: Enzyme Structure*, pp. 245–254, Academic Press
53. Suresh, P. *et al.* (2021) Phospholipid exchange shows insulin receptor activity is supported by both the propensity to form wide bilayers and ordered raft domains. *J. Biol. Chem.* 297, 101010
54. Yang, G.-S. *et al.* (2023) Lipid-driven interleaflet coupling of plasma membrane order regulates FcεRI signaling in mast cells. *Biophys. J.*, Published online August 1, 2023. https://doi.org/10.1016/j.bpj.2023.07.027
55. Lennon, J. and McCartney, P. (1970) The long and winding road. In *Let It Be*, Apple Records
56. Alberts, B. *et al.* (2014) *Molecular Biology of the Cell*, Garland
57. Schütz, G.J. and Pabst, G. (2023) The asymmetric plasma membrane-A composite material combining different functionalities? Balancing barrier function and fluidity for effective signaling. *Bioessays* 45, e2300116
58. Pautot, S. *et al.* (2003) Engineering asymmetric vesicles. *Proc. Natl Acad. Sci. USA* 100, 10718–10721
59. Hamada, T. *et al.* (2008) Construction of asymmetric cell-sized lipid vesicles from lipid-coated water-in-oil microdroplets. *J. Phys. Chem. B* 112, 14678–14681
60. Karamdad, K. *et al.* (2016) Studying the effects of asymmetry on the bending rigidity of lipid membranes formed by microfluidics. *Chem. Commun.* 52, 5277–5280
61. Doktorova, M. *et al.* (2018) Preparation of asymmetric phospholipid vesicles for use as cell membrane models. *Nat. Protoc.* 13, 2086–2101

62. Holzer, M. *et al.* (2010) Lipid transfer mediated by a recombinant pro-sterol carrier protein 2 for the accurate preparation of asymmetrical membrane vesicles requires a narrow vesicle size distribution: a free-flow electrophoresis study. *Langmuir* 26, 4142–4151
63. Drechsler, C. *et al.* (2018) Preparation of asymmetric liposomes using a phosphatidylserine decarboxylase. *Biophys. J.* 115, 1509–1517
64. Denkins, Y.M. and Schroit, A.J. (1986) Phosphatidylserine decarboxylase: generation of asymmetric vesicles and determination of the transbilayer distribution of fluorescent phosphatidylserine in model membrane systems. *Biochim. Biophys. Acta* 862, 343–351
65. Takaoka, R. *et al.* (2018) Formation of asymmetric vesicles via phospholipase D-mediated transphosphatidylation. *Biochim. Biophys. Acta* 1860, 245–249
66. Enoki, T.A. and Feigenson, G.W. (2019) Asymmetric bilayers by hemifusion: method and leaflet behaviors. *Biophys. J.* 117, 1037–1050
67. Krompers, M. and Heerklotz, H. (2023) A guide to your desired lipid-asymmetric vesicles. *Membranes* 13, 267