Biological Membrane Organization and Cellular Signaling

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ABSTRACT

To execute their many vital functions, cell membranes are highly organized. Here, we review how membrane structure shapes signal transduction across membranes. Recent experimental and computational advances have shed significant light on mechanisms linking the function of membrane signaling proteins to the composition and physical properties of the membrane lateral structures in which they are embedded. We provide an overview of the structural characteristics of membranes containing heterogeneous mixtures of lipids and other molecules, and summarize work on "raft" domains in model and cell membranes, as determined by microscopy, spectroscopy, neutron scattering and computer simulations. We discuss the principles of partitioning of proteins into membranes and how the structure, dynamics and function of membrane-embedded and peripheral proteins can be modulated by specific membrane components and physical properties of membranes and raft domains. Finally, we discuss challenges and future directions towards a molecular-level understanding of how membrane organization gives rise to various context-dependent cellular signaling.

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1. INTRODUCTION

Cell membranes, which are composed of a myriad of primarily lipid and protein molecules, are an indispensable element of prokaryotic and eukaryotic cells. Hundreds of types of cell membrane lipids have been found, and these can be roughly divided into three classes: phospholipids, glycolipids and sterols. Many of these lipids have now been recognized to be directly involved in cell signaling. Proteins typically occupy ~50% of the plasma membrane surface,[1] and again consist of three main types: integral, peripheral, and lipid-anchored. These proteins are the main protagonists of a variety of membrane functions, including molecular transport and signal transduction. In addition to the lipids and proteins, cell membranes also contain other biological molecules, such as, for example, saccharides displayed on the extracellular membrane surfaces that are essential for cell-cell recognition in eukaryotes.[2]

As well as defining the cell boundaries, membranes function as an essential medium for cellular communication. The function of biological systems is usually governed by their structures and dynamics, and cell membranes are no exception to this. A full understanding of the biological functions of cell membranes requires detailed knowledge about how lipids and proteins are organized and combine to determine the physicochemical characteristics of cell membranes. However, due to their dynamic, heterogeneous and relatively disordered nature, arguably much less is known about membrane structure and organization than for many other biomolecular systems.

The formulation of the lipid bilayer model by Gorter and Grendel in 1925 signified the beginning of the modern description of cell membrane structures.[3] Later, with the development of immunoelectron microscopy, proteins were found to span the lipid bilayer.[4] Based on these findings, in 1972 Singer and Nicolson proposed their influential 'fluid mosaic' model[5] that characterizes cell membranes as a randomly mixed liquid of lipids in which proteins are embedded. However, mixtures of unlike molecules are never randomly mixed, but instead show preferences for compositionally

distinct clusters, with either like neighbors being favored, or, as in the case of cholesterol in a membrane, for which the cholesterol-cholesterol interaction is unfavorable, with unlike neighbors being favored.[6] Thus, whereas entropy works toward random mixing, interaction energies between different types of neighboring lipid molecule favor non-random distributions. When the differences of interaction energies are large enough to overcome entropic mixing, distinct phases can coexist. This difference in interaction energy needs only to be very small, i.e., ~k_BT, for this to occur.[7]

Perhaps unsurprisingly, then, although early membrane models had assumed membrane lipids to be uniformly mixed, experimental evidence soon emerged suggesting that lipids could laterally segregate to form domains with distinct structural characteristics.[8] In native sarcoplasmic reticulum membranes, quasicrystalline clusters were detected at temperatures below 25°C, which also provided a microscopic explanation for the breaks observed in the Arrhenius plots of enzymatic activity at ~20°C for a number of enzymes.[9] X-ray diffraction studies also confirmed the coexistence of fluid and ordered lipids in two separate lamellar phases in microsomal lipids of *Tetrahymena*.[10,11] To reconcile these new findings, in 1976 the fluid mosaic model was extended to accommodate the possible existence of frozen or semi-frozen islands of less mobile lipids in a sea of fluid lipids.[12]

In the late 1970s, the importance of membrane environment in mediating protein-protein interactions also started to draw attention. For example, in 1976, using molecular field theory a model was formulated to describe how protein aggregation might be mediated by non-specific interactions between proteins and the surrounding lipids.[13] Then, in 1982, evidence was presented to show the existence of inhomogeneity in the lateral distribution of lipids. It was further postulated that the formation of this inhomogeneity might be driven by the segregation of either lipids or proteins that could order the packing of surrounding lipids.[14]

In the 1990s, detergent extraction experiments revealed that cellular membranes are laterally heterogeneous on the μm length scale and below, and, following extraction with detergents at lower

temperatures can be separated into two fractions: detergent-resistant membranes (DRMs) and detergent-soluble membranes (DSMs). DRMs and DSMs have distinct compositions, with DRMs enriched in cholesterol, sphingolipids and glycosylphosphatidylinositol (GPI)-anchored proteins.[15-17] These DRMs led to the proposal of 'lipid rafts' by Simons and Ikonen in 1997 to explain the lateral membrane inhomogeneity.[18] Almost ten years later, a consensus definition of lipid rafts was put forward,[19] describing them as being "heterogeneous, dynamic, cholesterol- and sphingolipid-enriched membrane nanodomains (10-200 nm) that have the potential to form microscopic domains (>300 nm) upon clustering induced by protein-protein and protein-lipid interactions". Meanwhile, in various model membranes with compositions mimicking cell membranes, increasing evidence was found that certain lipids interact preferentially with one another to form lateral membrane domains as a result of liquid-liquid phase segragation.[20]

The lipid raft hypothesis proposes that the selective clustering of certain lipids leads to the formation of relatively ordered membrane domains that can further recruit other proteins and lipids. The concept of lipid rafts provides a framework for understanding how membranes carry out many of their vital biological functions. Through the selective recruitment of certain proteins while excluding others, rafts can control protein-protein interactions and facilitate the formation of functional protein-protein or protein-lipid complexes in membranes. Moreover, the distinct physical properties of rafts and non-raft surroundings, such as membrane curvature, hydrophobic thickness, and tension, can also modulate the functional states of certain proteins. In this manner, lipid rafts are thought to mediate a range of cellular processes. Two types of lipid rafts have been proposed:[21] planar lipid rafts and caveolae that are flask-shaped invaginations containing caveolin proteins. This Review will discuss solely the non-caveolar type, focusing on the interplay between lateral membrane organization and signal transduction.[22]

Many questions about lipid rafts, including their size, composition and physical nature, remain hotly debated. At the heart of these questions lie molecular forces underlying the formation, stability

and fluctuation of raft domains, the characterization of which is required for a mechanistic understanding of the principles of membrane organization and its modulatory effects on the function of the membrane and membrane-associated proteins that are involved in cell signaling. These molecular forces include van der Waals, electrostatics, solvation, and steric effects acting on molecules located in raft, non-raft domains or the domain boundaries. However, even after more than two decades since the inception of the lipid raft hypothesis, a molecular-level understanding of how lipid rafts are formed and how they contribute to cell signaling, presumably through controlling membrane protein function, is still lacking.

Cell membranes are multiscale systems in which structural elements organize at different hierarchical levels to give rise to their complex biological functions. In this context, nanoscopic lipid rafts form an important bridge that connects molecular-level structures of proteins and lipids with cellular-level signaling processes. However, due to this mesoscale nature, the structures of lipid rafts are not well defined, and extremely challenging to characterize. For two recent reviews on the development of improved biochemical and biophysical technologies to study membrane organization, see Refs.[23,24] Here, we review recent progress in elucidating the role of membrane organization, and particularly lipid rafts, in cellular signaling. Specifically, we focus on the emerging molecular mechanisms underlying how various molecular forces and physical interactions govern membrane properties and raft formation, and how transverse and lateral membrane structures in turn regulate the function of associated signaling proteins.

2. MEMBRANE LATERAL ORGANIZATION

2.1 Lipids, Sterols, Carotenoids and Membrane Properties

The compositions of cell membranes are immensely complex and, during the cell cycle, dynamic. Even considering only lipids, there are hundreds of distinct species involved.[25] Further, specific raft domains have distinct compositional profiles depending on their location and functional state.[26] Thus, a critical step in uncovering the nature (and function) of raft domains is to understand their chemical compositions. Emerging lipidomic techniques are a promising tool for the identification and quantification of various molecular components in specific regions of the membranes at multiple time points,[25,27] and these have the potential to address fundamental questions about the roles of specific proteins and lipids in cellular signaling. Indeed, improvements in chromatographic separation and high-resolution mass spectrometry have enabled the identification of the lipid compositions of various eukaryotic membranes, revealing the differential compositions of cellular plasma and organelle membranes.[28] Based on the metabolic differential labeling of cells with light or heavy isotopic amino acids (Stable Isotope Labeling of Amino-acids in Culture, SILAC), a quantitative proteomic approach has been devised to discriminate raft from non-raft proteins and to construct comprehensive interaction networks of proteins that form transient raft signaling complexes.[29] This type of lipid compositional analysis was also instrumental in guiding the design and execution of the neutron scattering experiments that detected nanoscopic lateral membrane structures *in vivo*.[30]

Mapping out a **phase diagram** (*i.e.*, the phase dependence on lipid composition) for a specific lipid mixture represents an essential step towards understanding the phase behavior of the membrane. Phase diagrams show conditions at which thermodynamically distinct phases occur and coexist at equilibrium, and can thus reveal the underlying molecular forces that drive the domain formation.

Although whether fluid-fluid phase separation occurs in binary lipid mixtures is still debated,[31,32] phase diagrams for ternary lipid mixtures have been well established. Model membranes of ternary or four-component lipid mixtures can form both 'large' (micron sized) and 'small' (nanoscopic) domains. These lipid mixtures typically are composed of an unsaturated low-melting temperature lipid, a saturated high-melting temperature lipid and cholesterol. They can separate into

two distinct liquid phases: a liquid-disordered (L_d) phase comprising primarily unsaturated lipid species that is more fluid and disordered, and a liquid-ordered (L_o) phase enriched in saturated lipids and cholesterol that is more packed and ordered. Phase diagrams point to the thermodynamic feasibility of the coexistence of L_o/L_d phases in both giant unilamellar vesicles (GUVs)[20] and solid-supported bilayers of ternary lipid mixtures.[33,34] These diagrams also provided guidance for the choice of lipid compositions and the design of deuteration schemes in the recent neutron studies of nanoscopic membrane domains in model membrane systems.[35,36]

L_o and L_d model membranes have been widely used as surrogates to study the phase behaviors of cell membranes. However, it is worth noting that model and cell membranes differ in many ways. Raft domains are far more complex than in the L_o/L_d model systems, and not all phase behaviors observed in the simple systems at thermodynamic equilibrium can be directly translated into those in cell membranes. For example, it has been shown that some raft transmembrane (TM) proteins cannot reconstitute into L_o model membranes.[37,38]

Given the compositional complexity of cell membranes, it will be extremely difficult, and maybe even impossible, to extend phase diagram mapping to more biologically relevant systems of more than four lipid components, not to mention with other protein and saccharide components. It remains to be seen whether/how this compositional complexity may give rise to phase behaviors that are different from those occurring in the simple model membrane systems.[39] Nevertheless, although many detailed aspects obtained from the model systems may not be directly transferrable to biological systems, the fundamental principles governing the phase separation and the preferential association of proteins with specific membranes domains should be the same for both model and cell membranes.

Cholesterol, which is a type of sterol, is an essential component of lipid rafts in mammalian cell membranes, determining raft stability and organization.[40] Cholesterol alters physical characteristics of membranes, such as decreasing their fluidity and influencing permeability,[41,42] and also may regulate membrane protein function.[43] Moreover, apart from cholesterol, other sterols have

evolved to become essential components of certain biomembranes. Two of particular interest are ergosterol, which is found in the membranes of lower eukaryotes like fungi, and lanosterol, which is the major sterol constituent of prokaryotic membranes. Experiments have also shown that sterols that support ordered domain formation are both necessary and sufficient for the formation of membrane domains in the prokaryote *B. burgdorferi*.[44,45]

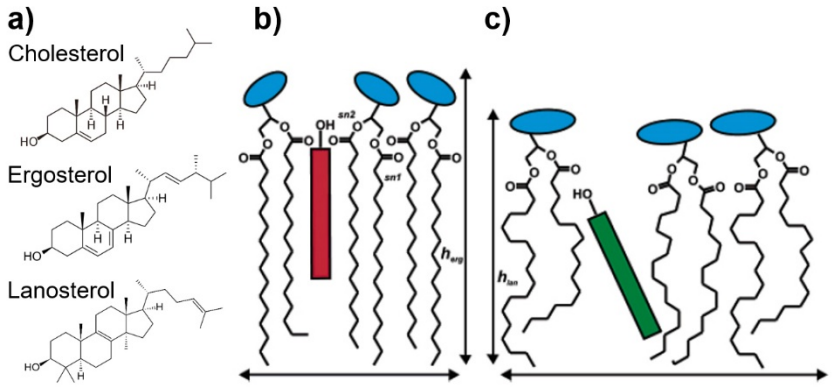

Figure 1. a) Cholesterol, ergosterol and lanosterol. Sketches depicting effects of b) ergosterol and c) lanosterol on a dipalmitoylphosphatidylcholine (DPPC) lipid bilayer as derived from MD simulations. In the DPPC-ergosterol system, the lipid acyl chains become more condensed (with a smaller area per lipid) and straighter (with more *trans* conformations), and the bilayer thickens. Ergosterol is positioned closer to the head-group region than lanosterol, and is less tilted. The behavior of cholesterol is intermediate between ergosterol and lanosterol. Adapted with permission from Ref.[46] Copyright 2007 American Chemical Society.

The chemical structures of cholesterol, ergosterol and lanosterol are very similar (Figure 1a). The main difference between the three is the number of methyl groups (lanosterol has three) that protrude from their otherwise flat faces. In addition, ergosterol has two conjugated C=C bonds in one of its steroid rings. Although these differences are very small, only cholesterol has been selected by

evolution to be a major component of mammalian cell membranes. Moreover, the conversion of lanosterol to cholesterol in mammalian cells is a long and energetically expensive path (18 enzymatic steps). An intriguing question, then, is, given its structural similarity to its precursors, why cholesterol was chosen by evolution for mammalian cell membranes.

Of particular interest in the present context, although cholesterol, ergosterol, and lanosterol have very similar chemical structures, only cholesterol and ergosterol can promote raft formation, whereas lanosterol has limited capacity to do so. These differences must originate in the way these sterols interact with and modify the physical properties of membranes.

Much information on detailed membrane physics has been provided by molecular dynamics (MD) simulation, which integrates Newton's equation of motion to calculate the trajectories of each atom in a molecular system. MD has become a very powerful tool to elucidate the structure and dynamics of biomolecular systems, and this is arguably especially true for intrinsically heterogeneous and disordered biomembranes. MD simulations of cholesterol, ergosterol and lanosterol in a dipalmitoylphosphatidylcholine (DPPC) lipid bilayer have provided atomistic detail on how these sterols modify the physical properties of membranes.[46] The differences between ergosterol and lanosterol observed in MD are schematized in Figure 1. Cholesterol is intermediate between the two.

All three sterols order the DPPC acyl tails and all three also condense the membrane. However, the degree to which they condense the membrane varies. The smooth face and tail unsaturation in ergosterol leads to this sterol interacting more closely with the lipids and tighter packing of the lipids with each other. Thus, ergosterol condenses the membrane and reduces the area per lipid. Furthermore, in the presence of ergosterol the lipids are in a higher proportion of *trans* conformers, the membrane is thicker (consistent with the reduction of the area per lipid) and the lipid order parameters are higher. Ergosterol is also aligned more closely with the membrane normal (less tilted) and also is positioned closer to the membrane/water interface. In contrast, the face of lanosterol is relatively rough, due to the presence of the above-mentioned methyl groups, leading to a less close interaction of the steroid

rings with the lipid acyl chains. Therefore, lanosterol packs the lipid chains less well and, consistent with this increased disorder, the sterol is more tilted with respect to the membrane normal. The above findings may explain why ergosterol is the most efficient of the three sterols at promoting raft formation, and may also provide part of the explanation as to why cholesterol is evolutionarily preferred over lanosterol in mammalian cell membranes.

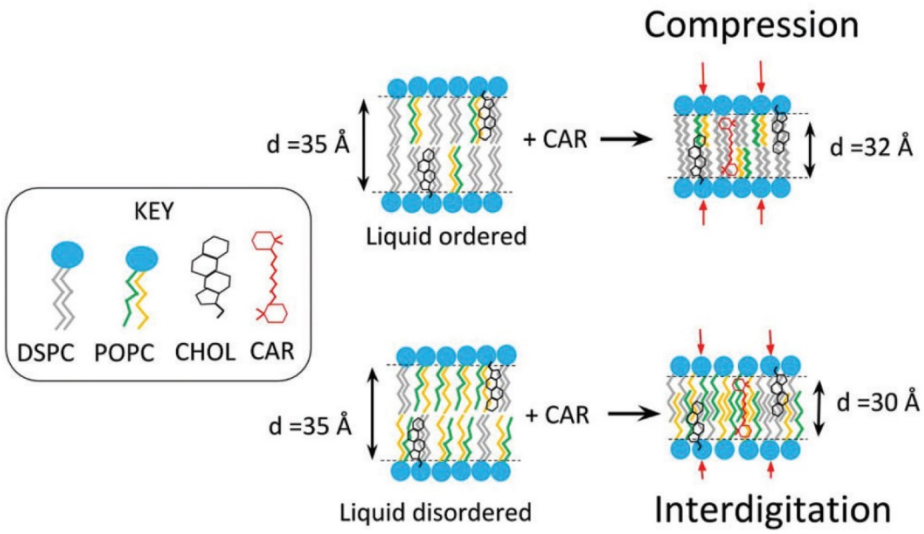

Figure 2. Carotenoids (CAR) thin the lipid bilayer via two competing mechanisms: the lipid tail compression (top) or the lipid tail interdigitation (bottom). Reproduced from Ref.[47] with permission from the PCCP Owner Societies.

Lipid rafts were originally thought to exist only in eukaryotic cells, because their formation is critically dependent on cholesterol, which is absent in prokaryotic membranes. However, growing evidence has shown that bacteria nonetheless contain raft-like structures called 'functional membrane microdomains' (FMMs), which compartmentalize cellular processes, such as signal transduction and membrane trafficking.[48] Many bacterial species produce carotenoids, and a carotenoid-deficient *Staphylococcus aureus* mutant showed mislocalization of FMM-related proteins, suggesting a

structural role of carotenoids in the formation of FMMs.[49,50] Carotenoids lack the sterol ring of cholesterol. In addition, unlike cholesterol, carotenoids span both leaflets of a lipid bilayer, and thus may mediate direct signal coupling across leaflets. It is therefore of interest to investigate whether, despite these differences, carotenoids have a similar condensation effect on lipid membranes as does cholesterol. Again, MD has provided an answer,[47] schematized in Figure 2. Carotenoids appear to vertically condense (thin) the lipid bilayer. This bilayer thickness reduction can occur *via* two distinct mechanisms: lipid-tail compression or lipid-tail interdigitation. Carotenoids compress an L_o bilayer primarily *via* the compression mechanism - the lipid tails bend so that both leaflets become thinner. In contrast, carotenoids compress an L_d bilayer mainly *via* the interdigitation mechanism - the lipid tails of both leaflets interdigitate so that the bilayer becomes thinner.

Cholesterol strengthens lipid bilayers by intercalating its planar steroid ring between lipids, thus enhancing the ordering and mechanical stability, increasing the thickness, and decreasing the area per lipid of the bilayers. The addition of carotenoids to an L_d bilayer shows a similar effect on the lipid ordering. However, along with this increase in order, a decrease in thickness and an increase in area per lipid are also observed, which are in contrast to what occurs to the membrane when cholesterol is added, with increased thickness and decreased area per lipid. Carotenoids vertically compress the membrane, while cholesterol induces a lateral condensation. In an L_o bilayer, carotenoids decrease the order yet also increase the area per lipid and decrease the bilayer thickness. Both carotenoids and cholesterol appear to condense a more fluid-like membrane while 'loosening' a more gel-like membrane. The MD results indicate that carotenoids enhance lipid-tail interdigitation across leaflets in the L_d bilayer, which may act as a mechanism for signal transduction across the membrane. These common effects of carotenoids and cholesterol suggest that carotenoids might indeed function as a cholesterol 'surrogate' in organisms whose membranes contain no cholesterol, regulating membrane structure and fluidity and facilitating membrane functioning.

Lipid rafts are a manifestation of fluid-fluid coexistence, whereas these nanoscopic domains are clearly different from phase separation in a thermodynamic sense. We now consider membrane structural, physical and mechanical properties, including lateral pressure (compressibility), bending rigidity and lateral diffusion. These properties often define the distinct phase state of the membrane, and, together with specific molecular interactions, can mediate how proteins and lipids organize to form cellular signaling complexes at the membrane.[51,52] The differences in these properties between domains have also been exploited to detect membrane lateral organization.[53,54] For example, recently ^{1}H-^{13}C solid-state NMR revealed L_o/L_d phase coexistence in a lipid mixture of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), DPPC and cholesterol based on differential local acyl chain packing in the two lipid phases.[55]

Lateral pressure determines important macroscopic and measurable membrane properties, such as the surface tension, the spontaneous curvature and associated bending rigidity. Although the membrane as a whole is at equilibrium, its different regions may deviate from an ideal (lowest energy) configuration, which creates stresses within the bilayer. Lateral pressure profiles describe the distribution of these local pressure stresses as a function of the distance into the center of the bilayer. For fluid isotropic membranes, the stress tensor, σ reduces to two components: $P_L = -(\sigma_{xx} + \sigma_{yy})/2$ (in plane) and $P_N = -\sigma_{zz}$ (normal). The lateral pressure profile is then $\pi(z) = P_L(z) - P_N(z)$. This quantity exemplifies the connection between microscopic models and continuum theories,[56] as its first and second moments result in the bending modulus times the spontaneous curvature and minus the Gaussian modulus, respectively.

Lateral pressure profiles and anisotropic stresses within a membrane are not easily measured or calculated. However, bulk mechanical properties of a membrane, such as the elastic area compressibility, K_a, is related to the lateral pressure as follows:

$$K_a = [\partial\gamma/\partial(lnA)]_T$$

where γ is the surface tension and A is the lipid area. The surface tension, defined as the energy per unit area, is related to the force needed to deform a membrane. For each lipid area A, the surface tension γ is given by,

$$\gamma = \int -\pi(z)dz = \int [P_N(z) - P_L(z)]dz$$

The area compressibility modulus, K_a, characterizing the resistance to area expansion or compression, can readily be obtained experimentally or computationally, such as NMR, X-ray diffraction, Atomic Force Microscopy (AFM) and MD simulations.[57]

Bending rigidity is an essential membrane property that is thought to be responsible for the shape of cells as well as influencing the raft formation.[51] It is quantified by two bending moduli in the two orthogonal directions for a planar membrane. The bending rigidity is strongly dependent on the lipid composition and phase state of a membrane. A number of experimental techniques have been employed to assess the bending rigidity in model and cell membranes.[58] However, most techniques measure only ensemble-averaged properties, and cannot isolate the contributions from coexisting systems. To overcome this limitation, a Neutron Spin Echo (NSE) approach was used to isolate the bending modulus of lipid domains from that of the surrounding bulk phase.[36] NSE probes the thermal undulational motions of a bilayer. Fitting the resulting dynamic structure factor $S(q, t)$ with a stretched exponential as $\sim e^{-(\Gamma t)^{2/3}}$ yields the relaxation rate Γ, from which the bending modulus κ can be extracted as $\Gamma \propto \kappa^{-1/2} q^3$ based on the Zilman and Granek theory.[59] By combining NSE with contrast matching, the bending modulus of nanoscopic L_d domains residing in L_d/L_o co-existing lipid vesicles was determined. This result revealed that the nanoscopic domains were dynamically decoupled from the surrounding L_o phase, maintaining bending properties similar to those of the bulk L_d phase.

The membrane bending modulus can be obtained from MD simulations by analyzing the membrane height fluctuations based on the Helfrich-Canham theory in which the total bending energy of a membrane can be approximated as contributions from bending (κ) and tension (γ) when the

membrane height fluctuations are small.[60] As such, this method is only valid for systems for which the *xy* dimension is far greater than the bilayer thickness. An improved method, which accounts for the contributions to the total lipid height fluctuation spectrum from lipid tilting, was subsequently proposed.[61] More recently, another, versatile method was devised in which the membrane bending modulus was calculated from an analysis of thermal fluctuations of lipid orientations.[62] This last method is compatible with multi-component membranes and has been widely used to compute bending properties for a number of membrane systems.[36,39,63-65]

Lipid diffusion, an important dynamical property of a membrane, provides information on the structure and physical state of the membrane. A variety of experimental techniques has been used to investigate how the membrane composition, phase state and structure influence the lateral mobility of lipids. Of these methods, Fluorescence Recovery After Photo Bleaching (FRAP), Single Particle Tracking (SPT), and Fluorescence Correlation Spectroscopy (FCS) are the three most commonly used.[66] These three techniques are complementary to each other, covering different spatial-temporal regimes with SPT being 10-20 nm, ~1 ms, FCS covering ~40 nm-1 μm, ~100 μs-100 ms and FRAP >1 μm, ~1 s.[67] SPT tracks the movement of individual particles using fluorescent or optical labels. FRAP first photo-bleaches a defined area in the membrane and then measures the rate at which fluorophores diffuse back into the bleached spot, while FCS measures correlated fluctuations of fluorescence intensity, from which the lateral diffusion of proteins or lipids can be determined.

Lipid diffusion has also been heavily investigated by MD simulations, which have provided fundamental insights into the molecular mechanisms of lateral lipid diffusion at multiple time and length scales. In a simulation, the diffusion coefficient, D is calculated from the time-dependence of the long-time mean square displacement (MSD), provided the linear regime is reached. A near μs-long all-atom MD simulation revealed that lateral diffusion in a lipid bilayer can be divided into two stages: the first stage occurs at short times ($t < 5$ ns), in which the lipid moves up to ~0.8 nm due to

conformational dynamics of its acyl tails, while in the second stage the lipid diffuses with random-walk-like displacements over length scales of ~100 nm with the diffusion coefficient reduced by more than one order of magnitude compared to the initial stage.[68] Later, based on extensive atomistic MD simulations of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) bilayers in the fluid phase, it was concluded that lateral diffusion of lipids in membranes is through collective flows; that is, lateral diffusion is continuous, and the motions of neighboring lipids are intimately coupled over tens of nanometers.[69]

Combining all-atom MD with stochastic analysis of individual trajectories, lipid molecules were found to exhibit non-Brownian diffusion in a variety of model membranes,[70] implicating a heterogeneous nature of the membranes. Cholesterol significantly affects lipid diffusion, leading to more pronounced and persistent sub-diffusion. Sub-diffusion is a diffusion process with a non-linear relationship in time, in which the MSD ~ t^{γ}, where the anomalous diffusion exponent, $\gamma < 1$. Sub-diffusion has been proposed as a measure of macromolecular crowding in the cytoplasm that affects a range of cellular processes, such as enzyme catalysis, protein assembly and trafficking, and regulation of signaling pathways.[71] Nevertheless, still very little is known about the diffusional dynamics in heterogeneous (phase-mixed) membranes, especially in the phase boundary regions. Is it distinct from the two populations in the domains? What is the dynamic signature when a lipid molecule moves across the phase boundary? As domains become smaller, the number of boundary lipids increases, and the boundary dynamics thus becomes more significant. Neutron-based techniques provide a unique way to measure lateral diffusional dynamics in different phases of a single lipid bilayer, and this may potentially reveal effects of the phase boundaries. The diffusion of lipids, cholesterol, and proteins into and out of lipid rafts is of high relevance to cellular signaling porcesses.[22,23]

Technical advances in MD as applied to membranes. MD simulations have been widely used to probe the local structure, dynamics and physical properties of model lipid bilayers in various phases. In particular, local packing, order parameters and dynamical properties have been extensively

studied for L_o and L_d phase membranes. For a detailed review on MD simulations of lipid membrane domains, see Ref.[72]

With advances in computer hardware and software, MD simulation has continued to extend both accessible time and length scales, progressively bridging the gaps between various types of experiment and simulation. For example, employing the Anton computer, which is a special purpose machine hard-wired to efficiently calculate MD, μs-long all-atom MD simulations were performed to study the dynamics of lipid mixing.[73] The simulations, however, failed to show complete phase separation from a randomly mixed lipid mixture. Nonetheless, although liquid-liquid phase separation has not yet been observed in all-atom MD, local lateral heterogeneity has emerged in μs-long all-atom MD simulations of a ternary mixture of DOPC, DPPC and cholesterol,[74] revealing substructures composed of saturated fatty acid chains packed with hexagonal order within the L_o phase of the bilayer.

To enable the observation of slow biological processes in all-atom MD, a number of **enhanced sampling** methods have been developed, such as umbrella sampling,[75] metadynamics,[76] the Wang-Landau algorithm[77] and generalized ensemble methods.[78,79] However, techniques such as these, that were designed for accelerating conformational sampling, have shown little or no success in simulating the formation of membrane domains, due partially to the difficulty in identifying a suitable reaction coordinate to describe the phase separation process, and thus with which to bias the dynamics. For example, a simulation study with the accelerated molecular dynamics (aMD) method[80] produced a 2-3 fold speedup in lipid diffusion rates relative to standard all-atom MD simulations,[81] but was still too short to show any clear sign of phase separation from a randomly mixed lipid mixture. In principle, aMD and generalized ensemble methods (e.g., replica exchange),[82] which use thermodynamic variables as a reaction coordinate, do have the potential to accelerate lipid phase separation in MD simulations. However, no such simulations have yet been reported, suggesting that considerable technical challenges still exist in reproducing phase behavior even in simple model membranes.

Furthermore, membrane domains are thought to form at a critical point,[54] and critical phenomena are extremely sensitive to external conditions; enhanced sampling methods that introduce extra noises (biases) to the system may therefore not be ideally suited for simulating phase separation in these systems.

Fortunately, another way of extending the time and length scales of MD simulations exists, through **coarse graining** (CG), in which multiple atoms are grouped into a CG bead to reduce the computational cost. Lateral phase separation has been observed in a number of CGMD simulations of lipid bilayer systems. For example, using the MARTINI force field,[83] various aspects of phase separation in planar bilayers and small vesicles of ternary lipid mixtures were investigated,[84] revealing the formation of membrane domains from uniformly mixed lipid mixtures, consistent with experimentally-observed L_o and L_d domains. Moreover, these membrane domains tend to be in register across the two leaflets of the bilayers (i.e., there is interleaflet co-localization of the domains). Other CGMD simulations showed that a high concentration of TM helices induced phase segregation in a ternary DPPC/dilinoleoylphosphatidylcholine (DLiPC)/cholesterol lipid mixture into L_o and L_d domains.[85] The TM helices were found to concentrate in the L_d domains, where their lateral diffusion was slowed down by a factor of 5-10 relative to the dilute case, also exhibiting anomalous sub-diffusive behavior, on the μs time scale.

A key, non-equilibrium feature of biological membranes is that they are asymmetric, i.e., different lipids make up the inner and outer bilayer leaflets. However, domain formation is thought to be coupled across leaflets,[86,87] and this coupling is essential for both the membrane structure and the cross-membrane signal transduction. Extensive CGMD simulations have confirmed the coexistence of L_o and L_d domains in both compositionally symmetric and asymmetric ternary bilayers.[88] Also, recently, CGMD simulations were performed of a plasma membrane comprising 63 different lipid species constructed to closely mimic the mammalian plasma membrane composition.[89] These simulations showed a large degree of lateral membrane heterogeneity, indicating that transient L_o-like

domains form and break on the μs time scale. In further CGMD simulations of the ternary (POPC/cholesterol/monosialotetrahexosylganglioside (GM1)) and quaternary (POPC/PSM/cholesterol/GM1) lipid bilayers,[90] it was found that GM1 tends to associate strongly with itself, and that the self-aggregation of GM1 molecules and co-localization of GM1 with cholesterol lead to the formation of L_o domains in the lipid mixtures. More recently, leveraging advances in computational capability and lipidomics, a realistic model was developed of the complex plasma membrane of the human brain.[91] Despite the lipid composition being significantly different from 'average' mammalian plasma membranes, with a higher cholesterol concentration and increased tail unsaturation, the CGMD simulations of these brain PM mixtures showed striking similarities to average membranes, with both exhibiting a range of dynamic in-plane heterogeneities. These lateral heterogeneities span a range of size and time scales depending on the lipid composition.

2.2 Detecting Membrane Domains

See it to believe it. Considerable effort has been expended in trying to 'visualize' membrane lateral organization (or phase separation) in lipid bilayers, especially in the context of 'lipid raft' phenomena in cell membranes. However, the direct detection of membrane domains, especially in live cells, has remained an enormous challenge, primarily due to their small suspected size (10-100 nm) coupled with their transient nature (in terms of both lateral mobility and association-dissociation dynamics), which are beyond the spatial and temporal limits of commonly-used optical microscopic techniques. Early evidence of membrane lateral structures was provided by X-ray and neutron diffraction experiments.[10,92] These techniques probe hydrocarbon chain packing and, by the analysis of Bragg peaks, can be used to detect the coexistence of lipid phases. However, diffraction techniques are usually

limited to simple model membrane systems and have been hitherto incapable of resolving domain structures in biologically relevant (disordered and heterogeneous) membranes.

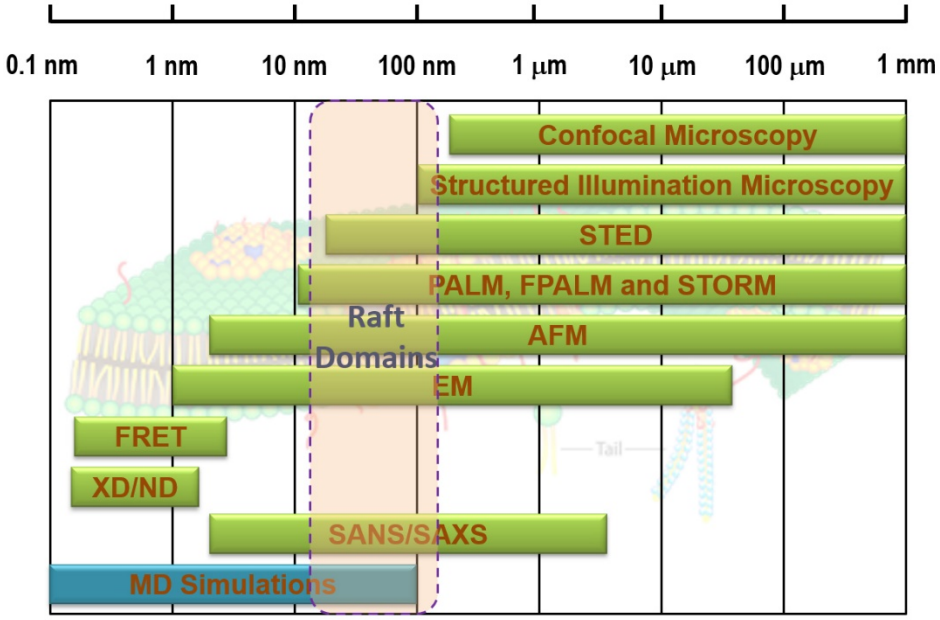

Figure 3. Length scales of various imaging and simulation techniques for probing raft domains in model and cell membranes. (STED: Stimulated Emission Depletion; PALM/FPALM: Photoactivated Localization Microscopy; STORM: Stochastic Optical Reconstruction Microscopy; AFM: Atomic Force Microscopy; EM: Electron Microscopy; FRET, XD/ND: X-ray/Neutron Diffraction; SANS/SAXS: Small-Angle Neutron/X-ray Scattering; MD: Molecular Dynamics)

Subsequently, fluorescence microscopy (FM) emerged as a popular technique to probe lateral membrane organization.[23,24] Compared to diffraction techniques, FM allows *in situ* observation of membrane structures in artificial and live cell membranes, visualizing membrane domains (*i.e.*, their size and shape) through the selective partitioning of dye molecules into different phases of the membrane. Unfortunately, functional membrane domains in cell membranes are believed to be smaller than the diffraction limit (<200 nm),[19,93] so cannot be detected with conventional confocal microscopy

techniques (Figure 3). Therefore, FM imaging has been mostly performed on model membranes of ternary or four-component lipid mixtures that can form large-sized domains. Over the past two decades, numerous FM experiments have shown definite evidence for the existence of micron-size domains in these model membranes on GUVs[94,95] and planar supported membranes.[96-98]

To overcome the diffraction limit, a range of super-resolution microscopy techniques has been developed, including Structured Illumination Microscopy (SIM), Stimulated Emission Depletion (STED), Stochastic Optical Reconstruction Microscopy (STORM), Photoactivated Localization Microscopy (PALM), and Total Internal Reflection Fluorescent Microscopy (TIRFM) (Figure 3).[23,24] An example of the use of one of these techniques was the determination of the lateral structure of the bacterial light-harvesting complex LH_2 in a model lipid membrane, that was observed using TIRFM.[99] Unfortunately, the full potential of super-resolution FMs has not been realized due to their limited temporal resolution, which prevents transient membrane domains (with lifetimes up to milliseconds)[24,100] from being resolved. Furthermore, as resolutions increase, higher intensity light sources are needed to excite fluorescent species in the sample, limiting the applicability of super-resolution FMs in live-cell imaging.

Atomic Force Microscopy (AFM) provides high-resolution membrane structures, in which membrane domains are distinguished by their difference in bilayer height (thickness).[101] AFM can reach lateral resolution of ~1-10 nm and transverse resolution of ~1 Å, thus capable of detecting nanoscopic domains. However, AFM is only applicable to supported bilayers and poorly suited to systems in which curvature affects the support lipid distance, and is thus again incompatible with *in vivo* imaging. Using AFM, both microscopic and nanoscopic domains have been found to coexist in mixed lipid bilayers of DPPC, dilauroylphosphatidylcholine (DLPC) and cholesterol.[102] Specifically, DPPC-rich domain sizes were found to be in the range of 26-46 nm in the cholesterol-free mixtures, while domain sizes vary between 33 and 48 nm in the ternary mixtures. These nanoscopic domains appeared to aggregate readily into microscopic domains.

Direct visualization of nanoscopic raft domains still presents a significant challenge for optical microscopic techniques. To overcome this challenge, a number of spectroscopic techniques have been developed that detect lateral membrane heterogeneities by distinguishing the differential dynamics (i.e., diffusion) of proteins and lipids in different domains, including FRAP, FCS and SPT.[23,24] The combination of FCS with super-resolution techniques, such as spot variation (svFCS) and STED microscope (STED-FCS),[103] has significantly improved the spatial and temporal resolution of FCS, allowing nanoscopic lateral membrane features with the size of 20-40 nm to be detected.[104,105] Taking advantage of significantly improved sensitivity and time resolution, recent interferometric scattering microscopy (iSCAT) experiments presented evidence for the existence of sub-20 nm domains in model membranes,[106] while their lifetime was determined to be longer than the μs timescale imaging speed.

Förster resonance energy transfer (FRET) is a single-molecule technique that determines, through measuring the FRET efficiency, if two fluorophores are within a certain distance of each other. FRET can probe much smaller distances (Å resolution) than the above techniques (i.e., FRAP, FCS and SPT), and has been widely used to investigate domain structure and phase behavior as well as raft-associating proteins in both model membranes and live cells.[107-110] Given their high spatiotemporal resolution, FRET-based approaches, in combination with super-resolution optical techniques, will be able to reveal the interplay between the molecular interactions and dynamics and the local phase states of the membrane, thus holding great promise in providing an integrative view of raft domain-mediated signaling events in cells.

Still, none of the fluorescence imaging techniques can resolve the structure or dynamics of clusters of up to 100 lipids, whereas these nanoscale clusters are thought to be characteristic of cell membranes,[111] constituting the building blocks of larger-scale signaling platforms. To address this problem, various neutron experiments have been developed that probe membrane structure and dynamics on nm length scales (from Å to ~10 nm) and ns timescales (from ps to ~100 ns).[112-114]

Exploiting the sensitivity of neutrons to the difference between hydrogen and deuterium, Small Angle Neutron Scattering (SANS) has emerged as a powerful tool for the characterization of membrane heterogeneities (domains) on nm length-scales.[115]

The SANS signal arising from unilamellar lipid vesicles (ULVs) comprises three parts: (i) a component reflecting the neutron scattering density contrast between the average bilayer composition and the solvent; (ii) a radial component from neutron scattering density variation in the membrane normal direction; and (iii) a lateral component from neutron scattering density variation in the membrane plane (surface). Through judicious selection of deuteration schemes, the former two contributions can be eliminated, and the remaining signal then comes solely from the neutron scattering density variation in the membrane plane, providing a description of lateral membrane heterogeneities. SANS thus determines nanoscopic membrane structure directly, without the need of models or extrinsic probes. SANS has been used to determine the size of nanoscopic membrane domains in a series of four-component model systems containing fixed amounts of cholesterol and saturated 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), mixed with varying amounts of unsaturated DOPC and POPC.[35] The domain size was found to vary from 6.8 nm to >22.5 nm in radius, depending on the DOPC:POPC ratio (*i.e.*, the extent of acyl chain unsaturation).

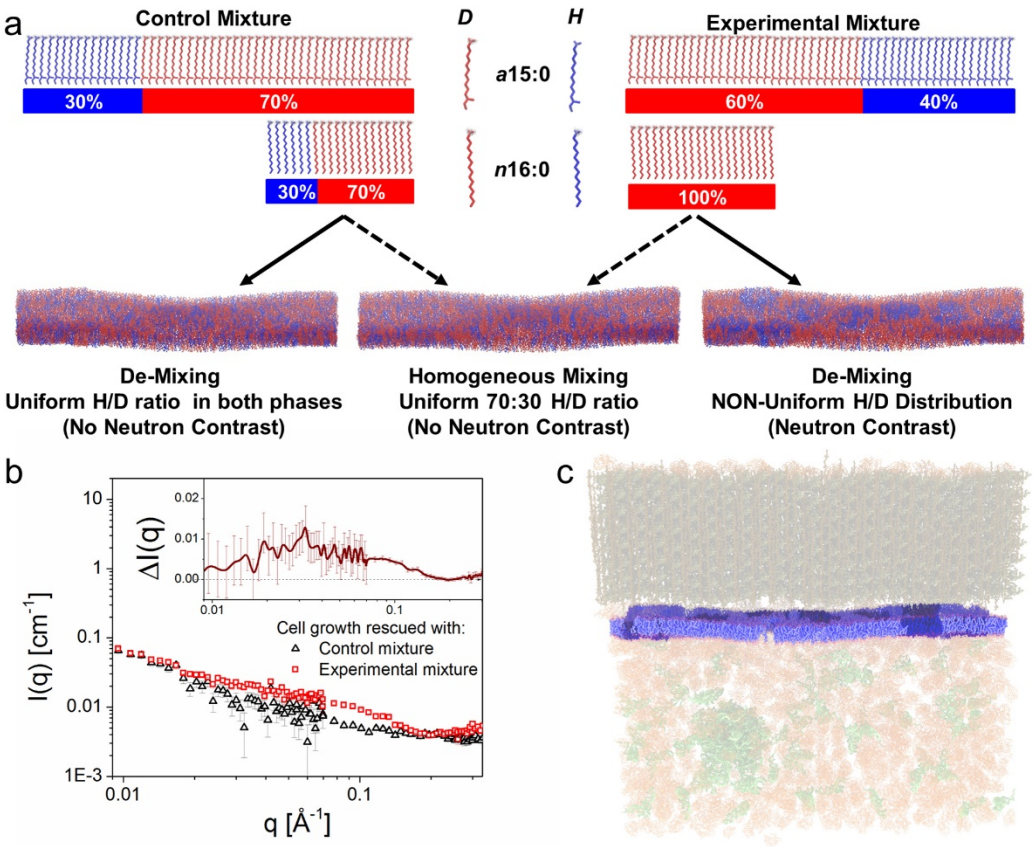

Figure 4. Detecting membrane lateral heterogeneities in *B. subtilis*. a) Schematic of the small-angle neutron scattering (SANS) experiments that use different blends of hydrogen (H)- and deuterium (D)-fatty acids (FAs) to control neutron contrast in the membrane. b) SANS spectra of experimentally manipulated *B. subtilis*, whose membranes are composed of experimental and control mixtures, respectively. The SANS signal for the experimental mixture shows clear excess scattering that indicates a non-uniform distribution of lipids in the membrane (inset). c) A schematic representation of the *B. subtilis* membrane as seen by neutrons under the contrast-matched condition. Reproduced from Ref.[30] under CC BY 4.0 https://creativecommons.org/licenses/by/4.0/.

Very recently, SANS has been used to observe, for the first time, lipid domains *directly* and *in vivo*, based upon deuterium labeling of the bacterium *B. subtilis* membrane (Figure 4).[30] By tuning the specific proportions of deuterium and hydrogen in two constituent lipids, the SANS spectra of

experimentally manipulated *B. subtilis* displayed clear excess scattering over the *q* range of 0.015–0.2 $Å^{-1}$ (Figure 4b), indicating a non-uniform distribution of lipids in the membrane with different neutron scattering density contrast. Given that the differential H/D ratio of lipid tails is the only source of neutron contrast in the experiment, the SANS result thus indicates that there exists lateral de-mixing of lipids containing the normal or branched acyl chains (with distinct H/D ratio), and that this lateral membrane heterogeneity is on the length scale of 3-40 nm. This observation of lipid segregation in the cell membrane of *B. subtilis* is on a comparable scale to that of 'lipid rafts' observed in eukaryotic cells,[18] consistent with the existence of raft-like domains in bacteria.[48] This experiment - performed under biologically relevant conditions - illustrates the potential of neutron-based techniques for *in vivo* interrogation of lateral membrane structures.

Although the existence of lipid rafts has been widely accepted, and membrane lateral heterogeneities have finally been detected in live cells, the chemical and physical nature of these heterogeneities *in vivo* remains controversial. The early concept of lipid rafts originated from the observation that a fraction of membranes composed of sphingolipid, cholesterol and a subclass of membrane proteins was resistant to cold detergent extraction, and these were thus called DRMs. However, recent findings have suggested that detergent extraction, key early biochemical methods used to identify lipid raft, might alter certain protein and lipid associations, leading to the extracted DRMs differing from native rafts.[116] In this vein, it has been shown that the detergent Triton might promotes domain formation in a homogeneous fluid membrane.[117] Also, FRET experiments have shown that most GPI-anchored proteins are uniformly distributed on the cell surface or exist as small (nanoscale) clusters,[107] whereas DRMs containing GPI-anchored proteins are often present in large (up to 1-μm diameter) domains.[116]

2.3 What Drives Membrane Lateral Organization

While raft domains have been widely accepted as functional platforms for cellular signaling, why and how they are formed, especially in living cells, remain hotly debated. This fundamental understanding is relevant because membrane phase transitions may be governed by the same physical principles as those underlying the assembly of protein-membrane complexes that give rise to cellular signal transduction. An excellent review on physical mechanisms of domain formation in model lipid membranes has recently been published by Schmid.[51] Here, we briefly review aspects that may also be involved in shaping cellular signaling.

A number of mechanisms have been proposed to explain how membrane domains can emerge. One obvious possibility is protein-protein interaction[118-120] in which lipids play only a passive role. In this model, proteins assemble at the membrane to form small and relatively stable clusters, which in turn recruit specific types of lipids to generate nanoscale membrane domains. This model is supported by the finding that a 'generic' GPI-anchored protein cannot be 'preferentially' recruited to a signaling complex,[121,122] as the assembly of the signaling complex seems to also require the interaction with a specific protein-interaction domain. This model, however, cannot explain why many proteins selectively interact with specific membrane domains through lipidation and/or binding of lipid molecules.

Another potential driving force is the interaction between the cytoskeleton and the plasma membrane. Cytoskeletons immobilize certain membrane inclusions (e.g., anchor proteins), which would then serve as pinning sites to promote the formation of membrane domains.[123-125] However, the fact that membrane domains can form spontaneously in pure lipid mixtures suggests that the lipid-lipid interactions are sufficient to drive membrane organization and thus are also crucial for raft formation *in vivo*. Indeed, the majority of our current knowledge about the mechanisms underlying the membrane domain formation has come from studies of well-characterized model membranes.

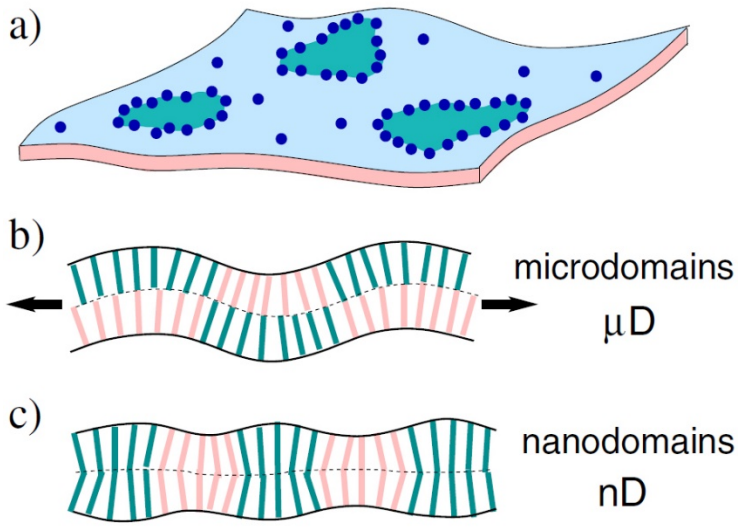

Figure 5. Two-dimensional microemulsions (micro- and nano-domains) can be stabilized via three mechanisms. a) line active molecules (linactants); b) bilayer curvature coupling (Leibler-Andelman mechanism); and c) monolayer curvature coupling. Reproduced with permission from Ref.[51] Copyright 2016 Elsevier.

One of these mechanisms is minimization of the line tension,[126] which is the energetic cost per unit length of the interfacial boundary line. A number of experiments have indicated that the L_o phases are thicker than the L_d ones.[127,128] This thickness mismatch at the domain edge could result in an unfavorable exposure of hydrophobic regions to water, and hence, the membrane would deform at the domain boundary to minimize it,[129] leading to line tension. Consistent with this idea, the effects of the line tension on the stabilization of membrane domains have been investigated and it was found that the domain size is strongly correlated with the thickness difference between the domains and the non-domain membranes.[126] Moreover, direct experimental evidence has shown that the line tension favors large membrane domains.[35] Given that the interface between domains is penalized by the line tension, and if line tension is dominant, membrane systems would phase separate in a thermodynamic sense (i.e., segregate to two or more bulk phases) so as to minimize the total length of phase boundaries.

Therefore, to form small, 'nanoscopic' domains the decrease of domain size, and thus the increase of the line tension, must be offset by other thermodynamic factors (Figure 5).

Leibler and Andelman investigated curvature-induced instabilities in membranes by introducing a general coupling between curvature and local composition under the framework of the Ginzburg-Landau free energy expansion approach.[130] This bilayer curvature coupling can lead to the formation of various undulated phases (on a mesoscopic scale) in fluid membranes. More intuitively, as illustrated in Figure 5b, lateral structure can be formed to compensate the membrane curvature stress, such that different domains (i.e., with different spontaneous bilayer curvature) alternate with each other across the two leaflets.[51,130] Schick carried out some further analysis on this membrane curvature-mediated mechanism and showed that the characteristic wave length of the mesostructures scales with the membrane surface tension γ, as $1/\sqrt{\gamma}$. For realistic biological membranes, the characteristic size of membrane domains is thus estimated to be on the order of 100 nm to μm.[131]

To explain why nanoscale domains are observed in membranes, Veatch et al. proposed that the domains are close to a critical demixing point,[54] such that the formation of small domains is due to an incomplete phase separation. In the vicinity of a critical point, lipid domains are characterized by a broad and fractal-like size distribution, and the average domain size should show a peculiar power law behavior while approaching the critical point, both of which were supported by AFM images of critical lipid clusters in supported multicomponent bilayers.[132] This critical phenomenon proposal provides a plausible explanation for the existence of nanoscale domains in lipid membranes, but raises the question of how biological systems control and maintain their lipid compositions close to a critical point. Nevertheless, increasing evidence is suggesting that the lipid composition of cell membranes is highly regulated and also maintained close to that of phase boundaries, such that small changes in lipid/protein composition or conformation can lead to dramatic effects on membrane organization, such as the coalescence of nanoscopic lipid clusters to form microscopic raft domains.[37,133]

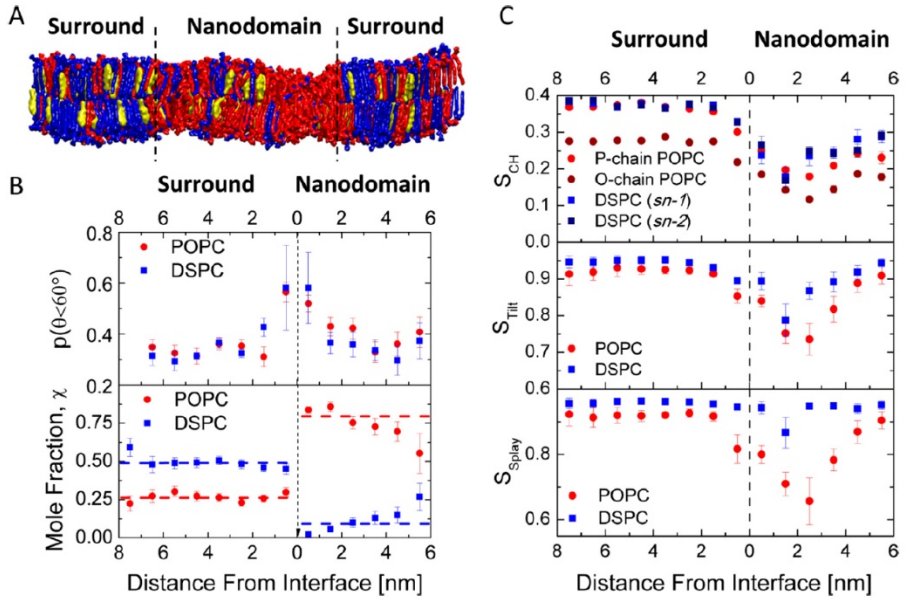

Figure 6. Molecular details of the domain interface revealed by MD simulations. A) Cross-section of the simulated lipid bilayer patch with a nanoscopic L_d domain embedded in an L_o phase after 150 ns of MD simulations (POPC: red, DSPC: blue, and cholesterol: yellow). B) Lipid orientation p (alignment of the *sn*-2 chain with respect to the domain interface, Top Panel) and composition χ (mole fractions of POPC and DSPC, Bottom Panel) and as a function of distance from the domain interface. C) Three order parameters, S_{CH}, S_{Tilt}, and S_{Splay} as a function of distance from the domain interface. Reproduced with permission from Ref.[36] Copyright 2015 American Chemical Society.

Analogous to surfactants that can lower the surface tension between two bulk phases, line active molecules (or linactants) have been proposed as another plausible explanation for the stabilization of nanoscale lateral membrane heterogeneities.[134] Linactants contain hybrid saturated/unsaturated chains that favor ordered or disordered lipid phases, respectively. As sketched in Figure 5a, linactants often concentrate at domain boundaries, where they can reduce the line tension and stabilize nanoscale membrane domains. MD simulations confirmed that certain hybrid

saturated/unsaturated chain lipids are enriched at the domain interface and thus lower the line tension.[135] The mixed acyl lipid POPC has been suggested as a line active molecule,[36,134] with its saturated *sn*-1 palmitoyl chain interacting preferentially with the L_o phase and its unsaturated *sn*-2 oleoyl chain favoring the L_d phase. In this way, the mixed acyl chains can bridge the two lipid phases more favorably, reducing the line tension and stabilizing the nanoscopic domains. Indeed, recent MD simulations of biologically relevant membranes provided molecular details of the distribution of lipid molecules in the domain boundary regions, revealing an increase in POPC concentration at the domain interface (Figure 6).[134]

The existence of nanoscale clusters in binary lipid mixtures, e.g., DPPC/Chol mixtures, has been demonstrated in a number of experiments, from early NMR diffusion[136] to recent neutron diffraction[137] and AFM,[138] but cannot be explained by the critical phenomenon mechanism. By noting the similarity between the nanoscale clusters in the DPPC/Chol mixtures and the ripple structure in one-component membranes, Meinhardt et al. proposed a monolayer curvature coupling mechanism.[139] As shown in Figure 5c, a mismatch between the spontaneous curvatures of the L_o and L_d monolayers causes membrane curvature stress; the larger this stress is, the smaller the domains become. The monolayer curvature can thus effectively offset the line tension at domain boundaries, leading to nanoscopic raft domains. Evidence of correlations between membrane curvature and clustering of lipid molecules has also been obtained from CGMD simulations.[140] In these simulations of complex multicomponent systems, the formation of nanoclusters of ganglioside GM3 was observed, especially in the concave regions of the outer leaflet. Similarly, recent neutron studies highlighted the difference in bending moduli between the two coexisting lipid phases that, together with their different spontaneous membrane curvatures, can potentially compete with the interfacial energy to stabilize small size domains.[36]

Current insight into the physical principles behind lipid phase separation has been gleaned mainly from studies of biomimetic lipid mixtures.[141] However, these simplified systems do not fully

capture the complexity of cell membranes. For example, recent combined simulation and experimental studies showed that non-canonical highly unsaturated lipids can promote liquid-liquid phase separation, stabilizing membrane domains by increasing the differences in cholesterol concentration and hydrocarbon chain order between the coexisting phases.[142] A path forward is thus to use biologically more relevant lipids, and even lipid extracts.[39] This, however, presents a formidable challenge, as no experiment has yet shown evidence of domain formation in these lipid extracts.

Given the complexity of cell membranes, the aforementioned mechanisms are not necessarily exclusive of each other, and they may actually act synergistically to drive domain formation. To tackle this problem, an integrated multiscale experimental approach will be required that interrogates various aspects of cell membranes across different length and time scales. Information on various physical properties (e.g., spontaneous curvature, bending rigidity) of specific membrane phases and especially those for isolated phases in the phase-mixed systems will be essential. When combined with theory and MD simulations, these results have the potential to elucidate at the molecular level how the myriad of interactions between lipids and proteins gives rise to membrane organization.

2.4 Partitioning of Proteins in Membranes

Having considered lipid heterogeneities and lipid effects on domain formation in model and cell membranes, we now turn our attention to proteins. A logical first area of enquiry is how peptides and proteins insert into membranes, and to derive the thermodynamic concepts behind this insertion. How proteins partition into membranes, and especially into different domains, has important implications for both raft formation and cell signaling.

2.4.1 Partitioning of Amino Acids in Membranes

An understanding of the factors determining amino acid side-chain positioning in membranes is fundamental to understanding membrane protein-lipid thermodynamics. Differences between protein-water and protein-lipid energetics lead to different positional preferences of the various amino acid residues in proteins. Some residues reside in the aqueous phase, some the head-group region, and others the membrane hydrophobic core. Indeed, variation in the positional preferences of residues has been found in experimental data on membrane proteins[143-145] and in studies on a number of synthetic peptides.[146-149]

A strong correlation exists between hydrophobicity scales and the probability of a given residue being present in the hydrocarbon region,[150,151] and therefore non-polar aliphatic residues have the strongest affinity for the center of the membrane. Tryptophan and tyrosine are frequently observed interacting with the head-group region, at the level of the lipid carbonyl groups,[149,152] and correspondingly in proteins often flank transmembrane segments. As would be expected, the head-group region is enriched in polar residues as well as the positively-charged residues lysine and arginine, which interact at the level of the phosphate group. In the bilayer, these positively-charged residues behave as 'snorkels', reaching into the lipid head-group region even when the C_α is in the membrane core. In contrast, the negatively-charged residues are rarely present within the membrane and, if present at all, are usually buried inside helical bundles.

The positional dependence of the solvation free energy of the twenty amino acid residues and their corresponding side chain analogues have been calculated. Initially, to avoid the computational expense of explicit lipid models, and for ease of interpretation, this was done using implicit membrane models[153-155] in which the lipid and solvent molecules are replaced by continuum models. For example, in a three-slab model, the membrane is represented as a slab of low dielectric constant embedded in a high dielectric solvent.[156-158] However, a drawback of this is that, the head-group energetics are not

accurately taken into account. Increasing complexity, 5-slab continuum dielectric models for the membrane distinguish between the solvent, head-group, and hydrophobic core regions.[159]

Given a slab membrane model, the Poisson-Boltzmann equation can be used to calculate the electrostatic free energy of transfer, ΔG_{elec}, of a side chain from the aqueous slab to any given position in the membrane. To improve the accuracy of the model, a non-polar surface-accessibility term, ΔG_{np}, can be added. The simplicity of this model allows an understanding of the key energetics involved. For example, a tradeoff can exist between ΔG_{elec} and ΔG_{np}, originating from the observation that whereas ΔG_{elec} favors polar environments, ΔG_{np} opposes entering the aqueous layer. A decrease of ΔG_{elec} on transferring from the center to the head-group region may be cancelled by an increase in ΔG_{np}. An example of this is in Ref.[160] in which the partitioning of tryptophan and tyrosine in the head-group region was found to arise from the above compensation whereas, in contrast, phenylalanine partitions in both the head-group region and the membrane core.

2.4.2 Partitioning of Peptides in Membranes

A major contribution to insertion thermodynamics is missing from side chain analogues - that of the peptide backbone. Therefore, we now discuss the partitioning of polypeptide segments into lipid bilayers. Unfortunately, experiments in this regard suffer from the fact that sequences that are sufficiently hydrophobic to spontaneously insert without disrupting the membrane have a tendency to aggregate in aqueous solution.[161,162] Nonetheless, a large body of indirect experimental and computational evidence has been collected and reviewed.[163,164] The trans-bilayer hydrophobicity profile guides suitably hydrophobic peptides, permitting them to bury themselves through helix formation. Unfolded insertion is unlikely due to the large energetic penalty associated with desolvating

an exposed peptide backbone (estimated at ~4 kcal/mol/bond).[164] Thus, a two-stage pathway exists, where helical segments fold at the phase boundary prior to insertion.

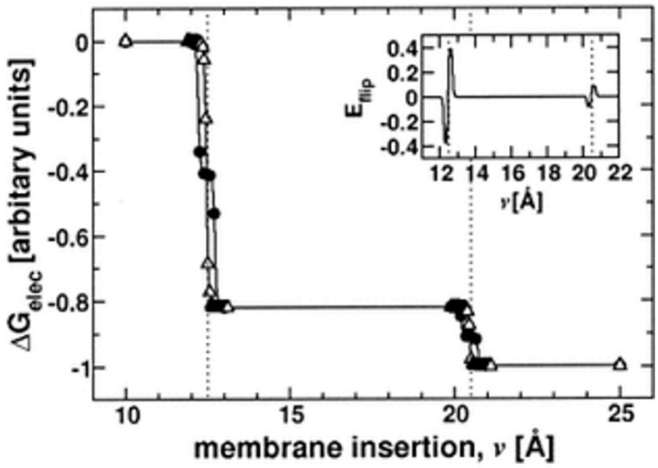

Figure 7. Electrostatic solvation energies (ΔG_{elec}) versus membrane insertion distance, *v*, for a dipole (dipole moment = 2.4 D). Two orientations, θ = 0° (●) and θ = 90° (Δ) are considered. The flipping energy of the dipole (E_{flip}) in the membrane is given by the difference of ΔG_{elec} between the two orientations. The two dotted lines depict the boundaries of the head-group region. Adapted with permission from Ref.[159] Copyright 2005 Wiley.

Dipole effects. Efforts have been made to understand the energetic factors determining the positioning of helical peptides in membranes. It is instructive in this regard to first consider a simple dipole, consisting of charges $q = 1.0e$ placed 0.5Å apart, crossing the above-described 5-slab membrane, for which the Poisson equation can be solved analytically. It is of interest to compare two orientations of the dipole, in which the angle between the dipole axis and the membrane normal is 0° or 90°. The difference in ΔG_{elec} between the 0° and 90° orientations is the barrier to flipping, denoted by E_{flip}, which indicates the relative stability of one orientation versus the other. Plots of ΔG_{elec} as a function of the membrane insertion distance, *v*, for the two orientations are shown in Figure 7.

As ΔG_{elec} is inversely proportional to the dielectric constant ε, this term decreases when traversing from the membrane core to the head-group region and then to the aqueous medium. According to this simple model, the difference in ΔG_{elec} between the membrane core and head-group region arises due to the variation of ε (and hence the potential) in the three media and is thus approximately 4 times the difference in ΔG_{elec} between the head-group region and water. In a homogeneous medium, there is no orientational preference of ΔG_{elec} and hence no tendency of the dipole to orient itself in a particular direction. This contrasts with behavior near the dielectric boundaries, i.e., when the dipole transits from the membrane core to the head-group region or from the head-group region to the aqueous layer. At these boundaries a difference in ΔG_{elec} of the two orientations occurs: if the axis of the dipole is positioned parallel to the membrane normal, then a reaction field appears in the medium with higher polarity that counters the charge closest to the boundary, and this lowers ΔG_{elec}. In contrast, if the dipole axis is perpendicular to the membrane normal, the reaction fields due to the two charges cancel, and there is no effect on ΔG_{elec}. As a result, near dielectric boundaries, the parallel orientation is favored. This effect is found to dominate the positioning of polyalanine helices in the membrane. With the addition of the surface-area-dependent term, ΔG_{np} to model cavity formation, Ref.[159] showed that the continuum electrostatics models can also quantitatively reproduce the tilt angles determined experimentally for a number of peptides in membranes.

Helix dipoles. Aligned α helix dipoles sum to a 'macroscopic' dipole that has been implicated in protein folding and function.[165] Poisson-Boltzmann models similar to those described above have been used to calculate the magnitude of the α helix dipole in various geometries and orientations.[166] In aqueous solution, the strength of the dipole is greatly reduced due to the reaction field generated by the solvent. Furthermore, whereas in vacuum the net effective dipole moment, μ_{eff}, increases with helix length, the opposite was found to be the case for helices in membranes: μ_{eff} decreases almost linearly

with helix length until the helices reach across the membranes. As the helix increases in length, the terminal charges meet the high-dielectric medium, which then screens them, leading to the inverse length dependence. Correspondingly, the reaction field in the surrounding solvent increases with increasing helix length, decreasing μ_{eff}. In contrast, when the helices are positioned parallel to the membrane plane and completely embedded in the membrane hydrophobic core, their dipole moments are much greater than when these helices stay perpendicular to the membrane plane. The μ_{eff} values of transmembrane helices are low as most of them have relatively small tilt angles with respect to the membrane normal (0°-40°).

Although continuum electrostatics calculations can be very informative, complex physiochemical properties of fluid phase lipid bilayers, especially at the interfaces, are best probed with all-atom MD. However, the timescales for spontaneous peptide insertion at room temperature are often much longer than MD has been able to access. Indeed, extrapolation of insertion rates obtained from simulations performed at high temperatures indicated that times needed for insertion at room temperature range from 8 μs to 160 ms. Fortunately, however, hydrophobic membrane-inserting peptides have been shown to be remarkably stable against thermal denaturation; circular dichroism experiments[167] revealed that these peptides remain fully helical and inserted in the lipid bilayer, even at temperatures as high as 90 °C. At these higher temperatures, MD sampling is estimated to be two orders of magnitude faster than at 25 °C, sufficient to directly simulate spontaneous peptide insertion. This enables direct MD determination of atomic detail information on the partitioning pathways, intermediate states, as well as insertion kinetics and related barriers. For the hydrophobic peptides examined in this way in Ref.[167], a folded insertion pathway was observed, consistent with a partitioning model consisting of three stages: helix binding to the membrane surface, intermediate and inserted. All systems studied showed first-order insertion kinetics, with barriers of ~23 kcal/mol for the tryptophan-flanked WALP peptides and ~15 kcal/mol for the other hydrophobic peptides.

2.4.3 Helix-Bundle Insertion via the Translocon

In eukaryotic cells, most helix-bundle membrane proteins insert into the endoplasmic reticulum membrane *via* the Sec61 translocon, a protein-conducting channel. Individual TM helices pass from the ribosome into the translocon channel and then exit the channel laterally through a gate into the surrounding lipid. In Refs.[168,169] the translocon was challenged with a large set of systematically designed TM sequences and the efficiency of membrane integration measured. The results suggested that the recognition of TM helices critically depends on direct interactions between the peptides and the membrane. In turn, this is consistent with descriptions based on partitioning the TM helices between the Sec61 translocon and the surrounding membrane. Interestingly, the code that the translocon uses for selecting nascent protein chains for membrane insertion is highly correlated with their hydrophobicity scales. This work provided the first quantitative experimental estimates of the insertion threshold and partitioning free energies of short TM helix forming polyleucine peptides.

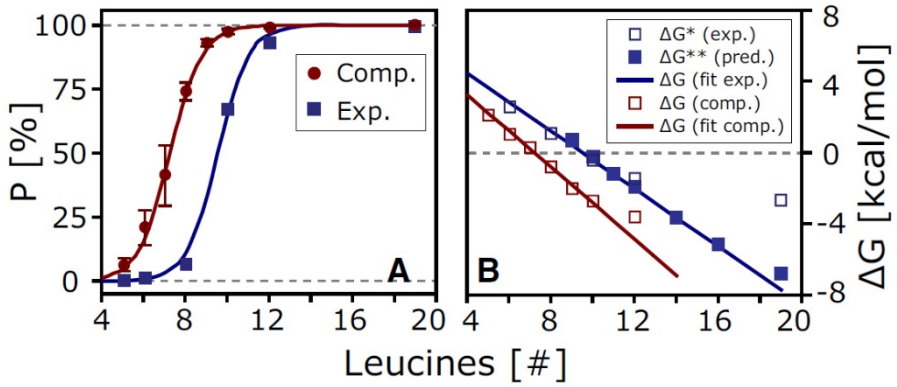

Figure 8. A) Membrane insertion efficiency *P* of polyleucine peptides as a function of peptide length *n*. B) Free energy of membrane partitioning of polyleucine peptides as a function of peptide length. The computed (red) and experimental (blue) values are shown for the individual peptides. The lines

indicate the two-state Boltzmann fit (A) and the linear fit (B) of the experimental and computed data points. Reproduced with permission from Ref.[170] Copyright 2010 Elsevier.

A simulation equivalent of the translocon experiments has been performed[171] using the high-temperature protocol of Ref,[170] with the free energy determined from populations of surface-bound and inserted GGPG-$(L)_n$-GPGG constructs. Figure 8 shows that the insertion free energy, ΔG_n increases perfectly linearly with the peptide length *n* in both simulations and experiment. Thus, all-atom MD simulations were used to directly determine peptide-partitioning properties, with excellent qualitative agreement with experimental observations. However, the offset and slope are slightly different from the experiments, reflecting a shift of the insertion probability towards shorter peptides by 2.3 leucine residues. This corresponds to an offset of $\Delta G_n = 0.25 \pm 0.02$ kcal/mol per leucine. The origin of this offset has yet to be determined. It may in part be due to a difference in the definition of the reference state between the experiment and the computation. In the simulations partitioning was between the membrane surface and the hydrocarbon core, whereas the translocon crystal structure suggests that hydrophobic peptides are expelled laterally from the protein conducting channel into the membrane.[172] Therefore, the experiments measured the partitioning between the translocon channel and the membrane, rather than between surface-bound and membrane-inserted configurations.

2.4.4 Partitioning of Proteins between Raft and Non-Raft Domains

The function of raft domains relies on the selective partitioning of certain proteins into the domains while excluding others from the domains. There has been an increasing number of experiments aimed at elucidating how proteins partition between raft and non-raft domains in biological membranes.[173-175] For a recent review on mechanisms for raft targeting, see Ref.[176]

The distribution of two model peptides between coexisting ordered and disordered lipid domains was determined using a fluorescence-quenching assay,[177,178] which measures the amount of Trp fluorescence quenched by a fluorescence quenching lipid (nitroxide-labeled) that is enriched in L_d domains. Two TM peptides were studied: one is a polyLeu family peptide (the LW peptide, acetyl-K2W2L8AL8W2K2-amide) and the other is a peptide derived from the raft-associating lymphocyte protein, LAT. Strong quenching of the Trp fluorescence revealed that the LW peptide preferentially partitions into L_d domains while being largely excluded from the L_o domains,[177] consistent with the notion that TM proteins are rarely observed in lipid rafts due to their inability to pack tightly within L_o domains.[179,180] CGMD simulations of the 7-TM protein bacteriorhodopsin in a phase-separating ternary DPPC/DLiPC/cholesterol bilayer also found that the protein preferentially partitioned into L_d domains.[85] To determine how acylation may influence raft targeting of TM proteins, raft association of palmitoylated and non-palmitoylated LAT peptides was examined and it was found that both acylated and non-acylated peptides have low raft affinities.[178] This suggests that other factors must be involved in targeting LAT to lipid rafts. Indeed, given that full-length LAT associated better with DRMs than the peptide and that some LAT (but not the TM domain construct) was isolated in a protein complex, protein-protein interactions may be an important factor contributing to the enhanced raft affinity of LAT.

Caveolin-1, which associates with cholesterol and sphingolipids, has been shown to play a role in the formation of caveolae, a special type of lipid raft. Therefore, using fluorescence microscopy, the partitioning of peptides derived from the scaffolding domain of caveolin-1 (CSD) into membranes was investigated.[181] The CSD comprises residues 82-101 of the N-terminal domain of caveolin-1 and was chosen because it is the region of caveolin-1 responsible for membrane binding. All CSD peptides were found to partition preferentially into L_d domains in model lipid bilayers composed of DOPC, brain sphingomyelin and cholesterol. This, somewhat surprising finding was attributed to a preference of caveolin-1 peptides for more fluid membranes and thus the exclusion from the tightly packed L_o

domains. It was further hypothesized that the CSD peptides' lack of lipid anchor moieties, coupled with their inability to oligomerize, may have resulted in their predominant partitioning in L_d domains.

To overcome the over-simplicity of synthetic lipid mixtures, the use of giant plasma membrane vesicles (GPMVs) in conjunction with fluorescence microscopy has emerged as a powerful approach for characterizing the partitioning of proteins between domains.[174,175] L_o phases in three- or four-component model membranes have been suggested to be more compact than in plasma membranes and thus less accommodating towards TM proteins.[182] GPMVs, obtained by chemically induced blebbing of isolated cells, reflect more accurately their phase properties in the plasma membrane of live cells with fewer caveats than model membranes. Baumgart et al. reported the first application of GPMVs to study the selective partitioning of proteins between domains.[183] It was found that while GPI-anchored proteins and the GM1-binding cholera toxin preferentially partitioned into L_o domains, all other proteins tested associated with L_d domains, including the dually palmitoylated Src-family kinase Lyn and the IgE receptor (FcεRI) that are known to preferentially associate with lipid rafts.

Sengupta et al. further explored the structural determinants for partitioning of selected proteins between L_o and L_d phases in GPMVs.[174] Their results showed that 1) GPI-anchored proteins preferentially partitioned into L_o domains, 2) both inner leaflet-anchored proteins, PH-dsRed and flotillin-2-EGFP, were, however, excluded from L_o domains, and 3) unlike LAT-EGFP studied previously in model membrane GUVs,[178] LAT-mEGFP, a peptide corresponding to the transmembrane domain of LAT, preferentially partitioned into L_o domains in GPMVs. The difference in partitioning of the inner leaflet-associated proteins in cells compared to in GPMVs suggests possible roles of cytoskeleton and leaflet asymmetry, both of which are absent in GPMVs, in protein partitioning, while the difference in partitioning for the two LAT peptides highlights that the oligomerization state of the proteins may have a significant effect on their membrane partitioning. Although specific TM residues and/or protein-lipid interactions have yet to be identified, a number of structural factors governing the

affinity of proteins for L_o domains *vs.* L_d domains are emerging, including the nature of protein membrane anchors, their oligomerization states, and their hydrophobic surface properties.

3. CELLULAR SIGNALING BY MEMBRANE-MODULATED PROTEINS

3.1 Membrane-Modulated Signaling Proteins

Back in the 1970s, it was already known that proteins are key players in shaping the signaling function of cell membranes. However, since the introduction of the lipid raft concept, it has become evident that not only the identity of proteins, but also the location and organization of proteins in membranes are integral to their function. These proteins can be roughly divided into two categories based on their location relative to the membrane: transmembrane (TM) proteins that transverse the membrane and peripheral proteins.

TM proteins include membrane surface receptors and membrane transporters. One important class of membrane transporter is ion channels that allow the rapid passage of ions across the otherwise ion-impermeable membrane. Ion channels thus maintain osmotic balance and modulate the membrane potential that is responsible for neurotransmission. In addition to their ion-conducting roles, ion channels often interact with a variety of signaling/scaffolding proteins and intracellular second messengers, and are thus essential for cell signal transduction.[184] Two most abundant classes of transmembrane receptors are G-protein coupled receptors (GPCRs) and single-pass transmembrane receptors (SPTMRs) that span the lipid bilayer seven times and one time, respectively. These transmembrane receptors play essential roles in cellular signal transduction by responding to extracellular signals and transmitting them to the inside of the cell.[185] On the plasma membrane, cell surface receptors are not only anchored by their TM domains, but the interaction and orientation of the TM domains also critically influence their function in signal transduction.[186]

Unlike TM proteins, peripheral proteins associate loosely with membranes. During cellular signaling, peripheral proteins can be recruited to the membrane through their membrane-binding domains (MBDs). A number of such MBDs have been identified, including the protein kinase C (PKC) homology-1 (C1)[187] and -2 (C2),[188] pleckstrin homology (PH)[189,190] and EEA1 (FYVE)[191,192] domains. Structural and biochemical analysis of these domains has shed light on their membrane-binding mechanisms through specific and nonspecific protein-lipid interactions, whereas how their membrane raft specificity (raft *vs.* non-raft) is achieved remains elusive. Nonetheless, it is known that some MBDs bind certain lipid species (e.g., phosphoinositides) with high affinity and specificity, and this may facilitate the recruitment of MBD-containing proteins to the specific regions of the plasma membrane in which these lipids reside; prominent examples of lipid-binding proteins include Akt[193] and PKC[194] kinases. For peripheral proteins that contain no MBD, some utilize part of their molecular surfaces (e.g., secretory phospholipase A2[195]) or amphipathic secondary structures (e.g., ADP-ribosylation factor[196]) to interact with the membrane. Other MBD-free proteins attach to the membrane through covalently-modified lipids;[197] lipid-anchored proteins come in three varieties: prenylated, fatty acylated and glycosylphosphatidylinositol (GPI)-anchored. Here, rather than trying to give a comprehensive catalog of membrane-modulated proteins, we have selected a few examples of membrane-associated proteins involved in signaling processes.

3.1.1 Lipid-Anchored Proteins

Glycophosphatidylinositol (GPI)-anchored proteins, which associate with the outer leaflet of cell membranes through a covalently attached glycolipid GPI, are important to cell signaling. These proteins played a role in shaping the early conception of lipid rafts,[16,93] and have been widely used as a marker to study the organization and dynamics of raft domains. GPI is a ubiquitous lipid anchor for many membrane-associated proteins in eukaryotic cells. The chemical structures of GPI anchors are

diverse, but the GPI core consists of a phosphatidylinositol, a glycan moiety, and a terminal phosphoethanolamine that is covalently attached to the carboxyl terminus of a protein.[198] A prominent feature of GPI-anchored proteins is that they preferentially associate with (are transiently confined to) the raft fraction of membranes enriched in sphingolipids and cholesterol,[93,199] and another is that they often exist in the form of transient homodimers.

Using chemical crosslinking, membrane microdomains of GPI-anchored proteins was demonstrated to exist on the surface of live cells. These microdomains contain >15 GPI-anchored proteins, but are much smaller than those microdomains isolated from cell membranes with non-ionic detergents.[200] Membrane domains involving GPI-anchored proteins have been extensively studied by a variety of biophysical techniques, including FCS,[104] FRAP,[201] and SPT.[202,203] Although these techniques do not directly image membrane organization, they do afford quantitative information on diffusional dynamics of GPI-anchored proteins, from which the nanoscale organization of these proteins has been inferred. Using FRET spectroscopy, the short-range interaction dynamics of GPI-anchored proteins has also been explored.[93,107] In these studies, the extent of energy transfer between GPI-anchored folate-receptor isoforms was measured and found to be independent of fluorophore densities in membranes, suggesting the existence of sub-μm-sized domains at the surface of live cells. Recently, technical breakthroughs in super-resolution FM have enabled visualization of lateral membrane clusters of GPI-anchored proteins of ~10 nm size.[204] These super-resolution experiments further revealed that these nanoscale clusters can coalesce to form larger scale signaling 'hotspots' of ∼100 nm size, supporting the notion of a hierarchical organization of the plasma membrane (containing structures on diverse scales from nm to μm).

Ras, belonging to a family of GTP-binding proteins, binds to the inner leaflet of cell membranes and is an important hub for a myriad of signaling cascades involved in diverse cellular processes. Mutations of Ras are key to several major cancers. Ras activation is strongly dependent on

guanine nucleotide exchange factors (GEFs), but also tightly regulated by its correct intracellular localization relative to the plasma membrane. Ras signaling relies on the selective partitioning of Ras proteins into specific membrane domains, where they form transient nanoclusters, which subsequently serve as the sites for recruitment and activation of various Ras effectors.[205]

Early biochemical experiments provided evidence that H-Ras and K-Ras partitioned into different membrane microdomains, and this was subsequently confirmed by direct EM imaging of the distribution of Ras proteins on membrane sheets.[206] EM provides nanometer spatial resolution, but no temporal information. Therefore, a variety of spectroscopic techniques, including FRET,[207,208] FRAP[209] and SPT[210] have been employed to examine the dynamic Ras localization in cell membranes. The results from these diverse techniques showed that H-Ras exists preferentially in a dynamic association with raft and non-raft membrane domains, while K-Ras localizes predominantly in non-raft regions. Recent experiments using ^{2}H solid-state NMR, FM, and AFM have shown that in model membranes N-Ras accumulates in the domain boundaries in a time dependent manner.[211]

3.1.2 Transmembrane Receptors

G protein-coupled receptors (GPCRs) constitute a large family of transmembrane receptors that are involved in virtually all signal transduction processes. GPCR signaling is part of one of the three major pathways through which extracellular signals are transduced to intracellular signals, and involves GPCRs, heterotrimeric G proteins (consisting of G_{α}, G_{β}, and G_{γ} subunits) and many key enzymes such as kinases and phosphatases, trafficking proteins, and secondary messengers, which are all organized and selectively partition into specific domains of the plasma membrane. As such, membrane structure and organization are crucial for the trafficking and signaling of GPCRs.

The function of GPCRs is primarily controlled by various signaling ligands, the binding of which triggers conformational changes that regulate the interactions of GPCRs with their downstream

effectors. However, the functioning of GPCRs also relies on their membrane environments, including the membrane curvature[212] and lipid composition.[213] For example, modulation of receptor function and organization by cholesterol has been reported for several GPCRs, including the β_2-adrenergic receptor (β_2AR),[214] the serotonin 5-HT_{1A} receptor[215] and the μ-opioid receptor.[216] Despite remarkable progress towards achieving a molecular-level understanding of how GPCRs work, much remains to be uncovered about the interplay between GPCR function and membrane environment and organization.

A number of studies have presented growing evidence that most types of GPCRs can form homo- or hetero-dimers, or even higher-order oligomers, and that this is required for signal transduction.[217-219] Therefore, the signaling function of GPCRs is also coupled with membrane-driven mechanisms that influence their dimerization and/or oligomerization. CGMD simulations showed dynamic oligomerization and clustering of GPCRs in a complex, multicomponent plasma membrane model, and further revealed an important role of cholesterol in promoting the GPCR clustering.[213] The co-localization of GPCRs in membrane raft domains has also been observed in several experimental studies. Experimental evidence was presented that the disruption of functional oligomers of the purinergic $P2Y_{12}$ leads to partitioning of the receptor out of lipid rafts,[220] suggesting that oligomerization is required for localization of the receptor in specific membrane regions for signaling. This phenomenon is reminiscent of that observed in Ras proteins.[221] The driving force for the observed oligomerization in raft domains is to minimize the free energy penalty arising from the hydrophobic mismatch between the protein and the membrane, a mechanism that will further discussed below in **Section 3.2.3**.

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels (LGICs) of the Cys-loop superfamily that respond to the neurotransmitter acetylcholine. nAChRs are involved in cellular signaling in two main ways: either generating second messengers or functioning as effectors by responding to such messengers. nAChR contains five pseudo-symmetric subunits, each consisting

of a conserved extracellular ligand-binding domain, a transmembrane pore domain and a variable intracellular domain. Upon agonist binding, the ligand-binding domain undergoes conformational rearrangements that are propagated to the transmembrane pore domain, leading to opening of the channel to allow ion flow. Gating, and thus the function, of nAChRs is sensitive to their membrane environments. Both cholesterol and anionic lipids have been implicated to promote nAChR gating, in a mechanism thought to selectively stabilize different conformational states of the receptor and perturb the transitions between these states.[222] However, direct experimental evidence supporting this hypothesis is still lacking.

Membrane lipid rafts are important for the function of many types of nAChRs. The neuronal α7 nAChR that has a property of high Ca^{2+} permeability is found to localize within lipid rafts.[223] Biochemical experiments suggested that α7 nAChR exists in distinct raft domains relative to other nAChR subtypes, where it regulates the function of adenylyl cyclase, a calcium-dependent isoform, coexisting in the raft domains.[224] Each nAChR subunit consists of four TM α-helices (M1-M4). Of these, the outmost M4 makes most extensive contact with the membrane, and is thus responsible for a direct association between nAChR and the rafts, which in turn modulates the receptor's function. In addition to the specific lipid-M4 interactions, the selective association of nAChRs with distinct raft domains is also governed by the physical properties of raft domains, such as hydrophobic mismatch and curvature stress, which will also be further discussed below in **Section 3.2.3**.

nAChRs also form oligomers on the membrane and this clustering is lipid raft-dependent.[222] Disruption of rafts by cholesterol depletion was found to result in reduced nAChR clustering and mobility on the cell surface.[225] Further biochemical experiments[226] showed that lipid rafts regulate nAChR clustering by facilitating the agrin/MuSK (muscle-specific receptor tyrosine kinase) signaling and the interaction between rapsyn and the receptor. Rapsyn, a scaffold protein required for nAChR clustering, is located constitutively in lipid rafts. Following MuSK's partitioning into lipid rafts and activation, agrin, a motor neuron-derived factor, stimulates the translocation of nAChR to lipid rafts.

These results provided insight into molecular events involved in nAChR clustering within raft domains, which is both biochemically and biophysically driven.

Cytokine receptors are involved in cytokine signaling, which has a number of biological roles. Cytokine receptors contain a signal-receiving extracellular domain, a single TM domain, and a signal-transducing cytoplasmic domain that does not possess intrinsic enzymatic activity but rather associates with tyrosine kinases to initiate intracellular signal transduction. Cytokine receptors are also regulated by raft domains,[227] which determine the specificity of the signaling and concentrate kinases and adaptor molecules. Disruption of lipid rafts alters cytokine signaling and attenuates the cytokine response.

Lipid rafts have also been implicated in the creation of discrete signaling platforms essential for some cytokine receptors, such as, for example, the erythropoietin receptor (EpoR).[228] Preformed homodimers of EpoRs exist in the absence of ligand,[229] which facilitate the formation of receptor-ligand complexes. For the EpoR receptor, signaling is initiated by a ligand-induced conformational change of the preformed dimer. Mutation of a single residue of the TM region shows an apparent change in subcellular localization and raft localization together with defects in eliciting biological responses and an increase in the inter-helical distance as shown by all-atom modeling of the TM dimer,[230] indicating that the dense packing of the EpoR TM domain and receptor oligomerization are crucial for efficient receptor activation and the selective amplification of downstream signaling cascades. Furthermore, lipid raft involvement with EpoR is of clinical significance. In patients with myelodysplastic syndromes EpoR signaling is impaired and the cells involved exhibit markedly diminished raft assembly compared to normal cells.[231]

Mechanosensing Channels respond to a mechanical deformation of the membrane and convert mechanical forces into electrical signals.[232] Fast mechanotransduction is a process by which many cells, especially sensory cells, rapidly transform (on a sub-millisecond timeframe) mechanical stimuli into graded electrical signals. This process likely requires that a mechanical stimulus directly gates an ion channel. Two models for this force activation have been proposed, and both have been

eventually shown to exist. In the 'membrane force' model, pressure on the plasma membrane alters its shape and/or surface area, causing opening of the protein channel. In contrast, in the 'tether' model molecular tethers convey force between the channel and the cytoskeleton or extracellular milieu.[233,234] Several examples of membrane force-activated channels exist.[235] In one example, the bacterial K^+ channel, MscL, undergoes helix tilting that opens the channel in response to membrane-tension forces concentrated in the interfacial head-group regions of the membrane.[236,237] Mechanosensing channels often reside compartmentalized in membrane domains, and their structure and function are sensitive to both their local (specific) lipid components and (bulk) membrane properties. Interestingly, a non-tension mechanotransduction mechanism was proposed for phospholipase D2 (PLD2) in which lipid rafts sequester PLD2 from its substrate, and mechanical disruption of the lipid rafts leads to substrate access and thus enzyme activation.[238]

The family of transient receptor potential (TRP) ion channels plays numerous physiological roles in signaling pathways. The mechanisms by which various extracellular and intracellular stimuli activate these channels are of interest. As an example, polycystin 1 (PC1), an adhesin-like receptor, and polycystin 2 (PC2), a nonselective ion channel, interact through their respective coiled-coil domains to form a mechanosensitive ion channel,[239] which mediates bending of the cilia to induce calcium flow into the cells. PC1 has a large extracellular portion made up of multiple domains in tandem array. The most common of these are the polycystic kidney disease (PKD) domains, which have a β-sandwich immunoglobulin-like (Ig-like) fold. Multi-domain proteins with Ig-like domains tend to resist unfolding when subject to force. Indeed, for PC1 to be an effective mechanosensor the extracellular domains must remain intact under physiological forces. Thus, AFM has shown that PKD domains exhibit a remarkable mechanical strength, suggesting that they have indeed evolved for mechanical stability and force transduction.[240] MD simulations suggest that the force induces a conformational rearrangement to an intermediate structure that possesses nonnative hydrogen bonds

and resists unfolding.[241] Furthermore, the engineering of mutations that prevent formation of these nonnative interactions was found to only moderately destabilize the native structure but reduce the mechanical strength dramatically. Very recently, a molecular mechanism was identified whereby PC1 and PC2 in the skeleton sense mechanical loading to regulate bone mass through the reciprocal control of osteoblastogenesis and adipogenesis.[242] Furthermore, structure-based high-throughput screening identified a small molecule that binds to PC2, enhances TAZ-mediated transcription, and mimics the effects of mechanical loading to stimulate osteoblastogenesis and inhibit adipogenesis *in vitro*. This may pave the way to therapies for osteoporosis and polycystic kidney disease.

TRP channels are believed to localize in lipid rafts. Although little is known regarding specific mechanisms of their raft localization, emerging data suggests that channel functions and properties are intimately coupled with their localization in and interactions with raft domains. Transient receptor potential channel 1 (TRPC1) is involved in store-operated Ca^{2+} entry (SOCE) by interacting with caveolin-1 (Cav1) and Ca^{2+} signaling proteins. Cholesterol depletion experiments showed that raft disruption dramatically reduced SOCE, supporting a role of raft in TRPC1 function.[243] Sághy et al. provided the first evidence for the importance of lipid rafts in TRPV1 and TRPA1 gating by measuring agonist-induced Ca^{2+} current with a ratiometric technique.[244] Further, the hydrophobic interactions between TRP channels and lipid rafts were proposed to modulate the opening properties of these channels. The same group also studied the inhibition of TRPV1 activation by a carboxamido-steroid compound (C1) and found that C1 inhibits the opening properties of TRPV1 by lipid raft modulation.[245] Recent electrophysiological and imaging experiments demonstrated that the activity of Transient Receptor Potential Melastatine 8 (TRPM8), a thermosensitive TRP channel, is also dependent on its raft association. Specifically, when lipid rafts were disrupted menthol- and cold-mediated responses of TRPM8 were found to be potentiated and the channel activation threshold shifted to a warmer

temperature.[246] Interestingly, it was also shown that glycosylation at the Asn934 residue facilitates partitioning of the protein into lipid rafts.

3.1.3 Lipid-Binding Proteins

Akt (**protein kinase B**) is a key enzyme involved in the phosphatidylinositide signaling pathway, or the phosphatidylinositol 3-kinase (PI3K)/Akt pathway that promotes cell survival and growth in response to extracellular signals. Akt, a serine/threonine kinase, resides in the cytosol in an inactive conformation. Upon the production of phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) by activated PI3K, Akt translocates to the plasma membrane by interaction with PIP_3 through its PH domain, such that Akt is fully activated to mediate downstream signaling. The Akt PH domain binds to PIP_3 preferentially over other phosphoinositides. FCS experiments provided evidence that efficient Akt membrane recruitment and activation is strongly correlated with the presence of raft domains in the membrane, and that the Akt PH domain drives the raft partition.[247] Given that phosphoinositide-dependent protein kinase 1 (PDK1) also resides in raft domains, the co-localization of Akt and PDK1 may thus promote phosphorylation (and thus activation) of Akt by PDK1. Using a genetic targeting strategy that allows perturbing a signaling event in specific membrane domains, it was demonstrated that Akt kinases are activated in membrane rafts, whereas the Akt inactivating (dephosphorylating) enzymes, phosphatase and tensin homolog deleted on chromosome 10 (PTEN), are primarily located in the non-raft regions of the membrane.[193] A similar mechanism was proposed to explain the tocopherol-mediated inactivation of Akt by the PH domain leucine-rich repeat-containing protein phosphatase 1 (PHLPP1): tocopherols selectively partition into non-raft membranes, where they co-recruit Akt and PHLPP1 through their respective PH domains to facilitate the dephosphorylation (thus inactivation) of Akt at Ser473 by PHLPP1.[248]

The ζ isotype of protein kinase C (PKCζ) belongs to the atypical PKC subfamily and is a key regulator of many intracellular signaling pathways, for example, signaling downstream of insulin receptors.[194,249] Unlike classical PKC isotypes, PKCζ is Ca^{2+} insensitive, nor does it bind diacylglycerol (DAG); instead the activity of these protein kinases is regulated by PIP_3 and ceramide. PKCζ contains three functional domains, including an N-terminal regulatory domain, a C1 domain and a C-terminal catalytic domain. The N-terminal domain normally binds to the C-terminal catalytic domain, thus self-inactivating the enzyme. PKCζ normally resides in the cytosol in an inactive form, and becomes activated by binding of PIP_3 to its C1 domain and phosphorylation of its activation loop residue T410, which translocates the enzyme from the cytosol to the membrane and triggers a conformational change, liberating the enzyme from auto-inhibition.[249] This activation of PKCζ eventually promotes glucose uptake through recruitment of the GLUT4 glucose transporter to the cell membrane.

Ceramides, a class of sphingolipids, are composed of sphingosine and a fatty acid. Generally, the fatty acyl chains are saturated or monounsaturated, and contain 16-20 (long) or 22-24 (very long) carbons. A number of studies have suggested that ceramides bind to the C1 domain of PKCζ and is essential for the kinase activation. It was demonstrated that hexanoyl-D-erythro-sphingosine, a ceramide analog, could enhance localization and phosphorylation of PKCζ in lipid microdomains, but reduce the association of PKCζ with a non-raft residing protein 14-3-3.[250] It was further shown that disruption of lipid microdomains led to the abrogation of PKCζ-dependent Akt inactivation, supporting the role of ceramides in localizing PKCζ to lipid rafts to activate the enzyme. Taken together, existing data highlights the involvement of the critical membrane lipids, ceramides, in organizing large signaling complexes (in membrane microdomains) necessary for activation of PKCζ.

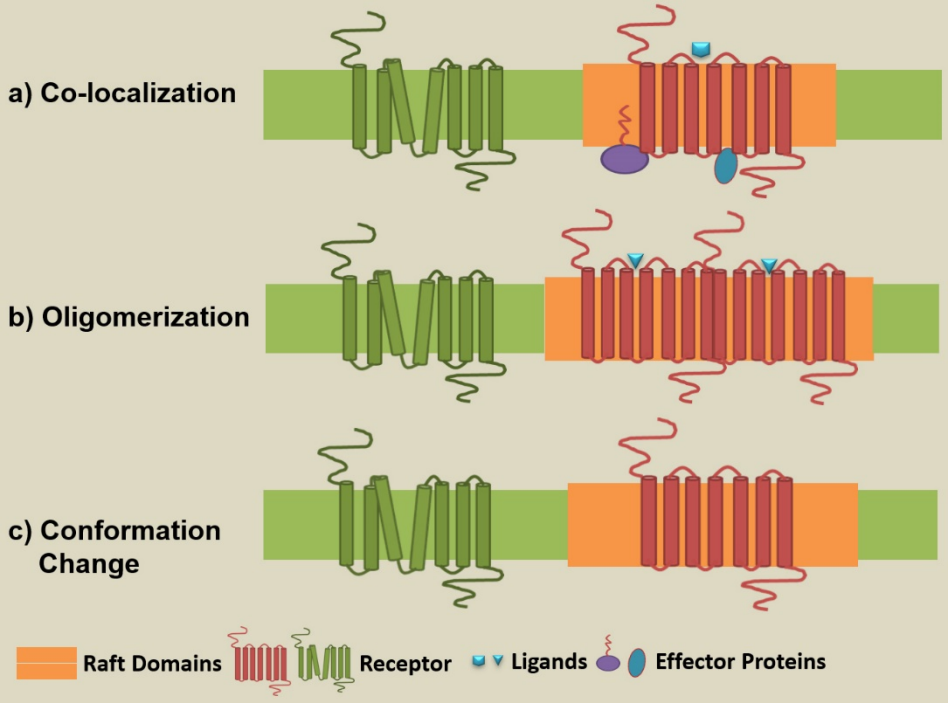

Figure 9. How can membrane organization modulate protein function? a) Lipid rafts spatially organize signaling molecules at the membrane to enhance protein-protein encounter kinetics; b) Lipid rafts facilitate the oligomerization of membrane surface receptors, triggering downstream signaling. c) Lipid rafts induce specific protein conformational changes that are necessary for signal transduction via the following two mechanisms: TM receptors can be gated by specific lipid components; or conformational equilibria and/or conformational transition kinetics of the proteins can be affected by the distinct physical properties of lipid rafts.

3.2 How Membrane Organization Modulates Protein Function

The structure, dynamics and function of the above-described and other membrane-associated proteins are all intimately coupled with their membrane environments. Membrane lateral organization can modulate protein function in two broadly-defined ways (Figure 9). Firstly, raft domains compartmentalize in the membrane plane, recruiting certain proteins while excluding others.

Therefore, the most prominent functional role of membrane organization appears to be through facilitating the co-localization (and possibly oligomerization) of signaling proteins and their relevant partners at the membrane (Figures 9a, b), which can enhance the efficiency and specificity of the signal transduction. For example, it has been shown with gramicidin that nanoscopic phase separation in the membrane affects the dimer dissociation kinetics,[251] providing direct evidence for the modulation of ion channel function by membrane organization.

Additionally, membrane organization can modulate protein function by the induction of specific conformational changes in proteins that are necessary for signal transduction (Figure 9c). This can be achieved via two main mechanisms: TM receptors can be gated by specific lipid components, such as cholesterol, by mechanisms analogous to classical agonists or allosteric modulators;[252] or conformational equilibria and/or conformational transition kinetics of the proteins can be shifted by the distinct physical properties of lipid rafts.[253,254]

While the coupling of protein function with the physicochemical state of the membrane provides a versatile mechanism for spatiotemporally coordinating signaling events from both the inside and outside of a cell, many molecular details remain to be elucidated. What are the driving forces for co-localization and induction of specific conformational changes in proteins? Why do proteins tend to cluster in specific membrane domains? How do distinct phase states of the membrane shift the conformational equilibrium of proteins? Addressing these questions is required to obtain a deeper understanding of the interplay between membrane organization and cellular signaling. Here, we select a few examples illustrating different mechanisms in play. While these underlying mechanisms vary, a few general principles are emerging. These principles are reminiscent of the fundamental mechanisms governing the formation of membrane lateral organization discussed in **Section 2.3**.

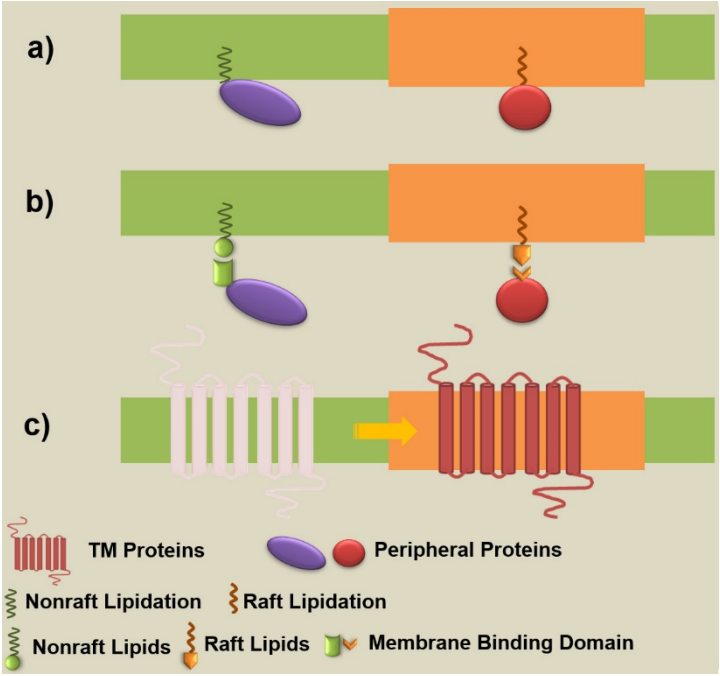

Figure 10. How do proteins preferentially partition into lipid rafts? a) Proteins are modified by different lipidations with distinct affinities for raft and non-raft fractions of the membrane. b) Proteins bind to different lipid species in the membrane that are concentrated in either raft or non-raft domains. c) TM receptors preferentially partition into the membrane domains that have comparable hydrophobic thickness to minimize the hydrophobic mismatch.

3.2.1 Lipid Anchors with Differential Raft Affinities

The majority of membrane co-localization can be attributed to the selective association (partition) of proteins with specific membrane domains. Many peripheral membrane proteins interact with membranes through their covalently attached lipid anchors (Figure 10a). Different lipid anchors have different raft affinities, which are determined largely by the nature of lipid anchors and the lipid tail packing of their local membrane environments.[176,255] Generally, saturated lipid anchors, such as palmitoyl, and myristoyl moieties, lead the corresponding lipid-anchored proteins to preferentially

segregate into more ordered raft domains, while branched or unsaturated lipid anchors, such as farnesyl and geranylgeranyl groups, prefer non-raft regions of the membrane.

GPI anchors have considerable diversity in the fatty acid chains attached to their phosphatidylinositol core, which has important implications for the partitioning and organization of GPI-anchored proteins in the membrane.[256] By incorporating streptavidin covalently modified with fully saturated or polyunsaturated lipid anchors into the Jurkat T-lymphoma cell membrane, confocal FM experiments revealed that the lipid anchors could elicit clustering-induced intracellular signaling.[257] The length and lipid structure (degree of saturation and branching) of GPI anchors were identified as determinants of the association of the GPI proteins with specific membrane domains. Further, using AFM to probe proteins in supported lipid bilayers, it was shown that the partition of GPI-anchored proteins into raft domains was influenced by sphingomyelin chain length: the TM-anchored angiotensin converting enzyme with a GPI anchor was found to associate with rafts in bilayers formed with brain sphingomyelin (mainly C18:0), whereas raft association did not occur in bilayers formed with the shorter-chain egg sphingomyelin (mainly C16:0).[258] In contrast, a membrane dipeptidase with a GPI anchor containing distearoyl phosphatidylinositol was excluded from rafts in egg sphingomyelin bilayers, but was associated with rafts in brain sphingomyelin bilayers. Clearly, given the vast variations in the composition and linkages of the diacyl- or alkyl-acylglycerol moieties of GPI proteins, much remains to be uncovered about the biophysical principles determining the selective incorporation of GPI proteins into distinct membrane domains.[259]

Lipid anchors are essential for localizing Ras proteins to specific membrane domains. Ras attaches to the membrane through its prenylation and palmitoylation (H-Ras and N-Ras) or the combination of prenylation and a polybasic sequence adjacent to the prenylation site (K-Ras). It has been shown that K-Ras4B attached with a farnesyl group can readily partition into the more loosely-packed non-raft fraction of the membrane, but not into raft domains that are more tightly packed with saturated phospholipids and cholesterols.[260] In addition to lipid anchors, neighboring protein sequences

(adjacent to the lipidation site) and protein conformations have also been recognized to govern the raft localization of Ras proteins.[261]

The spatial organization (nano-clustering) of Ras in the membrane is also controlled by lipid anchors.[262,263] The exposure of farnesyl groups to raft domains was found to promote the K-Ras4B oligomerization, thus increasing protein's cooperativity.[260] Detailed studies with extensive MD simulations revealed that the triply lipidated membrane-targeting motif of H-Ras assembles into clusters and segregates to the boundaries of membrane domains, driven by the differential preference of the palmitoyl and farnesyl anchors for L_o and L_d domains, respectively. H-Ras clusters prefer the domain boundaries, while the monomers do not, suggesting an interplay between Ras clustering and its domain-specific distribution.[221] Further, each Ras isoform has been shown to interact with a distinct set of lipid molecules within a Ras nanocluster, which may lead to isoform-specific Ras signaling.[264,265] More specifically, it was proposed that the precise amino acid sequence and lipid modification of the Ras proteins define a combinatorial code for their membrane binding, governed by distinct dynamic tertiary structures of the lipid anchors. This distinct Ras-lipid interaction then gives rise to differential membrane domain localization and nanoclusters with different lipid compositions that together shape the signaling output of Ras.

3.2.2 Specific Binding of Lipid Molecules

Lipid composition determines specific membrane functions and homeostatic mechanisms, and maintains these functions by regulating physical properties of membranes *via* lipid compositional adjustments.[266] Organisms unable to maintain thermal homeostasis, such as bacteria, must regulate their membrane lipid compositions in response to temperature; without regulation, temperature would affect membrane fluidity and thus function. Bacterial membranes regulate their fluidity in response to the environment through embedded thermosensors.[267] These sensors mediate the cold transcriptional

induction of acyl lipid desaturase enzymes that introduce *cis* double bonds into preexisting fatty acids, thus optimizing membrane fluidity at the new temperature. What membrane properties do these proteins sense? Lowering the temperature will decrease the area per lipid, and may then increase membrane thickness, and this can be detected by membrane proteins. A histidine kinase (DesK) has been well studied in this regard. Intriguingly, two hydrophilic amino acids (Lys10 and Asn12) are critical for DesK cold activation.[268] Given that these side chains should be able to snorkel to the membrane-water interface, these amino acids could act as a buoy, stabilizing the position of the transmembrane segment of DesK. In this 'sunken buoy' model of thermosensing the unstable state involves dehydration of the polar cluster, resulting in membrane expansion associated with a kinase activity, whereas hydration accompanying membrane narrowing would promote phosphatase activity.

Lipid composition also plays an important role in shaping the function of membrane-associated proteins. Certain membrane (lipid) components, such as sterols, ceramides, phosphoinositides (PPIns), diacylglycerol (DAG) and lyosphospholipids have emerged as important regulators of cellular signaling (Figure 10b). Most of these lipids cannot form bilayers alone and must be mixed with other lipids. Therefore, the presence of these lipids in planar bilayers causes local membrane deformations and stresses, which may dictate membrane's ability to interact preferentially with certain proteins or certain regions of proteins. Importantly, many of these lipids have distinct binding affinities (or abundance) for different membrane domains (raft *vs.* non-raft) as well as for different proteins, potentially triggering membrane-domain-specific signaling events.

Some lipids, such as phosphoinositides, facilitate the recruitment and/or activation of proteins to form spatially localized functional complexes. A large number of proteins can selectively bind phosphoinositides through their PH domains. For example, the PH domain of phospholipase C-δ1 (PLCδ1) that binds to the head-group of phosphatidylinositol 4,5-bisphosphate (PIP_2) with high specificity is required for localization of the enzyme in the membrane.[269] Similarly, PIP_3 binds to the

PH domains of protein kinases, e.g., Akt and PDK1, and recruits both proteins to the PIP_3 site (raft domains) in the membrane in response to PI3K activation.[270,271]

Lipids can also act as second messengers or through specific lipid-protein interactions in signaling cascades. Since specific lipid species are enriched in distinct membrane domains, selective protein-lipid interactions thus manifest as specific protein-membrane domain interactions. Increasing experimental evidence suggests that lipids can bind to and modulate the function of ion channels in a manner analogous to classical agonists or allosteric modulators.[252] Lipids have been found in the heat-gated ion channel, TRPV1 (Transient Receptor Potential cation channel subfamily V member 1) and contribute to its activation mechanism: phosphatidylinositol is a competitive antagonist of TRPV1.[272] In Polycystin-2 (PC2), a TRP channel, described above, that is involved in Ca^{2+} signaling pathways, phosphatidic acid (PA) forms a tight complex with the protein, and structural data indicates that the type of lipid bound may be crucial for the conformation of the selectivity filter.[273] The conserved lipid-binding site in PC2 is a potential lipid-mediated activation mechanism. The preferred locations of PC2 outside the endoplasmic reticulum, such as in the ciliary membrane or the plasma membrane, differ in the content of negatively-charged lipids: the ciliary membrane contains elevated levels of phosphoinositides and sterols, and these differences in lipid composition may directly contribute to the functional diversity of PC2 at different locations. The binding of different membrane lipids would help to partially explain the location-dependent functional diversity reported for PC2.

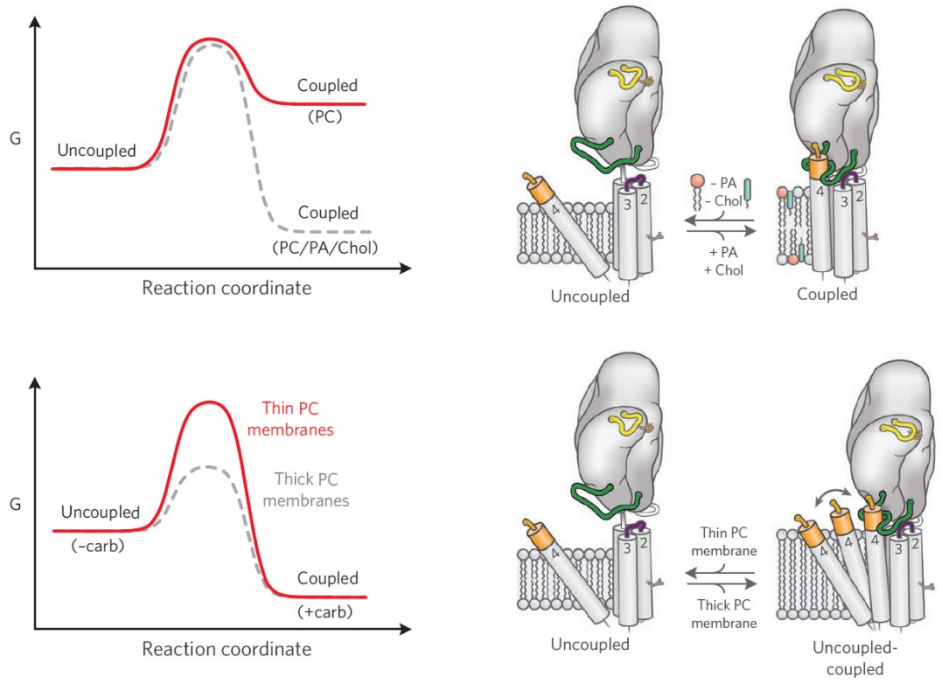

Figure 11. Two speculative mechanisms by which lipid-nAChR interactions can modulate the nAChR activation (the conversion of an uncoupled conformation to a coupled conformation): conformational selection (top) and a kinetic mechanism (bottom). Adapted with permission from Ref.[254] Copyright 2013 Springer Nature.

Cholesterol has been shown to influence the structure, dynamics and function of a large number of membrane proteins, including GPCRs,[274] nAChR,[222] inwardly-rectifying potassium (Kir)[275] channels, and TRPV.[275,276] Multiple potential binding sites of cholesterol have been identified in these proteins by docking calculations and MD simulations. Non-annular cholesterol binding sites are important for protein function in several different protein families, whereas no consensus has been reached concerning the importance of cholesterol consensus motif (CCM) in cholesterol recognition.

Crystallographic structures of β_2AR revealed a CCM, which was predicted to be present in 21% of Class A GPCRs.[277] However, subsequent crystal structures of several other GPCRs showed no bound cholesterol at this site.[278] Recent extensive atomistic simulations showed that the human β_2AR

is allosterically modulated by cholesterol.[279] Specifically, cholesterol binds to specific high-affinity sites on the transmembrane helices 5-7 of the β_2AR to alter its conformational dynamics. CGMD simulations have been employed to explore the correlation between cholesterol binding and dimerization of the β_2AR.[280] It was found that cholesterol prevents dimerization of the receptor *via* binding to transmembrane helix 4 that forms the dimer interface.

The importance of cholesterol in nAChR function has been documented in a number of studies.[281] Back in 1980, it was first shown[282] that the inclusion of cholesterol in a reconstituted phosphatidylethanolamine (PE)/phosphatidylserine (PS) membrane not only enhanced agonist-induced ion conduction, but also promoted transitions from the resting to the desensitized state. Currently available structural and functional data on nAChRs reveal that cholesterol can modulate TM α-helix/α-helix packing between M4 and the adjacent M1 and M3, which in turn can either stabilize different conformational states and/or alter the allosteric transition pathway of the receptor.[222] A recent study of nAChRs reconstituted into PC membranes showed that cholesterol and anionic lipids can alter the rates of transitions between activatable and non-activatable conformation states (Figure 11).[254] Specifically, cholesterol and anionic lipids thicken the PC bilayers, lowering the energetic barrier so that agonist-induced conformational transitions can occur on an experimentally accessible time scale. Given the great complexity in nAChR conformational landscapes and nAChR-lipid interactions, unraveling the molecular mechanisms underlying lipid-nAChR interactions thus remains an immense challenge, and it has thus been proposed that these mechanisms must be elucidated under a comprehensive thermodynamic framework.[281]

Phosphoinositides (PPIns) are critical for the recruitment and activation of effector proteins in plasma membranes.[283] There are seven possible PPIns based on which inositol positions are phosphorylated: phosphatidylinositol 3-phosphate (PtdIns3P), PtdIns4P, PtdIns5P, PtdIns 3,4-bisphosphate (PtdIns(3,4)P_2), PtdIns(3,5)P_2, PtdIns(4,5)P_2 and PtdIns 3,4,5-trisphosphate

(PtdIns(3,4,5)P_3). As discussed copiously above, PtdIns(3,4,5)P_3, or PIP_3, is a key modulator of the PI3K/Akt signaling pathway. Its other effector proteins include small GTPases of the Arf family (ADP-ribosylation factors), PDK1 and phospholipase γ (PLC_γ). All these effectors interact with PIP_3 through their PH domains.[284]

PtdIns(4,5)P_2, or PIP_2, is the most abundant PPIns in the inner leaflet of mammalian plasma membranes, representing ~45% of total PPIns. Unlike PIP_3, PIP_2 mostly preferentially partitions into non-raft regions of the membrane. PIP_2 is essential for the activation of the MAPK (Mitogen-Activated Protein Kinase) Rho1/Pkc1-mediated signaling cascade, on which actin cytoskeleton organization critically depends.[284] It functions through interacting with an ENTH (Epsin N-terminal homology), ANTH (AP-180 N-terminal homology) or PH domain of effector proteins.[285] PIP_2 also activates Kir, a tetrameric potassium channel containing a TM channel domain coupled to a large cytoplasmic domain. PIP_2 binds to four identical interaction sites on the surface of Kir to modulate the channel activity.[286,287] Additionally, PIP_2 has been shown to influence clustering of ion channels. The effect of PIP_2 on membrane organization has been investigated with CGMD simulations of a plasma membrane model containing >100 copies of Kir.[288] It was found that Kir channels tend to cluster in a PIP_2-dependent manner, thus organizing the membrane into dynamic compartments, which may in turn affect the channel function.

Using a large-scale mammalian plasma membrane model, recent CGMD simulations have explored how specific-protein lipid interactions mediate the organization of sphingosine-1-phosphate receptor 1 (S1P1) in the membrane. Specific interaction sites, particularly for PIP_2 molecules, were identified, which might facilitate the co-clustering of the lipid and the S1P1 receptor. The simulations also showed a strong interaction pattern between S1P1 and cholesterol. It was found that the hydroxyl group of cholesterol interact specifically with residues in TM1, TM2 and TM3 of the receptor. Interactions between cholesterol and TM6 were also observed; this is relevant because the TM6 helix

undergoes the largest conformation changes during activation, so it is most likely to be functionally coupled to the activation of the receptor.[213]

Ceramides exert significant influence on various membrane physical properties, and this has been thoroughly reviewed recently.[289] Ceramides tend to spontaneously aggregate, resulting in the formation of ceramide-enriched microdomains.[290,291] These microdomains often form projections with high curvature, which can trigger not only protein clustering but also conformational changes of certain proteins. Confocal microscopy experiments revealed the formation of micron-sized highly ordered gel domains in model membranes induced by ceramides, which can be partially attributed to the extensive hydrogen bonding interactions in the polar head-group regions of the bilayers.[292] Ceramide-rich membrane microdomains have also been observed in living cells by fluorescence microscopy.[293,294] These ceramide-rich membrane domains were demonstrated to facilitate CD95/CD40 signaling just like the lipid rafts do - to recruit certain proteins while excluding others and stabilize the large signaling complexes.[291]

In addition to serving structural roles in membranes, ceramides can also directly modulate protein function and thus signal transduction through specific lipid-protein interactions. Ceramides have been particularly implicated in the potentiation of signaling cascades that lead to cell death.[295] This ceramide signaling involves protein phosphatase 2A, p38, JUN N-terminal kinase (JNK), Akt, protein kinase Cζ (PKCζ) and survivin. It has been shown that the activity of protein phosphatase (PP)1 and PP2a and the protein kinase C family is regulated by ceramides, possibly by direct binding of them to the proteins, although no specific binding sites for ceramides have been identified.[249] Ceramides have been shown to specifically bind to and activate protein kinase c-Raf, leading to subsequent activation of the important MAPK cascade.[296]

3.2.3 Membrane Physical Properties and Protein Function

The structure and physical properties of membranes have important implications for the function of both the membranes themselves and membrane-associated proteins involved in cellular processes. Membrane domains with distinct physical properties thus influence protein partition and function in different ways (Figure 10c). Local lipid binding events in the proximity of proteins can also modify the physical properties of the membranes, such as the shape and tension, which can in turn modulate the behaviors of constituent proteins. In the following, we briefly discuss important mechanical and physical membrane properties of relevance to cross-membrane signaling.

The **membrane potential** comprises three components: 1) the transmembrane potential due to any charge gradient across the membrane, 2) the surface potential resulting from any net excess charges on the membrane, and 3) the membrane dipole potential, which arises from the molecular dipoles located on the membrane. Changes in the electrical potential across a membrane can affect cell growth and proliferation. For example, dividing cells are more depolarized than quiescent cells, and oncogenically transformed cells are generally more depolarized than normal cells.[297] A mechanism for this effect has been recently suggested, again involving Ras proteins. As described above, nanocluster assembly of the Ras proteins requires complex interactions between membrane lipids and the Ras lipid anchors and amino acid residues. Recent work demonstrated that plasma membrane depolarization induces rapid and substantial changes in the nanoscale organization of anionic phospholipids on the inner leaflet, leading to increased K-Ras nanoclustering. Thus, K-Ras nanoclusters may allow the membrane to act as a field-effect transistor to control the gain in Ras signaling circuits.[298]

The origin of the **dipole potential** is the preferential alignment of interfacial water dipoles with dipoles in the lipid molecules, to which a negative contribution comes from phospholipid head-group P^--N^+ dipoles. The interior of the bilayer has a large positive dipole potential, of several hundred millivolts.[299,300] Theoretical considerations, and experimental evidence obtained from model membranes, indicate that in L_o domains the dipole potential is stronger than in L_d domains. Therefore, the heterogeneity of the dipole potential in plasma membranes would be correlated with the presence

of lipid rafts.[301] Moreover, it has been shown that the dipole potential affects the insertion and folding of an amphiphilic peptide in the membrane.[302] In this vein, the study of a drug molecule, saquinavir interacting with membranes and membrane receptors[303] revealed that the dipole potential modulates membrane-protein interactions, contributing to the altered behavior of membrane receptors likely located in membrane rafts.

Hydrophobic mismatch refers to the difference between the thicknesses of two adjacent hydrophobic regions. In phase-separated model membranes, hydrophobic mismatch was shown to regulate the size of the coexisting domains.[35] It is also thought to cause domain anti-registration observed in CGMD simulations of model membranes.[88] The hydrophobic thickness of TM proteins normally should match that of the hydrophobic (lipid acyl chain) regions of the surrounding lipid bilayers (Figure 10c). If a thickness mismatch does occur, both the protein and the lipid bilayer will adapt themselves to minimize this mismatch. For example, the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) can be accommodated in membranes of different hydrophobic thickness by inducing conformational changes in the protein as well as local deformation of the lipid bilayers.[304] Hydrophobic thickness compatibility thus provides an additional mechanism by which membrane organization can modulate protein function, *i.e.*, thickness mismatch induces conformational changes or shifts of conformational equilibria of proteins (Figure 9c). Hydrophobic thickness has been shown to influence interactions between membrane-reconstituted γM4 α-helices of nAChR by altering the α-helix/α-helix packing.[305] Recently, hydrophobic thickness has also been demonstrated to modulate the relative populations of activatable *vs.* nonactivatable conformations and control the transition kinetics between the two conformations to shape the nAChR activity (Figure 11).[254]

In a further effect, if a membrane segregates into multiple domains with distinct hydrophobic thicknesses, a protein will preferentially partition into those domains with a comparable hydrophobic thickness, resulting in clustering of specific lipids and proteins (Figure 10c).[306] In addition to TM proteins, the mechanism with which lipid anchors influence the distribution of lipid-anchored proteins

in membrane domains is also partially rooted in matching the hydrophobic thickness of acyl chains.[258] FRET experiments showed that the association of rhodopsin in membranes is promoted by a reduction in membrane thickness (hydrophobic mismatch).[307] The study further suggested that the functional state of rhodopsin depends on the clustering of the protein in the membrane, and long-range lipid-protein interactions due to curvature deformation (strain) and hydrophobic solvation forces drive rhodopsin oligomerization. Interestingly, it was found that, in both model membranes and simulations, structural adaptation at the peptide-lipid interface in response to hydrophobic thickness mismatch is significantly constrained by the presence of cholesterol.[118] This constraint in hydrophobic thickness will then facilitate the selective lateral segregation of proteins and lipids, suggesting a complex interplay between protein sorting/clustering and membrane phase separation.

In search of structural determinants for raft association, Lorent et al. quantified the raft affinity of dozens of transmembrane domains (TMDs).[308] Three physical properties were identified, which collectively determine raft partitioning: TMD palmitoylation, thickness and surface area. As discussed above in **Section 3.2.1**, palmitoylation facilitates raft partitioning of palmitoylated proteins because its saturated acyl chains have high affinity for the more ordered L_o phase. Lipid rafts are usually thicker than coexisting non-raft domains. To avoid hydrophobic mismatch, proteins with thicker TMDs would thus preferentially partition into rafts. Although this hypothesis was challenged by earlier experiments in DRMs and synthetic lipid mixtures,[309,310] recent experimental evidence in GPMVs has shown that the TMD length was indeed strongly correlated with raft phase partitioning.[311] The raft partition coefficient decreased from 1.1 to ~0.65 when an α-helix was reduced from 24 to 18 residues. This correlation held true for most four single-pass transmembrane proteins assayed.

Besides palmitoylation and thickness, TMD surface area was also identified as a determinant for raft partitioning.[308] This was supported by a strong correlation between the raft affinity and total solvent accessible surface area (ASA) of a TMD. Proteins with smaller TMD ASAs bind more strongly

to lipid rafts than those with greater TMD ASAs, consistent with the observation that TM proteins tend to oligomerize when moving from non-raft to raft domains. The surface area-dependent partitioning suggests that local packing of TMDs into an ordered (*vs.* disordered) lipid environment is associated with an energetic cost - the differential surface tension ($\Delta\gamma_{TMD,Lo\text{-}Ld}$) for a TMD solvated between a raft ($\gamma_{TMD,Lo}$) and non-raft ($\gamma_{TMD,Ld}$) phases. Based on the measured partition coefficients, $\Delta\gamma_{TMD,Lo\text{-}Ld}$ was estimated to be 1.1 pN/nm, in good agreement with a previous computationally predicted value of 0.6 pN/nm.[84]

Lateral pressure affects many membrane-related cellular processes by triggering conformational changes of membrane proteins or by shifting the equilibrium of their conformational states. Steric repulsion between the hydrophobic core and between the polar head-groups gives rise to high (repulsive) lateral pressures in these regions, balanced by attractive hydrophobic forces at the acyl chain-water interface. As such, the lateral pressure tends to compress proteins embedded within the membrane, especially those located at membrane domain boundaries, which can induce conformational changes or alter conformational (or aggregation) equilibria of these proteins that reduce their cross-sectional area within the high-pressure regions of the membrane, thus modulating their function.

Work elucidating the effect of varying the sterol on membrane lateral pressure profiles showed significant changes when cholesterol is replaced by desmosterol, 7-dehydrocholesterol or ketosterol.[312] Thus, the intriguing idea exists that, by altering the lipid composition the lateral pressure profile can be adjusted and, as a result, the functional state of a membrane protein. Several membrane proteins are known to be structurally and functionally altered by changes in membrane lipid composition that modulate the lateral pressure profile.

Interestingly, it has been suggested that the mechanism of anesthetic action may arise from the modulatory effect of some general anesthetics on membrane lateral pressure profiles:[313] channel opening increases the cross-sectional area of postsynaptic ligand-gated ion channels, and anesthetic-

induced lateral pressure in the membrane will thus favor the closed state, which effectively desensitizes the ion channels. A combined X-ray diffraction and all-atom MD simulation study on effects of the anesthetic drug ketamine on membrane structures partially confirmed this lateral pressure-mediated hypothesis,[314] showing that the presence of ketamine in the membrane at clinically relevant concentrations caused a significant shift of lateral pressure toward the center of the bilayer. This effect was estimated to be large enough to subsequently modulate the activity of membrane-bound nAChR channels by affecting their opening probability. Recently, a family of mechanosensitive ion channels has been identified that senses lateral stress forces from the lipid bilayer to open and close the channels,[236] which will be further discussed below.

Membrane curvature. Most cellular membranes have regions of high curvature, induced by the local composition of lipids and proteins. Membrane curvature stress as well as non-equilibrium reshaping of the membrane exerts direct influences on protein functions by affecting conformational dynamics, ligand binding and allostery.[254] Membrane curvature also contributes to raft formation[315] by stabilizing nanoscale domains.[139] Contrariwise, raft formation can induce membrane curvature at the phase boundaries, which in turn can influence the sorting, partitioning and functioning of proteins and lipids. Recently, it was demonstrated that the nanoscale lipid assembly on stem cell membranes is mediated by lipid raft formation *via* cell geometry changes,[316] and that these changes activate Akt signaling pathways, which provides a mechanism describing how cell shape (curvature) regulates cellular processes such as cell differentiation, growth and survival.[317,318]

Generating high local membrane curvature is usually mediated and controlled by specialized proteins[319-321] that can either directly induce curvature by interactions with the membrane or act as indirect scaffolds interacting with membranes via linking proteins. These proteins can either embed their small hydrophobic or amphipathic regions into the membrane matrix[320,321] or attach the membrane surface to their intrinsically curved protein scaffolds via electrostatic interactions.[322] For the direct interaction case, a quantitative mechanism of membrane bending by hydrophobic or amphipathic

helices has been suggested. In this mechanism the membrane monolayer is modeled as an anisotropic elastic material coupled with the internal stresses and strains arising from the embedded inclusions.[323] An example of the latter, indirect scaffold mechanism is the *Drosophila* amphyphsin BAR domain, a peculiar banana shaped protein that senses membrane curvature by binding preferentially to highly-curved, negatively-charged membranes.[324]

Membrane tension is kept stable through the physical feedback of membrane bending energy, which controls membrane lipid composition through modulating the conformation of phosphocholine cytidylyltransferase, a membrane-embedded enzyme that catalyzes a key step in the phosphatidylcholine synthesis.[325] Caveolae - small cup-shaped membrane invaginations rich in sphingolipids and cholesterol - have also been shown to regulate membrane tension through their disassembly/reassembly cycles.[326]

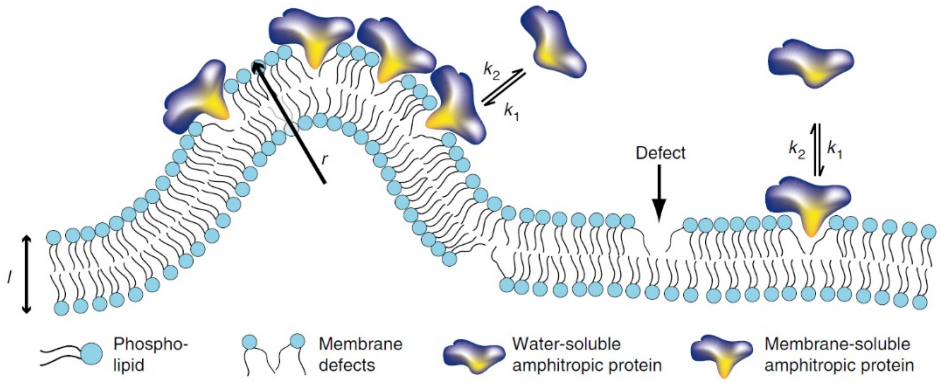

Figure 12. A schematic model of how membrane curvature is sensed by amphiphilic molecules. Membrane bending introduces packing defects on the membrane surface that function as binding sites for amphiphilic molecules. Adapted with permission from Ref.[327] Copyright 2009 Springer Nature.

Membrane curvature can drive subcellular localization and functioning of proteins. By specific amino acid substitutions, it was demonstrated that the preference for strongly curved membranes of

the classical chemoreceptor TlpA arises from the curved shape of chemoreceptor trimer of dimers, highlighting the importance of the intrinsic shape of TM proteins in determining their subcellular localization.[328] Combined experiments and molecular theories showed that membrane curvature is essential for the enrichment of the N-Ras lipid anchor in raft-like phases, driven by the relief of curvature stress upon anchor insertion. underscored the role of in determining the partition of N-Ras lipid anchor and palmitoyl chain into the membrane.[329] CGMD simulations have shown that the curvature stress caused by the local deformation in the membrane produces long-range attractive forces that facilitate membrane-mediated protein assembly.[330] It has further been suggested that this curvature-mediated attraction can promote cooperation in membrane remodeling between proteins that even lack any specific interactions.

Employing quantitative FM, curvature-dependent binding of amphipathic α-helices and protein anchoring motifs on single liposomes was measured, revealing that curved membranes contain a higher density of defects that can serve as binding sites for the recruitment of proteins to the curved membranes (Figure 12).[327] These curvature- and stress-induced defects in lipid packing have emerged as a major mechanism underlying many modulatory effects of membranes on protein function. Remarkably, the above study also concluded that curved membranes rather than anchoring proteins dictate membrane curvature sensing – protein recruitment is achieved through extensive weak interactions instead of strong specific interactions with the curved membranes, consistent with the fact that membrane curvature can be sensed by a number of unrelated motifs, such as amphipathic α-helices, alkyl chains, cytochrome b_5, and dynamin.[327] Recently, fluorescence imaging was used to establish a quantitative relationship between membrane curvature and the sorting of three types of GPCRs in live cells.[212] It was further demonstrated that the curvature-dependent sorting could be fine-tuned by ligands, revealing an intricate biomechanical coupling mechanism for GPCR sorting.

While conformational transitions of many membrane proteins are known to be sensitive to membrane curvature,[331] detailed information on how proteins adapt their structures in response to

membrane curvature is still sparse. One better studied example is, protein kinase C (PKC), a key enzyme in cellular signaling cascades, whose activity has been shown to be tightly controlled by curvature stress due to the weakened lipid head-group interactions induced by PE and cholesterol.[332,333] The C1 domain of PKC acts as a sensor of membrane curvature and undergoes conformational rearrangements to allow the kinase to access the site of phosphorylation in response to the change of its local membrane environment. Recent solid-state NMR experiments indicated that in the influenza M2 proton channel, an amphipathic helix induces membrane curvature, which in turn distorts the TM helices of the protein to interfere with drug binding.[334] The same research group also reported that the backbone conformations of both hydrophobic domains of a parainfluenza virus fusion protein are membrane-dependent, adopting a β-strand predominant structure in negative-curvature membranes.[335]

3.2.4 Inter-leaflet Coupling

A key feature of cell membranes is that they are asymmetric, i.e., different lipids make up the inner and outer bilayer leaflets. The outer leaflet of the plasma membrane is often made of sphingolipids, saturated and unsaturated glycerolphospholipids together with cholesterol, which exhibit nanoscopic phase behavior characteristic of rafts in reconstituted model membranes. In contrast, the cytoplasmic leaflet consists mainly of unsaturated PC, PE, PS and cholesterol, which typically fail to phase separate and remain mixed. However, evidence shows that domain formation in one leaflet is coupled to the other leaflet, and that this coupling may play a role in transmitting cellular signals across the membrane. Physical mechanisms of inter-leaflet (transverse) coupling have been reviewed in Refs.[86,87]

For robust and controllable cellular signaling, the interactions of membrane surface receptors and their cytoplasmic effector proteins need to be coordinated. The coupling of domains across leaflets provides a way to co-localize various signaling components on both sides of the membrane. With this coupling, domains in the outer leaflet can induce domain formation in the inner leaflet, which will

bring proteins that are exclusively associated with the inner leaflet to the raft in response to an extracellular signal. For example, GPCRs detect molecules outside a cell and then activate G proteins, such as Ras, inside the cell to transmit signals from outside the cell to its interior. In response to the extracellular signal, G proteins can traffic into or out of lipid rafts. Like their cognate receptors, many G proteins also preferentially localize to lipid rafts.[185] The dominant mechanism by which G proteins can be targeted to raft domains is through fatty acylations, such as palmitoylation and myristoylation.

4. CONCLUDING REMARKS

Cell membranes constitute an essential platform for receiving, processing and propagating cellular signals. In recent years, it has become clear that microscopic and nanoscopic domains are formed in cell membranes. Although these membrane domains have been studied for decades, their nature and roles in cellular signaling have only recently come to light. With the availability of proteomic and lipidomic information on nanoscopic regions of the cell membranes, enormous amounts of information on protein and lipid species involved in specific cellular signaling pathways are being unveiled. Leveraging this information has dramatically advanced our understanding of how cellular signaling complexes are assembled in membrane domains. However, the recent work also manifests the complexity of biological systems, which utilize a range of mechanisms in the spatiotemporal hierarchy to achieve robust and tunable cellular signaling. Thus, elucidation of the structures, forces and dynamics determining these membrane signaling events, particularly the interplay between cellular signaling and membrane organization, is an enduring, but fascinating, challenge.

Recent advances in a range of fluorescence microscopic and spectroscopic techniques, have offered tremendous opportunities to directly and indirectly probe the size and dynamic properties of membrane functional domains as well as membrane-protein interactions in living cells. Another exciting line of research is the use of neutrons to elucidate the nanoscopic membrane domains in both

model membranes and living cells. Furthermore, although not suitable for live cell studies, structural biology techniques such as X-ray crystallography, NMR and Cryo-EM have continued to provide critical molecular-level insights into how individual signaling molecules and/or signal complexes work on the membrane. The ongoing challenge is how to combine all these different techniques to come to an integrative understanding of how functional membrane domains selectively recruit, organize and activate a wide range of signaling proteins.

Computational approaches, such as molecular simulations, which can simultaneously reveal the behavior of all molecules in a system at a high spatial and temporal resolution, provide an ideal framework for integrating various experimental observations. Yet, there remain gaps in both time and length scales between experimental and computational techniques (Figure 3). As experimental techniques continue to scale down (higher spatial and temporal resolution) and MD simulations keep scaling up (longer time and length scales), these gaps are narrowing. However, to eventually eliminate this gap, it will be necessary to continue to improve the experimental techniques, and at the same time develop new simulation tools to integrate the experimental results across multiple time- and length-scales. In this way, the myriad of dynamic interactions between lipids and membrane proteins will be able to be revealed, dissecting the key principles underlying the roles of membrane organization in complex cellular signal transduction.

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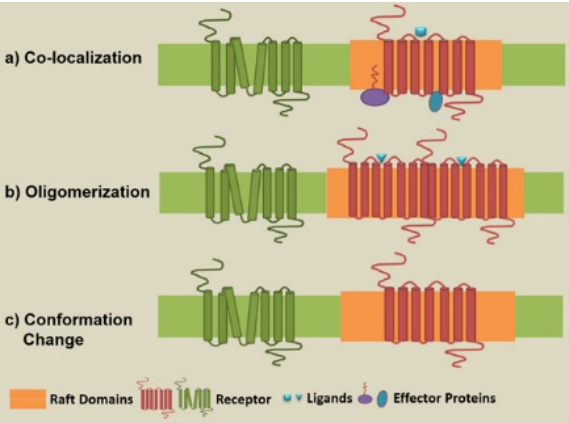
TOC Graphic


a) Co-localization
b) Oligomerization
c) Conformation Change
Raft Domains
Receptor
Ligands
Effector Proteins