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Lipid–Protein Interactions in Plasma Membrane Organization and Function

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Abstract

Lipid–protein interactions in cells are involved in various biological processes, including metabolism, trafficking, signaling, host–pathogen interactions, and transmembrane transport. At the plasma membrane, lipid–protein interactions play major roles in membrane organization and function. Several membrane proteins have motifs for specific lipid binding, which modulate protein conformation and consequent function. In addition to such specific lipid–protein interactions, protein function can be regulated by the dynamic, collective behavior of lipids in membranes. Emerging analytical, biochemical, and computational technologies allow us to study the influence of specific lipid–protein interactions, as well as the collective behavior of membranes on protein function. In this article, we review the recent literature on lipid–protein interactions with a specific focus on the current state-of-the-art technologies that enable novel insights into these interactions.

Contents

1. INTRODUCTION

Plasma membranes (PMs) are composed of hundreds of distinct lipid and protein species. This complexity produces a dynamic ensemble that collectively coordinates the structure and various functions of the PM. Our understanding of PM composition and organization has evolved over the past century since the discovery of lipid bilayers, the semipermeable barriers that isolate the cell from its exterior. The fluid-mosaic model (127) was a cornerstone of this evolution, which postulated that the PM is a two-dimensional liquid with embedded proteins in constant motion. This model, however, has been continuously revised. First, the protein concentration of the PM was shown to be significantly higher than was previously thought (34). Second, the PM was shown to be laterally more heterogeneous, with lipids playing active roles in determining PM structure and function (56, 122, 125). Third, the prominent role of the cortical actin cytoskeleton in PM structure and function became evident (67, 72). Together, these insights have produced a revised model of a highly complex, dynamic, and compartmentalized system intertwined with the ever-changing cortical actin network. Importantly, most processes occurring at the PM rely extensively on these dynamic and structural aspects (33, 120).

2. DIVERSITY OF LIPID–PROTEIN INTERACTIONS

Interactions of lipids with proteins are major determinants of PM structure and function. Lipids are generally divided into three subclasses according to their interactions with the membrane proteins: bulk lipids, annular lipids, and nonannular lipids. Bulk lipids are lipids that do not directly interact with proteins. Nevertheless, these lipids are not totally passive entities and still play a role in protein function. For instance, membrane heterogeneity is driven by preferential interactions between certain membrane components. Cholesterol has a greater affinity for saturated lipids than for unsaturated ones. While these interactions are quite weak at the individual lipid level, collectively they create a heterogenous landscape that can sort proteins into distinct lipid

environments (59). Importantly, these bulk lipid environments can influence membrane protein geometry and thus function in processes including signaling (10, 101, 118), transport (15, 75), and motility (80, 139). Annular lipids are lipids that surround the immediate environment of the proteins. They nonspecifically interact with the proteins and make a ring around the transmembrane domains (TMDs) of proteins. Nonannular lipids, in contrast, specifically interact with certain proteins. Such specific lipid–protein interactions can occur due to specific lipid-binding motifs (17, 130) or more generic electrostatic interactions (61, 124) between charged protein sequences and oppositely charged membrane surfaces. These lipid interactions often directly modulate protein conformation and function (21, 152).

Means of functional regulation of proteins via lipid–protein interactions vary. For instance, as mentioned above, bulk lipids can influence the protein geometry and change protein accessibility for their interaction partners (106). Alternatively, specific interactions can directly impact the protein function (28). This can be achieved via allostery (lipid interaction on one side of the protein causing conformational changes on another side) (28, 147) or the oligomerization state of the protein (2). Finally, lipids themselves can be the substrates for proteins, for instance, during lipid transport (55).

2.1. Phospholipid Binding to Proteins

Structural studies of lipid–protein interactions have revealed several protein domains responsible for specific binding to different lipid species. For phospholipid-binding proteins (for a detailed review, see 77), prominent representatives include pleckstrin homology (PH) domains, PHD fingers, and PX domains, all of which recognize phosphatidylinositol (PI), FYVE, and C1 domains. PH is a domain characteristic for kinases (PI3K, AKT, BTK). The PX domain is present in sorting nexins (47) involved in membrane trafficking. FYVE (104) is a zinc-finger domain that was initially identified in the proteins Fab 1 (the yeast ortholog of PIKfyve), YOTB, Vac 1 (a vesicle transport protein) and EEA1 (134). C1 domains are zinc-finger domains specific to phorbol esters and diacylglycerol that were found in protein kinase C (29). Such phospholipid-specific domains are abundant in cytosolic proteins that can be bound to the intracellular PM leaflet, the membrane of organelles, or endosomes.

2.2. Cholesterol Binding to Proteins

Cholesterol is well-known for forming complexes with membrane proteins, including ion channels, neurotransmitters, and hormone receptors. Specific recognition of cholesterol by proteins is granted by cholesterol consensus motifs (CARC, with preferential localization in the outer membrane leaflet and its mirror motif, CRAC, which preferentially localizes to the inner membrane leaflet). CRAC (cholesterol recognition/interaction amino acid consensus) typically follows the pattern of L/V-(X)(1–5)-Y-(X)(1–5)-R/K. CARC and CRAC were found in multiple G protein–coupled receptors (GPCRs) (e.g., rhodopsins, β2 adrenergic receptors), ion channel proteins, caveolin, and some proteins involved in cholesterol intracellular trafficking [e.g., NPC1 (54)]. Moreover, the CARC domain was found to mediate interactions of proteins of HIV-1 (73) and the influenza virus (37) at the initial steps of host cell infection.

2.3. Sphingolipids Binding to Proteins

Interactions of proteins with sphingolipids have been poorly studied; however, there are important examples of such interactions. Sphingolipids can bind to specific pockets in the TMDs of membrane proteins (25). VXXTLXXIY is the best-characterized sphingolipid-binding motif, and

new motifs are constantly being found (9). Such sphingolipid-binding domains have been found in Alzheimer, prion, and HIV-1 proteins (85). GPCRs, such as cholecystokinin, oxytocin, and secretin receptors, and subtypes of human serotonin receptors also bear evolutionarily conserved sphingolipid-binding motifs (9, 123).

3. METHODS FOR INVESTIGATING SPECIFIC LIPID–PROTEIN INTERACTIONS

Investigation of specific lipid–protein interactions and deciphering the homology of lipid-binding domains largely rely on structural biology techniques. There are other techniques such as nuclear magnetic resonance, electron spin resonance, and force spectroscopy; however, due to space restrictions, we focus mostly on structural methods in this review. We briefly address the state-of-the-art technologies that have contributed immensely to our current understanding of specific lipid–protein interactions, as well as of collective membrane behavior. We apologize for leaving out important work in the field performed using other technologies not mentioned in this review.

3.1. X-Ray Crystallography

X-ray crystallography allows one to obtain the structure (atom positions and chemical bonds) of crystallized material by studying the diffraction of X-rays by electron clouds of the crystal. Although this approach is widely applicable to deciphering the atomic structure of proteins, it requires protein crystallization, which has historically been challenging for membrane proteins. Membrane proteins contain hydrophobic residues, which require detergents to extract them from the membrane for subsequent purification and crystallization (93). This requirement is particularly problematic for isolation of proteins together with their lipid ligands because detergents interact strongly with lipids. The selection of detergents is of crucial importance for crystallization, and research on the applicability of different types of detergents has made it possible to identify the best detergents for crystallography (102). As such, several proteins have been successfully crystallized together with their lipid ligands, which enabled the resolution of structures of lipid-binding pockets. For instance, interactions of the pore-forming protein lysenin with sphingomyelin (**Figure 1*a***) or of β2 adrenergic receptor with cholesterol (**Figure 1*b***) were confirmed by structural studies. As an alternative, crystallization in lipid mesophases (148) or nanodiscs (14, 31) was used to study the structure of bacterial outer membrane proteins, photosynthetic proteins, and GPCRs (23, 58, 109). Another alternative is electron crystallography (107, 110), which enables the solution of the structure of proteins together with their lipid ligands (annular lipids) by the formation of two-dimensional crystal arrays.

3.2. Cryogenic Electron Microscopy

Over the past decade, cryogenic electron microscopy (cryo-EM) (6, 22, 42, 98) has emerged as a promising alternative to X-ray crystallography for elucidating protein structure. In cryo-EM, vitrified protein solutions are imaged with electron microscopy to obtain multiple single-molecule images of the same protein in different orientations. Many such images can be processed to reveal protein topography with atomic precision. Unlike X-ray crystallography, cryo-EM does not require protein crystallization; instead, vitrification is achieved by plunge freezing a protein solution in liquid nitrogen. This approach has been extensively employed to solve structures of various membrane proteins, including GPCRs (153), pore-forming toxins (11), assembled dynamin polymers (66), clathrin cages (90), and Bar domains (136). Since crystallization is not required, many proteins that could not be studied with X-ray crystallography can now be solved with cryo-EM.

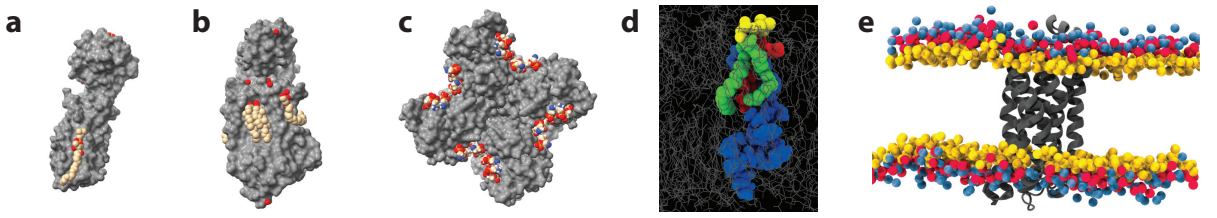


Figure 1

Structural studies of protein–lipid interactions. (*a*) The structure of the pore-forming protein lysenin (*gray surface*) with its sphingolipid ligand (*colored spheres*) determined by X-ray crystallography (30), available in the Protein Data Bank (PDB) as 3zxg. (*b*) The structure of β2 adrenergic receptor (*gray surface*) with its cholesterol ligands (*colored spheres*) determined by X-ray crystallography (49), available in the PDB as 3d4s. (*c*) The structure of the transient receptor potential protein TRPV1 (*gray surface*) with its phospholipid ligands (*colored spheres*) determined by cryogenic electron microscopy (cryo-EM) (41), available in the PDB as 5irz. (*d*) Sphingomyelin (SM) interacts with the transmembrane domain (TMD) of COPI machinery protein p24, as determined by mass spectrometry and molecular dynamics (MD) simulations. Blue indicates the TMD of p24; red indicates the SM-binding pocket; yellow indicates the SM 18:0 head group; and green indicates the SM 18:0 backbone and *N*-acylated fatty acid. Panel *d* adapted with permission from Reference 25. (*e*) The position of β2 adrenergic receptor in the lipid bilayer, obtained by coarse-grained MD simulations. Lipids are shown with glycerol beads in yellow, phosphate beads in red, and choline beads in blue. Image available in the MemProtMD database (94) as 3d4s.

One example is the transient receptor potential (TRP) superfamily, the structures of whose proteins could not be solved by X-ray crystallography due to the difficulties in crystallization. Since 2013, developments in cryo-EM enabled researchers to solve at least one protein for each of seven subfamilies of TRPs (22). Particularly important for this review, the structure of TRPV1 resolved by cryo-EM identified the distinct amino acid residues that bound phospholipid hydrophobic tails in the outer leaflet of the PM, as well as the hydrophilic residue in the extracellular domain that targeted phospholipid headgroups (**Figure 1*c***).

Unfortunately, isolation of membrane proteins for vitrification still relies on detergents to extract proteins from their native membrane. A potential alternative is protein isolation via nanodiscs, which appears to be advantageous when the aim is to resolve the lipid ligands of the proteins (41, 153). Even in these cases, lipid ligands tend to provide low electron microscopy contrast, which introduces difficulties for their resolution with atomic precision.

3.3. Structure Prediction and Simulations

The progress of computational technologies has enabled structure prediction of proteins based on their amino acid sequences. Clearly, the prediction of protein secondary structure and the geometry of protein folding domains ab initio (i.e., physics-based) is challenging, so methods of protein structure prediction often rely on existing knowledge of 3D structures obtained for similar proteins by experimental techniques. Several approaches are employed. Homology modeling (138) compares amino acid sequences of unknown structure to those of well-resolved proteins and maps unknown residues onto the known structural template of the related homologous protein. This approach often provides an efficient starting point for sequences with at least 30% similarity (144). An alternative fold recognition approach (100) relies mainly on the notion that the number of folds is much lower than the number of sequences, and it attempts to identify the most likely fold for a given sequence. Similar fragment-based methods (126) allow for assembly of protein structure based on structures of sequence fragments similar to various proteins available in the protein data bank. Finally, the most difficult protein structure prediction approaches rely only

on physical potentials of atomic interactions (64, 105, 129). The modern methods of structural predictions often combine several of the abovementioned approaches, and they became especially powerful with the development of neural networks and machine learning algorithms. The most relevant example is the recent AlphaFold project (117), which uses a convolutional neural network trained on Protein Data Bank structures to estimate the distances between the atoms of residues of a protein. Therefore, it can accurately predict the structure of a protein given its sequence by minimizing the potential by gradient descent.

Structure predictions can be highly useful for determining lipid binding sites from the structures of membrane proteins. Lipid binding pockets can be recognized from known amino acid sequences (97), characteristic protein folds (86), hydrophobicity (89), or charge (8). The orientation and affinity of lipid ligands can then be further assessed by molecular docking (149). Molecular dynamics (MD) simulations enable the elucidation of possible lipid binding pockets reconstituted together with membrane lipids in silico (16, 25, 116) (e.g., a p24 transmembrane domain interacting with sphingomyelin, as in **Figure 1*d***). Such molecular- or atomic-scale dynamic simulations provide the unique opportunity to explore molecular interactions at the Angstrom- and nanosecond-scale.

Transmembrane proteins can be simulated as embedded in the dynamic ensemble of the multicomponent PM (20) to resolve specific lipid–protein interactions involved in membrane transport (35), viral infection (65), or antimicrobial activity (128). However, simulation of thousands of molecules for multiple microseconds can be computationally demanding, necessitating alternative methods such as standard residue interaction networks (147) to interpret protein structures and resolve molecular interactions. An alternative to computationally demanding full-atom simulations is the use of coarse-grained simulations where molecules are simplified (131). Using coarse-grained simulations, the duration of the simulation, as well as the number of simulated molecules, can be increased (39). The combination of structural studies and MD simulations of the membrane proteins in the lipid bilayer have led to the establishment of the MemProt database of simulations of transmembrane proteins embedded in the lipid bilayer (94) (as an example, β2 adrenergic receptor in the lipid bilayer is shown in **Figure 1*e***). Compared to the other techniques discussed above, MD simulations can provide unique information on the energetics and kinetics of lipid–protein interactions. Since certain time windows can be simulated dynamically, free energy calculations can be performed to test lipid binding affinities (and thus relative probabilities of binding) to certain proteins or protein binding affinities to certain lipid environments (for reviews, see 26, 27).

4. METHODS TO STUDY COLLECTIVE LIPID–PROTEIN INTERACTIONS

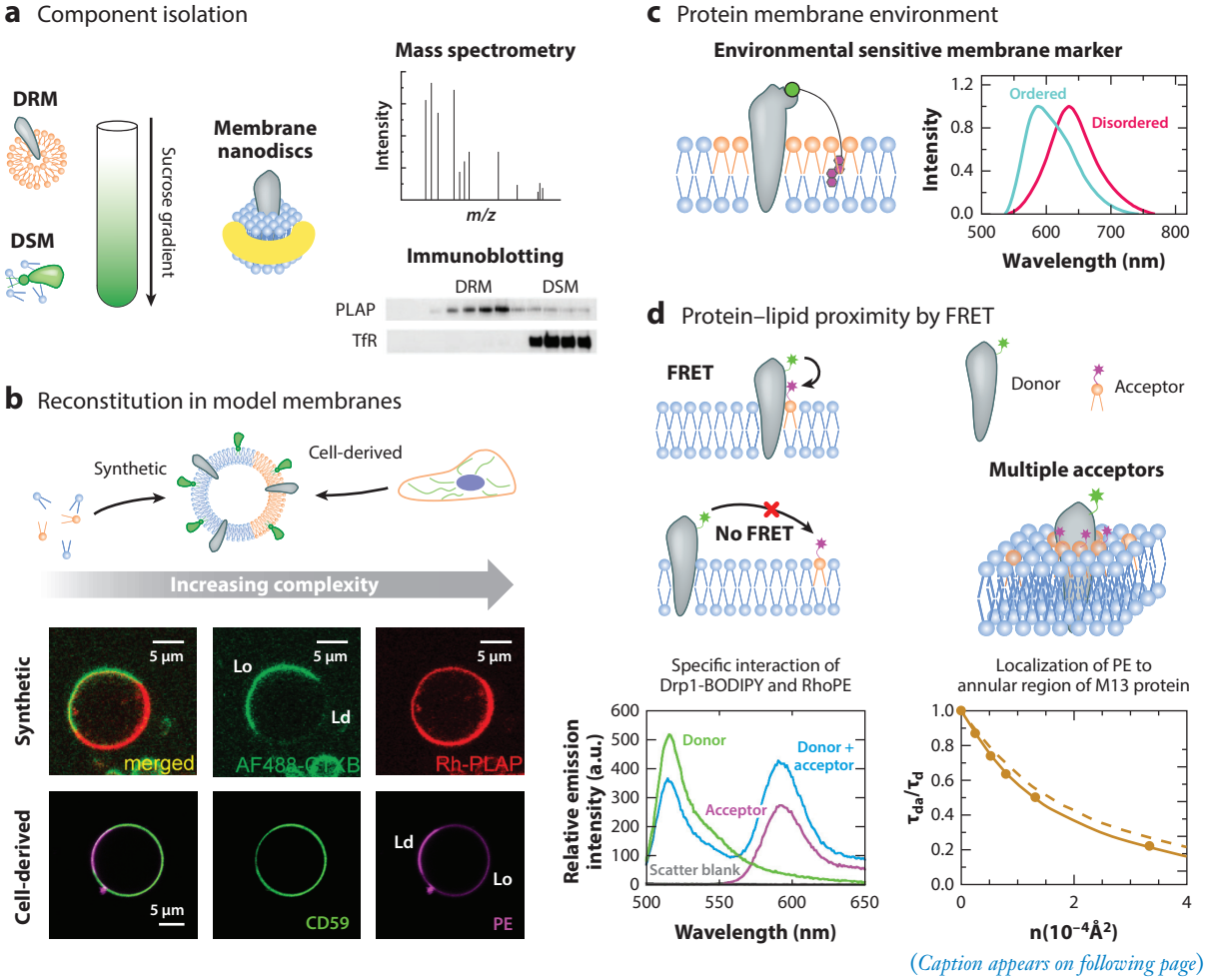
In addition to their roles as individual ligands for protein interactions, membrane lipids collectively determine the biophysical properties of membranes. Therefore, it is crucial to consider membranes as more than the sum of their individual components. Technologies that enable us to see nanoscale biophysical properties of membranes help us understand how these collective properties take part in protein functionality. We discuss various aspects of collective membrane properties in the following sections.

4.1. Protein Sorting (Enrichment) into Certain Lipid Environments

Multiple mechanisms have been proposed for protein sorting into particular lipid environments independent of specific lipid–protein interactions. One such mechanism is based on membrane thickness matching. Hydrophobic transmembrane domains of membrane proteins (or their

post-translationally acylated parts) can attract lipids of optimally matched chain length and/or saturation degree (88). Furthermore, for acylated (e.g., palmitoylated, myristoylated) proteins and GPI-anchored proteins (GPI-APs), compartmentalization largely relies on the length and saturation degree of the lipid anchor. Another sorting mechanism is lipid-driven assembly of ordered domains (lipid rafts) that sequester membrane proteins and define their transmembrane positioning and spatial organization. In this context, lipid rafts are usually defined as nanoscale signaling platforms rich in cholesterol, sphingolipids, glycolipids, and raft proteins, i.e., proteins that prefer more ordered membrane environments (32, 81). Despite the debatable nature of membrane lateral domains, it is evident that lipid–protein interactions go far beyond specific recognition of lipids by certain proteins and should also be considered in the context of collective behavior. To obtain the comprehensive picture of such complex, collectively driven lipid–protein interactions, the PM has been studied using various biochemical and analytical techniques, as detailed below (**Figure 2**).

4.1.1. Detergent resistance to study membrane heterogeneity and protein sorting. The content of ordered domains was first studied by using detergents to extract detergent-resistant



(Caption appears on following page)

Figure 2 (*Figure appears on preceding page*)

Methods to study collective lipid–protein interactions. (*a*) Lipids and proteins are isolated from plasma membranes by detergents or nanodiscs. The content of detergent-resistant membrane (DRM) and detergent-soluble membrane (DSM) fractions can be analyzed by biochemical methods or mass spectrometry (12). Representative Western blot showing DRM association of marker proteins [placental alkaline phosphatase (PLAP) and transferrin receptor (TfR)] from MDCK cells extracted with Triton X100. Panel *a* adapted with permission from Reference 115, copyright National Academy of Sciences (2003). (*b*) Reconstitution of proteins in synthetic [giant unilameller vesicles (GUVs)] or cell-derived [giant plasma membrane vesicles (GPMVs)] model membranes. Such vesicles are widely studied by fluorescence microscopy. The GPI-anchored proteins PLAP (62) and CD59 reconstituted in a GUV and a GPMV, respectively. In the synthetic GUV, the Lo domain is labeled by Cholera toxin B subunit (AF488-CTXB; *green*). PLAP is labeled with Rhodamine (Rh-PLAP; *red*), which incorporates preferentially into the Ld domain. In the cell-derived GPMV, Abberior Star Red-lipid (PE; *magenta*) localizes preferentially to the Ld domain. In contrast, GPI-anchored protein CD59-EGFP (CD59; *green*) incorporates preferentially to the Lo domain. Panel *b* adapted with permission from Reference 62, copyright American Chemical Society (2005). (*c*) Probing of local membrane order at the proximity of specific proteins with the environmentally sensitive membrane marker Nile Red. Nile Red is modified with a linker to specifically recognize a protein of interest and incorporate it into the lipid bilayer at the proximity of the protein. The fluorescence spectrum of Nile Red is sensitive to membrane order. (*d*) Elucidation of lipid–protein interaction by Förster resonance energy transfer (FRET). When the protein (donor) and the lipid (acceptor) are in close proximity (1–10 nm), energy transfer occurs. Lipids can localize to the annular region of the protein and act as multiple acceptors for a single protein donor. (*Bottom left*) Representative emission spectra of BODIPY-labeled wild-type (WT) Dynamin-related protein 1 (Drp1) (the donor) in the absence and presence of 1 mol% Rhodamine PE (RhoPE) lipid (the acceptor) in DOPC/DOPE/cardiolipin liposomes. FRET was detected by a decrease in donor emission intensity in the presence of the acceptor accompanied by a FRET-sensitized increase in acceptor emission upon donor excitation. The acceptor-only trace shows direct excitation of the acceptor at the donor excitation wavelength, which represents the background. Panel adapted with permission from Reference 84, copyright National Academy of Sciences (2021). (*Bottom right*) Donor (DCIA-labeled protein) fluorescence quenching by energy transfer to acceptor [(18:1)2-PE-NBD] in the DMoPC bilayer at different concentrations of the acceptor. The FRET efficiency is represented as a ratio between the fluorescence lifetime of the donor in the presence of the acceptor and the fluorescence lifetime of the donor in the absence of the acceptor. Circular points indicate experimental energy transfer efficiencies; the solid line indicates theoretical simulations obtained from the annular model for protein–lipid interaction; and the dashed line indicates simulations for random distribution of acceptors. Fitting indicates the localization of PE lipids in the annular region of the protein. Panel *d* adapted with permission from Reference 40, copyright Biophysical Society (2004).

membrane (DRM) and detergent-soluble membrane (DSM) fractions, with further analysis of these fractions conducted using biochemical methods (e.g., western blotting) or mass spectroscopy (13, 44) (**Figure 2*a***). This approach showed the association of some proteins [such as GPI-APs (15)] with sphingomyelin- and cholesterol-rich membrane fractions. Moreover, for caveolin (103) or GPCRs (71) that were found in DRMs, the association with sphingomyelin and cholesterol is preferential due to specific lipid interactions mediated by consensus structural motifs. Despite being useful in demonstrating the general heterogeneity in PMs, DRM fractions showed major discrepancy in the composition when different detergents were used (115). This made clear that information on membrane compartmentalization obtained by application of detergents should be addressed with caution, and more sensitive approaches must be employed. Therefore, alternative approaches were developed, including lipid bilayer nanodiscs (31) using membrane scaffold proteins (7) or synthetic polymers (53, 99). Membrane nanodiscs can resemble some features of the native membrane environment (87), which allows for characterization of membrane order and protein orientation, in addition to the content of an extract. For example, order parameters of lipid chains are widely assessed by nuclear magnetic resonance (57). Notably, the developments in nanodisc extraction contributed to the sample preparation methods required for structural studies (14, 153), namely crystallization (X-ray crystallography) or plunge freezing (cryo-EM).

4.1.2. Liquid–liquid phase separation as a biophysical basis for lateral protein organization. Extensive investigation of self-assembly and self-organization of lipids in synthetic multicomponent lipid bilayers supports the concept of lipid-driven compartmentalization of the PM. Specifically, lipid bilayers undergo phase transition from a liquid-crystalline phase (low lipid

order and high mobility) to a gel phase (also sometimes called the solid phase; low lipid mobility and high order). PMs at physiological conditions adopt a liquid-crystalline phase, which is a fundamental basis of the fluid-mosaic model.

Two-component lipid bilayers composed of lipids with distinct phase transition temperatures and saturation exhibit formation of spatially separated domains of the liquid-crystalline phase and gel phase at intermediate temperatures. Interestingly, introduction of cholesterol can fluidize the gel phase and convert it into a liquid-crystalline phase. Because this phase is distinct from the cholesterol-poor liquid phase, lipid bilayers composed of saturated lipids, unsaturated lipids, and cholesterol can exhibit liquid–liquid phase separation (LLPS). Such bilayers contain two liquid-crystalline phases of distinct lipid order and mobility: a liquid-disordered (Ld) phase (low order, high mobility) and a liquid-ordered (Lo) phase (higher order, lower mobility). Classically, the lipid bilayer composition that was used to model biologically relevant LLPS was a combination of phospholipids with low transition temperatures (e.g., DOPC or POPC), phospholipids with high transition temperatures (e.g., DPPC, sphingomyelin), and cholesterol. Such LLPS can be observed in a variety of model membrane settings. In particular, spherical cell-sized vesicles have been applied to investigate the biophysical determinants of LLPS and incorporation of membrane proteins into lipid domains (62, 143). These synthetic vesicles provide certain advantages that make them ideal for studying lipid–protein interactions. First, their lipid composition (e.g., amount of charged lipids, cholesterol, saturation) can be tightly controlled to evaluate interactions of isolated proteins with different lipid species (113, 140). Furthermore, transmembrane proteins can be incorporated via detergent dilution into free-standing model membranes (5), whereas extramembrane domains can be anchored to lipids via His-Tags or biotin-avidin coupling (19, 60). Despite these advantages, the limited complexity of these synthetic systems restricts their use in studying native membrane interactions.

As an alternative to fully synthetic membranes, cell-derived vesicles can be exploited for studying protein compartmentalization. These membranes are extracted from living cells by application of vesiculating agents, thus largely conserving PM lipid and protein content. Moreover, phase-separated cell-derived vesicles display LLPS between microscopic ordered and disordered domains. Due to the easy reconstitution of desired membrane proteins in these cell-derived systems (by forcing the cells to express these proteins), they have been used to investigate the structural determinants of ordered-domain association for membrane proteins (81).

Liquid phases in artificial and cell-derived membranes differ in composition and organization: Synthetic systems are simpler in composition with extreme difference in order between ordered and disordered phases. In contrast, cell-derived membranes are more complex in composition with marginal difference in membrane order between ordered and disordered phases (137). As protein sorting into lipid-driven domains is a function of the packing of the domains, which varies between different model membrane systems (119, 137), compartmentalization of proteins can differ in artificial and cell-derived membranes (5, 62). As such, a GPI-AP (one of the bona fide ordered domain components), placental alkaline phosphatase (PLAP), partitions into Ld domains when incorporated into synthetic vesicles, presumably due to extreme order difference between the domains. Another GPI-AP, CD59, clearly prefers ordered domains in phase-separated cell-derived vesicles (**Figure 2*b***). Therefore, synthetic and cell-derived systems can provide complementary insights into the comprehensive picture of lipid and protein compartmentalization.

4.1.3. Environment-sensitive probes to predict the lipid environment of membrane proteins. Biophysical properties of the PM can be assessed using fluorescence lipophilic probes sensitive to membrane order (69, 112), viscosity (70), and tension (24). When used in combination with fluorescent proteins, these probes can report on the immediate environment of fluorescently

labeled proteins (142). Notably, such probes can also be modified with ligands of specific proteins to measure the biophysical properties of the protein microenvironment (48, 141) (**Figure 2*c***). Using this method, Hanser et al. (48) conjugated an oxytocin receptor (a GPCR) ligand to the environment-sensitive fluorescent dye Nile Red and reported different lipid packing around the oxytocin receptor compared to the bulk membrane environment. With a similar strategy, Umebayashi et al. (141) developed a Nile Red construct that specifically binds insulin receptor. Similarly, the membrane environment of insulin receptor was distinct compared to the average PM order (141). Both papers highlight the importance of a linker between the protein binding site and the Nile Red molecule, as this linker changes the probe localization in the membrane.

4.1.4. Protein–lipid proximity sensing by Förster resonance energy transfer. Förster resonance energy transfer (FRET) provides a unique opportunity to measure the molecular distance between two molecules, a donor and an acceptor (a FRET pair), with a sensitivity of units of nanometers. The main requirement for FRET is that molecules of interest are tagged with fluorescent moieties, and the fluorescent spectrum of the FRET donor overlaps with the absorption spectrum of the FRET acceptor. Notably, the intrinsic fluorescence of proteins (e.g., via excitation of Trp) can also be used as a FRET donor. FRET effectively reports on the position and orientation of membrane proteins (92), as well as on protein–protein interactions and oligomerization in the PM (63, 88, 151). In the context of lipid–protein interactions, such a FRET pair can obviously be constituted of a single protein and lipid species of interest (**Figure 2*d***). As FRET acceptors, lipids can be modified by a fluorescent moiety at the headgroup or fatty acyl chain. Lipid–protein FRET can report on specific lipid–proteins interactions (25), where FRET occurs between a single protein donor and a single lipid acceptor. Moreover, FRET between a single protein donor and multiple lipid acceptors can provide information on the recruitment of lipids to the protein annular region (40). Finally, FRET is employed to resolve protein incorporation into nanodomains enriched in specific lipids (1, 83, 111). Deciphering the characteristics of lipid–protein interactions (affinity, avidity, presence of lipids in the annular region of a protein, or protein incorporation into the lipid domains) from data obtained by FRET experiments requires the development of appropriate models by analytical solutions or simulations (82, 111, 135). A major advantage of the FRET approach is the possibility to monitor energy transfer in living cells employing live-cell fluorescence microscopy techniques. FRET measurements are highly sensitive to lipid and protein orientation and concentration, as well as to spectral overlap between the donor and the acceptor; therefore, these measurements require very reliable experimental controls.

4.1.5. Lipid environment of proteins by native mass spectrometry. Mass spectrometry (MS) has been a key tool to study both specific and collective lipid–protein interactions. Several MS methods have been applied to observe lipid–protein interactions, with native MS being the most common. In native MS, biomolecules are converted from a 3D, condensed-liquid phase to the gas phase via the process of electrospray ionization MS (ESI-MS) (78). The term native in the name of this method implies that the former liquid phase should be as physiologically relevant (in terms of pH, osmolarity, and ionic strength) and functional as possible to keep the biomolecules in their native state prior to gas phase transition and MS analysis (78). Being a gas-phase method, native MS has mild experimental conditions that enable the noncovalent interactions and functionality of the biomolecules to be largely preserved. This allows one to obtain information on stoichiometry, binding partners, and biomolecule topology and dynamics. Therefore, it is widely used to study lipid–protein interactions. Advanced sample preparations such as native nanodiscs or styrene maleic acid lipid particles (SMALPs) make it even more attractive for such applications, since native lipid–protein interactions can be preserved in these polymer–lipid combinations (52).

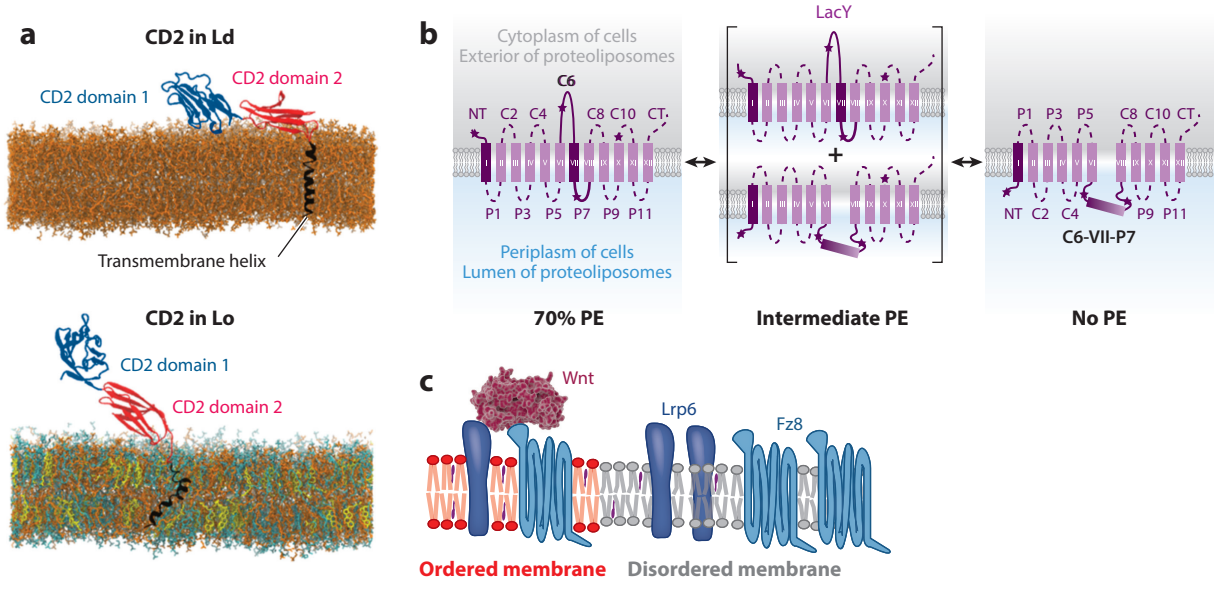


Figure 3

Membrane biophysical properties determine protein geometry and interactions. (*a*) Simulation snapshots of the equilibrium configurations of CD2 in liquid-disordered and liquid-ordered bilayers, showing DOPC (*orange*), SM (*cyan*), Chol (*yellow*), CD2 domain 1 (*dark blue*), CD2 domain 2 (*red*), CD2 transmembrane helix (*black*), and lipid carbohydrate chains (*light blue*). Panel *a* adapted with permission from Reference 106. (*b*) Effect of changes in PE content on the orientation of lactose permease (LacY) in lipid bilayers. Transmembrane domain orientation is summarized for LacY in proteoliposomes containing 70% (*left*), intermediate (*center*), or 0% (*right*) PE. Panel *b* adapted with permission from Reference 145, copyright National Academy of Sciences (2015). (*c*) Wnt binding to its coreceptors (Fz8 and Lrp6) in the plasma membrane. Although the coreceptors are enriched in disordered domains, the ligand (Wnt3a) binds to the ordered domain pool of the receptors. Panel *c* adapted with permission from Reference 118.

Through the use of native MS, different aspects of lipid–protein interactions have been studied. For example, Laganowsky et al. (74) showed that different proteins are bound selectively by different lipid classes. Gupta et al. (44) showed that interfacial lipids are required for protein oligomerization. Landreh et al. (76) demonstrated the role of annular lipids on protein folding and conformation. Native MS and its applications have been reviewed in detail in Reference 12, and the elaborate protocols can be found in Reference 45.

4.2. Protein Geometry Influenced by the Membrane Properties

Protein recruitment to certain lipid environments, discussed above, leads to increased local concentrations. Such enrichment might ensure a concentration threshold for triggering biological processes. However, enrichment is not a requirement for lipid environment-selective activation of proteins. Even a small fraction of a given protein can be the active pool if it resides in the lipid environment that ensures a favorable geometry for molecular interactions. The CD2 protein, for instance, shows different geometries, and thus different accessibility, in different lipid environments (106) (**Figure 3*a***). Another example is the bacterial protein lactose permease, which can undergo rapid postassembly TMD flipping in response to changes in the lipid environment (145) (**Figure 3*b***). Finally, Frizzled, a receptor for the Wnt ligand, has been shown to partition preferentially into the DSM fraction, as does its coreceptor, LRP6. However, the Wnt ligand binds exclusively to the minor DRM pool of these receptors (118) (**Figure 3*c***). These examples show

that protein function can be modulated by the biophysical properties of the local lipid microenvironment.

5. BOTTLENECKS FOR STUDYING DYNAMIC LIPID–PROTEIN INTERACTIONS IN LIVE CELLS

Investigating membrane proteins in model membranes or in cellular isolates can provide useful information on protein compartmentalization. Moreover, tight control of complexity in model systems allows us to study the interactions between proteins and specific lipid species (28). However, model systems are artificial systems devoid of intracellular dynamics, endo- and exocytosis, and other energy-driven processes. These limitations prevent a comprehensive understanding of collective lipid–protein interactions in fully physiological settings. To further this understanding, imaging techniques based on fluorescence microscopy, spanning from conventional imaging to spectroscopic techniques like FRET, fluorescence lifetime imaging, or fluorescence correlation spectroscopy (FCS), are employed for direct observation of PM dynamics under physiological conditions. Fluorescence imaging relies on the introduction of fluorescent tags to the molecules of interest. For proteins, the discovery of fluorescent proteins several decades ago enabled efficient tagging of proteins without disrupting their native functions. For lipids, on the contrary, introduction of fluorescent tags presents some challenges. Lipids are relatively small, with sizes often comparable to organic fluorophores; thus, modification of lipids with the fluorescent moieties often alters their shape, localization, and dynamics in lipid bilayers and, consequently, their interactions with membrane proteins. Another challenge arises from the huge variety of lipid species present in the PM. Lipids differ in headgroup, length, and saturation of their fatty acyl chains, and even small differences in lipid structure can affect lipid–protein interactions (4, 95, 96). Thus, identifying the lipid structures required for interactions among hundreds of different types of lipid species is a major challenge. Another limitation is the spatiotemporal regime of lipid–protein interactions. Most lipid–protein interactions are highly transient in nature (microsecond timescales). While this regime can be studied using MD simulations, accessing lipid–protein interactions in cellulo with conventional experimental methods is difficult. To this end, techniques of high spatiotemporal resolution must be employed to evaluate these interactions in a live-cell context.

The spatial resolution of conventional imaging techniques is limited by the diffraction limit, a physical barrier which typically limits resolution of two objects to more than a few hundred nanometers. However, in the past decades, super-resolution imaging techniques have been developed to provide insights an order of magnitude beyond the diffraction limit. Such methods could allow us to directly access nanoscale organization and dynamics in cellular membranes, thus yielding direct ways to visualize lipid–protein interactions. Stimulated emission depletion (STED) microscopy is among the most-used super-resolution techniques. While each super-resolution technique has its own advantages and disadvantages, STED is the only technique that provides super-resolved images without postprocessing, i.e., directly accessing the spatiotemporal regime needed to study molecular interactions. This makes STED a suitable technique to investigate processes in live cells.

The concept of STED was formulated in the 1990s and experimentally realized in later years (38, 51). Its underlying procedure is to shape the detection volume by depleting fluorescence emission around the immediate vicinity of the focal spot. Briefly, fluorophores are first excited by confocal laser excitation volume, with subsequent donut-shaped illumination by a second laser (**Figure 4*a***). This overlay of a second laser input allows the peripheral excited fluorophores to be depleted, while the center of the focal spot is unaffected by the depletion laser. The efficiency of this scheme lies in the nonlinear dependence of depletion with respect to the STED pulse

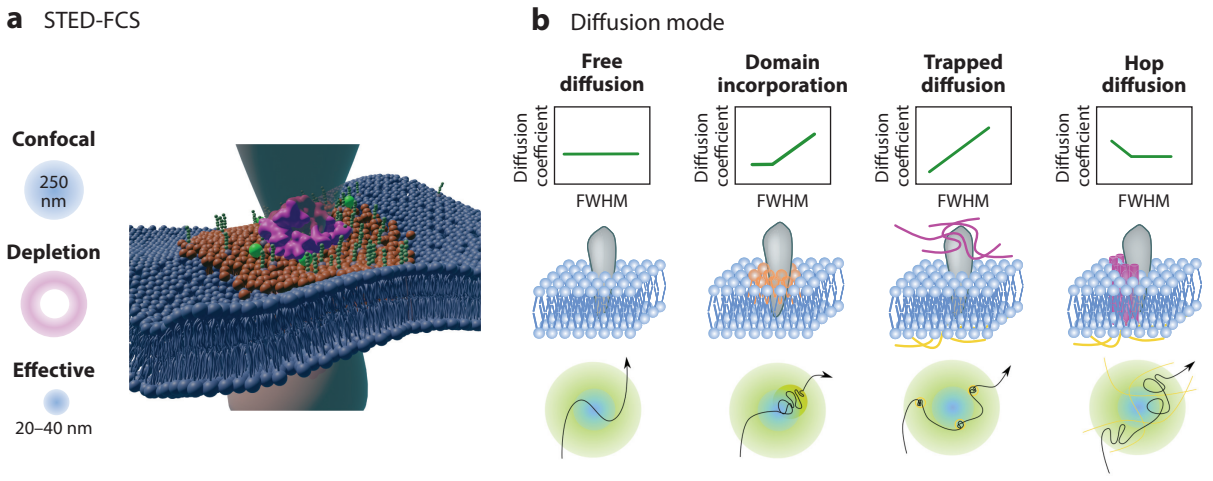


Figure 4

Stimulated emission depletion fluorescence correlation spectroscopy (STED-FCS) to resolve lipid–protein interactions. (*a*) Principle of STED-FCS. Stimulated depletion reduces the effective focal spot down to 20–40 nm, increasing the resolution by an order of magnitude. (*b*) Lipid or protein diffusion modes can be determined by quantification of the protein diffusion coefficient (*D*) at different effective focal point diameters [apparent full width at half max (FWHM)]. Protein diffusion modes are characteristic for the types of interactions of the protein in the lipid bilayer. At free diffusion, *D* remains constant. At transient domain incorporation, *D* decreases together with FWHM, but when FWHM approaches domain size, *D* saturates or, in some cases, slightly increases due to incorporation in domains with a specific *D*. At trapped diffusion, *D* decreases with decreasing FWHM due to transient binding to lipids, the actin cytoskeleton, or the extracellular matrix. Hop diffusion manifests when the protein diffuses fast within plasma membrane compartments (introduced by actin meshwork, for example), but hopping between compartments is significantly slower. Thus, *D* increases toward small FWHM.

intensity; as the STED laser intensity increases further, the depletion region expands, but the center of the focal spot remains largely unaffected. Therefore, the effective excitation length scale (after depletion) can be tuned continually from a diffraction-limited spot down to approximately 20 nm (**Figure 4*a***).

STED microscopy provides excellent spatial resolution; however, like most other super-resolution microscopy, the temporal resolution is limited because scanning of the sample by the laser is required. To gain temporal resolution, STED can be combined with other spectroscopic methods discussed above. For example, FCS provides a toolset for observations of molecular diffusion and intermolecular interactions in the microsecond regime. FCS detects the fluctuation of the emitted fluorescence intensity of molecules diffusing through the observation volume. Intensity fluctuations are analyzed by calculating and fitting their autocorrelation function, which allows quantification of the number of diffusing particles, their brightness, and their diffusion (50, 114, 121).

Interactions occurring in membranes, such as lipid–protein interactions, alter the diffusion characteristics of the molecules (146, 150). As such, molecules in the membrane can not only diffuse with different velocities, but also follow different diffusion modes. The diffusion mode defines how the diffusion coefficient of a certain molecule changes with respect to the size of the observation spot (usually measured as full width at half maximum of point spread function). For example, a molecule undergoing free (Brownian) diffusion has a diffusion coefficient independent of the size of the observation spot. Notably, recent studies show that most PM components do not exhibit free diffusion. Some molecules undergo trapped (confined) diffusion, where the diffusion coefficient is smaller with decreasing observation size, whereas other molecules undergo

hop diffusion, where the diffusion coefficient increases with smaller observation spots. When a molecule is trapped in a domain, the diffusion coefficient decreases until spot size approaches to the length scale of a domain, then remains constant thereafter (**Figure 4*b***).

Such heterogeneous molecular diffusion characteristics provide insights into the nanoscale spatiotemporal organization of molecules in the PM, which can be revealed by measuring the diffusion law. Combining STED with FCS (STED-FCS) (**Figure 4*a***) enables access to tunable observation sizes. The diffusion mode can then directly be obtained by investigating the dependence of the diffusion coefficient on the observation diameter (3, 36, 91) (**Figure 4*b***). This approach successfully revealed anomalous diffusion of membrane components: Many phospholipids undergo hop or free diffusion, whereas sphingolipids and glycolipids exhibit trapped diffusion. Intriguingly, GPI-APs undergo domain-like diffusion in a cholesterol-dependent manner (114). Similarly, Ras protein nanodomains were shown using STED-FCS (46).

While STED-FCS provides a direct way to measure the nanoscale behavior of molecules, it is not the only such technique. Optical nano-antennas coupled to FCS successfully showed the nanoscale diffusion modes of lipids and proteins in the PM (108). Similar measurements have been performed using spot variation FCS. In this method, instead of making the observation spot smaller (e.g., using STED or nano-antennas), the observation diameter is enlarged (79). The dependence of diffusion on spot size makes it possible to extrapolate toward the nanoscale regime. Spot variation can be achieved with laser scanning confocal microscopy by changing pinhole (132) and objective filling or with camera-based imaging (such as total internal reflection or single plane illumination microscopy) by binning the adjacent pixels (68). Using spot variation FCS, lipid-dependent interaction of the Wnt protein with membrane have been shown (4). Interestingly, nanoscale interactions between the Wnt protein and its receptors in the PM and lipid membrane determine the signaling potency. Moreover, interferon gamma receptor 2 (IFN-γR2) interaction with sphingolipid and cholesterol nanodomains has been demonstrated using spot variation FCS (10). A disease-causing mutation in IFN-γR2 that leads to aberrant glycosylation of the protein removes the receptor from lipid nanodomains and places it in actin nanodomains. This change in nanoscale localization is sufficient to change interferon signaling. These examples are clear evidence of nanoscale lipid–protein interactions and their contribution to cellular signaling.

6. CONCLUSION AND OUTLOOK

Lipids and membranes were at the center of research for many decades, from the early 1900s until the DNA revolution in the 1960s, when lipids started to lose popularity. For a long time, they were considered to be the molecules providing passive membrane platforms for proteins to reside. As a result, lipids have been considered to be specialized molecules and studied by significantly smaller numbers of labs compared to nucleic acids or proteins. Even today, ironically, while the study of proteins responsible for lipid synthesis or regulation pathways are seen as high-impact research, lipids themselves might not be equally interesting for a broader audience. However, the study of lipids is becoming more popular. Research in past decade, as we try to cover in this review, has found evidence for functions of lipids that we did not comprehend before. This reviving attention is fueled by several factors. First, technological advances enabled us to see that many proteins possess lipid-binding motifs, which suggests that specific lipids are needed for protein function. Second, lipidomics studies showed that lipids can be indicators of cellular states, health, and disease. Finally, the importance of collective behavior in cells has started to be more broadly appreciated, and membranes are the best examples of such collective behavior in cells.

The list of specific lipid–protein interactions is growing constantly. Large classes of proteins, such as GPCRs, tetraspanins, channels, and transporters, provide examples of proteins bearing motifs for lipid binding. The upcoming years will provide clearer evidence for such interactions, their structural determinants, and their role in protein functionality. Methodologies will also be developed further to see what was previously unseeable. For example, we are far from fully utilizing diffusion mode analysis by super-resolution spectroscopy to understand lipid–protein interactions. The main barrier is the investigation of multiple species simultaneously to visualize direct interactions. Future developments of multicolor diffusion mode measurements will be invaluable to visualize transient lipid–protein interactions in living cells.

Lipidomics has recently gained popularity and has already provided evidence on lipid markers of health and disease (43). Soon enough, it will be a routine complementary measurement to other omics approaches to obtain a comprehensive picture of cells and cellular states. Recently developed single-cell lipidomics approaches will be important methods for future lipid research, as well (18).

Modern omics approaches allow temporal, longitudinal, and spatial analysis of single-cell genomics, transcriptomics, proteomics, and lipidomics. However, even if we know all of the components of a cell, the collective behavior of these components cannot be easily predicted. Therefore, biophysical measurements of membranes provide unique information on the environments that proteins need to function optimally. Again, methodological advances will significantly change our understanding of the role of collective behavior in molecular functions. For example, recent chemical biology tools allowed us to study the immediate environment of membrane proteins (48, 141). Progress in this area to make this approach easily applicable to all proteins (e.g., via targeting generic tags such as HALO and SNAP) will be revolutionary in the field. Correlative technologies that can measure, dissect, and connect different biophysical properties of membranes (133) will shed light on the biophysical principles underlying cellular functions. MD simulations will complement experimental approaches and help us access the mechanistic consequences of collective lipid membrane properties.

We will discover many novel roles of lipids in the upcoming years. Many more examples of specific lipid–protein interactions, as well as roles of collective membrane properties in protein functions, will be revealed. This line of research, enhanced by interdisciplinary approaches, holds promises for very interesting findings and even more interesting future questions. Understanding lipid–protein interactions better will help us understand diseases better, and some disease processes (such as virus entry and budding and abnormal lipid trafficking in lysosomal storage disorders) will give us important insight into novel lipid–protein interactions and their role in cellular physiology.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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