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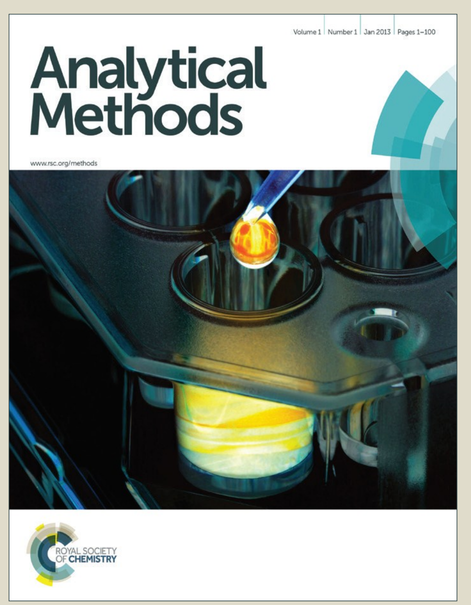

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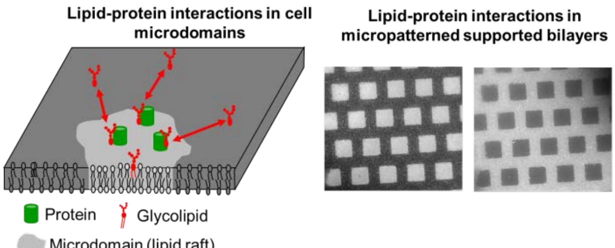
Table of contents



Abstract

Current understanding of the cell membrane suggests that it is a patchwork structure composed of many proteins and lipids that are not all freely diffusing, but rather can take part in dynamic microdomains within the plane of the membrane. These domains can form or be maintained in several ways, such as the coalescence of "lipid shells" around proteins and/or cytoskeletal compartmentalization. Interactions within a micro-environment not only co-localize multiple components of some functional unit, but also may be involved in the regulation of that unit's activity. However, studies of protein-lipid associations and their impacts on protein activity are challenging for a number of reasons. In this review we describe the salient features of classical and emerging methodologies for studying protein-lipid interactions and their limitations.

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A review of traditional and emerging methods to characterize lipid-protein interactions in biological membranes

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Abstract

Current understanding of the cell membrane suggests that it is a patchwork structure composed of many proteins and lipids that are not all freely diffusing, but rather can take part in dynamic microdomains within the plane of the membrane. These domains can form or be maintained in several ways, such as "lipid shells" around proteins and/or cytoskeletal compartmentalization. Interactions within a micro-environment not only co-localize multiple components of some functional unit, but also may be involved in the regulation of that unit's activity. However, studies of protein-lipid associations and their impacts on protein activity are challenging for a number of reasons. In this review we describe the salient features of classical and emerging methodologies for studying protein-lipid interactions and their limitations.

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Introduction

The cell plasma membrane is a fundamental biological barrier that encapsulates the cell cytoplasm and nucleus and plays the important role of regulating the interaction between the cell and the outside environment. This membrane is comprised of a bilayer of amphipathic lipid molecules and proteins arranged to shield their hydrophobic parts from the aqueous external environment. Membrane proteins embedded within the lipid bilayer solvent take part in many critical functions including transport, signaling, and recognition between the cell and its environment. Not surprisingly then, over 60% of drugs on the market target membrane species[1], [2]. However, recently it has become appreciated that not only is the protein interaction with the drug important for altering its activity or function, but that the level of modulation, or even the final biological outcome, may be coupled to the lipid interactions the protein also makes with the surrounding membrane[3]–[5]. Beyond drug discovery, understanding of such interactions may prove critical in other biotechnology applications where membrane proteins are key elements, for example in biosensing[6], tissue engineering[7], biofilm formation[8], and so on.

An important manifestation of these interactions is the way lipids and proteins tend to separate into micro-domains through direct association among themselves instead of distributing uniformly as a homogeneous, two-dimensional fluidic environment. Micro-domains compartmentalize distinct kinds of lipids and membrane proteins and are an important paradigm of cell membrane organization. One notable, but still enigmatic, type of micro-domain that results from phase separation is the lipid raft, which is enriched in cholesterol and sphingolipids relative to the surrounding phospholipid-rich membrane. Specific kinds of proteins and glycolipids are enriched in rafts, while others are excluded. It is hypothesized that raft domains

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play a key role in regulating the functions the membrane proteins and their biological activities through selective partitioning of species into and out of rafts[9]–[13]. While there is a lot of circumstantial evidence to support the raft hypothesis, characterizing these lipid-protein interactions has been difficult. Furthermore, correlating these interactions with biological function has been even more of a challenge.

This review article will focus on several key aspects of membrane lipid-protein interactions. First, a brief summary of evidence to support micro-domain existence in cell plasma membranes will be given. Second, a few principles of lipid-protein interactions will be provided with a discussion of the roles of these interactions on biological events. Third, current methods to understand and analyze these interactions will be discussed. Though the review does not exhaust all the approaches, it will cover traditional and emerging methods that are predominantly used to assess the specificity of lipid-protein interactions in the aspects of: a) structural characterization of lipid-protein complexes/interfaces, and b) biochemical characterization of proteins within their local membrane environment, especially focused on protein-raft interactions. Finally, the challenges and opportunities that lie ahead in this field will be left as parting views.

1. Experimental support for existence of lipid rafts in cells

While phase segregation in model membranes were postulated as early as the 1960's [14], [15] (see also Veatch 2007 [16] and references therein), direct detection of lipid rafts in cell membranes has proven elusive partly because of their small scale and dynamic nature. However, there is a wealth of evidence supporting their existence. Here we provide a brief synopsis of this evidence, but for a thorough review see Lingwood and Simons[17]. Detergent resistant membrane (DRM) fractionation assays isolate membrane fractions that are enriched in cholesterol, sphingomyelin, and raft-associating species[18]. Surface labeling with antibodies or

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toxins binding to raft-associated proteins are used to tag raft membrane domains[19]. Single particle tracking methods[20], [21] detect changes in protein diffusion, which indicates the presence of more viscous raft domains. Finally, plasma membrane vesicles devoid of cytoskeletal proteins have been shown to readily undergo phase separation into large, observable raft-like and fluid-like domains[22], [23]. In parallel to these techniques, recent advances in spectroscopy[24] and super-resolution imaging[11], [25], such as photoactivation localization microscopy (PALM)[26], stochastic optical reconstruction microscopy (STORM)[27], and stimulated emission depletion (STED) fluorescence microscopy[28], [29] have revealed dynamic, nanoscale lipid raft assemblies in living cells. As the lipid raft hypothesis has gained acceptance, the number of hypotheses for the mediation of cell processes through lipid-protein interactions involving rafts in has increased.

Although membrane protein interactions within their local membrane environment seem critical to their functions, they are not easy to probe because traditional protein characterization techniques may disrupt the native lipid-protein interaction in the cell or artifactually change the native associations. For example, detergents used in DRM fractionation can coalesce rafts and could kinetically trap non-raft species in them during this process. DRMs are also dependent on choice of detergent as different detergents may cause changes in clustering and fraction compositions[30]. Surface labeling of cells requires antibodies or toxins to bind to specific species, often crosslinking them and causing artifactual enrichment[31]. An alternative is to label fixed cells, but membrane organization and lipid-protein associations of dead cells are not necessarily indicative of live conditions[32], [33]. Isotope labeling methods have some utility[34], but can only be applied to cultured cells, are expensive, and still require isolation methods to identify residents.

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Therefore, to expand our current knowledge of how membrane proteins function and are regulated, a variety of techniques including novel membrane platforms are needed to expand the characterization of lipid-protein interactions and minimize possible artifacts.

2. The principles of lipid-protein interactions

Lipid species interacting with membrane proteins can be classified into two categories: annular lipids and non-annular lipids[4]. Annular lipids solvate membrane proteins by forming a shell around their surfaces (Fig.1A), while non-annular lipids are found buried within protein helices (or protein complex subunits) forming lipid-protein complexes (Fig. 1B). The critical differences between annular and non-annular lipids are their degrees of affinity for, and their residence times with, membrane proteins. Annular lipids exhibit lower affinity to membrane proteins compared to non-annular lipids and possess the motional freedom to exchange with the bulk environment; in contrast, non-annular lipids are more restricted from exchanging with the surrounding lipids and may bind directly to specific sites on membrane proteins. The following sub-section will expand on the principles of annular and non-annular lipid-protein interactions and provide biological examples of each type.

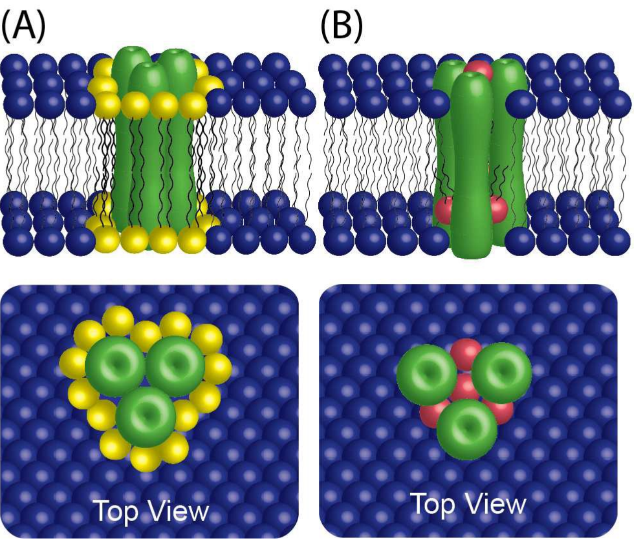

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Figure 1. (A) Annular lipid arrangement (yellow) around a protein complex (green). (B) Non-annular lipids (pink) arranged within a multi-subunit protein complex. The surrounding lipids (in blue) represent non-raft membranes enriched in phospholipids.

2.1. Annular lipid – protein interactions

The effects of annular lipids on membrane protein structure and their functions are mainly ruled by the following interaction principles[4]:

1) The degree of hydrophobic matching between membrane protein core and the surrounding lipid acyl chains.

2) The structure and charge distribution of lipid headgroups and protein amino acid residues located near the lipid-water interface.

To avoid exposing the hydrophobic domain of membrane proteins to aqueous solution, the hydrophobic thicknesses of lipid bilayers and proteins must match. Mismatching results in the distortion of protein structures, which can influence their functions. One well-known example is the mechanosensitive channel of large conductance (MscL) from *Escherichia coli*. Hydrophobic matching is controlled by a shift in bilayer thickness (via acyl chain length), which results in a distinct MscL conformational change and its gating function: a thin bilayer favors channel opening while thick bilayer favors a closed structure[35]. Another important effect of hydrophobic matching is to sort/cluster membrane proteins into specific domains. Hydrophobic matching between SNARE protein transmembrane domains and surrounding bilayers is mediated by cholesterol and is believed to cluster SNARE proteins into thicker raft domains[36].

The headgroup region of annular lipids also has important influence on membrane protein conformation and activity. Hydrogen bonding and charge-charge interaction between the specific amino acids of proteins and lipid headgroups can largely affect the structure of the protein. A clear case is in rhodopsin photoactivation[37]. Phophatidylethanolamine lipids (PEs) were found

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to interact tightly with rhodopsin and promote the formation of MII, a major intermediate of the rhodopsin photocycle. One of the possible mechanisms to explain the observation is the formation of hydrogen bonds between the PE headgroups and Glu-134, which is exposed upon MII formation[38]. Other studies also detected "hotspots" on membrane proteins for binding by anionic lipids due to their electrostatic interactions[4], [39]. An example is the characterization of a subset of annular lipids around ABC transporters[40], where negatively charged phosphatidylglycerol lipids (PGs) were demonstrated to exhibit higher affinity toward TmrAB dimer than zwitterionic PEs.

2.2. Non-annular lipid – protein interactions

Lipids exerting higher affinity to specific binding sites on protein transmembrane domains are referred to as non-annular lipids. These lipids may be integrated within the protein core structure, especially between protein subunits. Due to their high-affinity binding to membrane proteins, non-annular lipids can be resolved in membrane protein crystal structures by high-resolution crystallography, and many examples of non-annular lipid-protein interactions have been reviewed in literature[41], [42]. Non-annular lipids serve diverse purposes in modulating protein structure and function on cell membranes. For instance, lipids can act as co-factors for membrane proteins to function, and a typical example is the potassium channel KcsA[43]. Anionic phospholipids, such as PGs, were found to bind at the monomer-monomer interface in the KcsA homotetramer. It is believed that the interaction of PGs with the non-annular binding sites of KcsA helps the packing of KcsA structure and supports its conducting state.

Another well-known function of non-annular lipids is their allosteric effect in regulating membrane protein activities. For example, binding between cholesterol and many G-protein-coupled receptor (GPCR) allosteric sites were found to be critical for to GPCRs biological

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pathways, such as the full activation of the oxytocin receptor[44] and the ligand binding and G-protein coupling of serotonin$_{1A}$ receptor[45]. A second example of regulation comes from protein interaction with the acyl tails, in particular, the influence of the flexibility of chain conformations. These have been shown to mediate annular lipid/protein interactions, such as between polyunsaturated lipids and GPCRs[46]–[48].

Finally, non-annular lipid-protein interactions have been suggested to play a role in targeting proteins to distinct lipid domains. For example, binding between the metabotropic glutamate receptor (a type of GPCR found in *Drosophila melanogaster*) and cholesterol was shown to target the receptor to lipid rafts[49]. In another example, binding of amyloid precursor protein (APP) and cholesterol has been suggested to promote localization of APP in lipid rafts, and the association of APP to lipid rafts is believed to be essential to the progression of Alzheimer's disease[50].

3. Approaches for identifying and characterizing lipid-protein interactions

3.1. Structural characterization of protein-lipid complexes/interfaces

Membrane protein structures, and particularly lipid-protein interaction sites, provide significant insights into how lipid-protein interactions might impact function. Among structural characterization tools, crystallization is the standard method that analyzes lipid-protein complex structures with atomic level resolution. X-ray crystallography (XRC) has been used extensively for defining the structure of three-dimensional lipid-protein complexes and recognizing lipid-protein binding sites (Recently reviewed in Shi, 2014)[51]. For example, cholesterol has been observed in crystal structures of the β2-adrenergic receptor by using XRC[52], [53]. However, to achieve high-resolution structural information, high quality protein-lipid complex crystals are needed, which makes crystallization a critical step for successful XRC performance. The

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traditional method (*in surfo* method) to crystallize protein-lipid complexes requires the use of surfactants (detergents) to solubilize protein-lipid complexes from the native bilayer environment and form detergent-protein-lipid micelles. Membrane proteins may be denatured during this process and lipids bound to the protein-lipid assembles may be replaced by detergent molecules. As an alternative, *in meso* methods have been developed in the past decade to overcome this limitation (recently reviewed in Caffrey et al, 2012)[54]. The *in meso* methods crystallize protein-lipid assembles in lipidic mesophases, which preserves assembled protein-lipid structures and reflects more realistic lipid-protein interactions. This method has been especially useful in determining structures for G protein-coupled receptors, with 55 distinct GPCR structures crystallized and resolved by the *in meso* method[55].

Two-dimensional electron crystallography resolves 2D protein crystal structures in lipid bilayers, where proteins are purified, reconstituted and crystallized in a lipid bilayer environment (recently reviewed in Wisedchaisri et al, 2011)[56]. The crystal resolution achieved by electron crystallography is now comparable with XRC. For example, a 1.9Å resolution structure was successfully determined for aquaporin and its annular lipids[57]. In addition, electron crystallography has been able to reveal membrane protein structures that were not solved by 3D XRC. For example, the conformational change resulting from ligand binding to cyclic nucleotide-modulated potassium channel, MloK1, was recently reported using 2D electron crystallography[58]. With both techniques now having comparable resolutions, a distinct advantage of electron crystallography over XRC is the lower amounts of proteins required, which is critical for membrane proteins with typical low yields due to expression limitations and purification processes[59].

Although crystallization is a powerful method of resolving lipid-protein complex structure,

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membrane proteins do not readily crystallize, consequently few protein-lipid complexes have been solved. As of February 2015, membrane proteins (1,604 entries, retrieved from http://blanco.biomol.uci.edu/mpstruc/) only account for 1-2% of all the protein structures (99,147 entries) released in the protein data bank (PDB)[60]. As an alternative approach, nuclear magnetic resonance (NMR) can be used to obtain protein-lipid structural information, but with molecular-scale resolution. NMR has rapidly improved from the traditional solution NMR, which was limited to small molecules (< 40 kDa) in solution, to solid-state NMR. Solid-state NMR has the ability to examine macromolecule structure (> 100 kDa), such as purified proteins reconstituted into lipid bilayers. Thus NMR is now routinely used to determine lipid-protein complex structures (reviewed in Tycko, 2001 and Huster, 2014)[61], [62]. For example, the non-annular binding sites of the potassium channel KcsA and anionic phospholipids (previously mentioned in section 2.2) were also well-characterized by solid-state NMR,[43] corroborating results of the previous x-ray crystallography study[63].

Despite the ability to resolve structures with high resolution, the structural characterization tools stated above cannot often capture the fast dynamics of lipid-protein interactions. For instance, annular lipids are highly dynamic and interact with the surrounding bulk lipid bilayer at the rate of 10^{-7} s[64]. Moreover, the complexity of lipid-protein environment makes it challenging to study lipid-protein interactions with high time resolution. Other techniques like electron paramagnetic resonance (EPR) spectroscopy and 2D Infrared (2DIR) spectroscopy can better capture the dynamics of lipid-protein interactions (recently reviewed in Smith, 2012)[65]. EPR has the potential to probe protein-lipid interfaces with high sensitivity and fast timescales (nanosecond to microsecond). This temporal resolution permits characterization of the dynamics of annular lipid shell surrounding membrane proteins and thus the residence time of lipid-protein

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complexes[66]. Speed and sensitivity are also advantages of 2D IR spectroscopy. By probing electronic and vibrational dynamics at the time scale of femtosecond to picosecond[67], researchers are able to measure the strength of lipid-protein interactions and define the orientation and conformation of proteins in lipid bilayers[68] [69].

Besides probing specific interactions at the molecular level, the characterization of structural motifs of lipid-protein complexes, such as between proteins and lipid raft domains, can provide insight on biological function. Structural characterization of raft lipids (sphingolipids and cholesterol) bound to proteins has provided significant insights on: 1) how these proteins may be targeted to rafts, and 2) how their conformation, function, and interactions with other species are influenced by interaction with raft lipids. For example, evidence supports that influenza virus proteins (hemagglutinin, neuraminidase, M2) associate with lipid rafts of host cells as a way to organize prior to budding to form viral progeny[70]. Both crystallographic and NMR studies have clarified the M2 protein structure and its cholesterol-binding domains[71], [72]. From these studies, M2 protein is believed to be raft-anchored through its binding with cholesterol and localizes at the edge of the bud zone to carry out a crucial role in mediating the particle scission process[73].

In summary, structural characterization tools receive much attention owing to their capacity to determine lipid-protein complex structure with high resolution, detect the dynamics of lipid-protein interactions, and probe the role of lipid rafts on protein conformation and function. However, these techniques are low throughput and the sample preparation techniques are often difficult and can give rise to artifacts. To develop a more complete picture, cell-based studies are also needed to complement structural information.

3.2. Cell-based techniques to study lipid-protein interactions and raft function

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A primary objective of raft investigations is often to determine how species are targeted to these domains. Specific structures of membrane species have been found to dictate their raft-association preferences[74]. For instance, it was found that differences in the structure of glycophosphatidylinositide (GPI) anchors could be correlated to the differences in raft-association of GPI-linked proteins[75]. Diaz-Rohrer et al. further explored this concept[76]. They devised an array of protein constructs based around a single pass protein, trLAT, with variations in transmembrane domain lengths and sequences as well as number of palmitoylation sites. By observing the trafficking behavior of these fluorescent fusion protein constructs in cell membranes and the raft partitioning behavior in model giant unilamellar vesicles, they proposed a unique raft pathway whereby raft-associated proteins are recycled to the plasma membrane.

It is worth bringing to the reader's attention that the labeling strategies used in experiments can potentially influence the interactions between lipids and proteins, and the dynamics and stability of membrane domains. In many of the papers reviewed herein, careful controls (to the degree possible) have been performed to understand and minimize the effects of labels used. Nonetheless, labels are known to significantly influence partitioning behavior. For instance, antibody labeling can crosslink species and cause of stabilization of domains [21] and acyl chain labels can disrupt the usual association of saturated lipids with lipid rafts [77].

Another objective of raft investigations is to determine how raft environments can affect protein function. Many of these studies use disruption of rafts in live cells to evaluate their influence on protein function, that is, to observe differences in protein function before and after raft disruption. Rafts can be disrupted by either removal of cholesterol using cyclodextrins or using knockouts of sphingolipid biosynthesis enzymes. Depletion of cholesterol by cyclodextrin has been shown to reduce levels of raft marker proteins in detergent resistant membrane

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fractions. Disruption methods also revealed stimulation of signaling through the tyrosine phosphorylation and Ras-ERK pathways, promoting the idea that raft-association regulates protein activity (particularly LCK) in these cascades[78]. Although cyclodextrins are useful tools to deplete cholesterol, their mechanisms of action are still unclear and may promote raft formation instead of eliminating them[79], may remove cholesterol from raft and non-raft domains at different rates, and, at high enough concentrations, may extract other molecules including phospholipids from the membrane[80].

In another study of cell migration induced through the CXCR4/CXCL12 pathway, sphingomyelin was shown to play an important role. By knocking out sphinogmyelin synthases, it was concluded that sphingomyelin acts as a selective regulator of GPCR signaling: low sphingomyelin concentration in rafts leads to an increase in CXCR4 levels in rafts and increased dimerization, both correlating to increased cell migration[81]. Although the strategy of genetically deleting an individual enzyme targets the lipid-protein interaction directly, it is important to note that there will also be changes in all lipids "downstream" of that enzyme in the pathway, which can also impact other cellular functions.

A third objective of raft investigations is to characterize the dynamics of biomolecules partitioning into and out of rafts within the membrane plane. Biomolecules move laterally within the lipid bilayer by Brownian diffusion[82]. With the advent of single molecule microscopy techniques to track individual proteins embedded in cell plasma membranes, it has been revealed that protein diffusion is strongly influenced by partitioning into raft domains as well as by interactions with other membrane species, particularly those immobilized by the cytoskeleton. Such studies have highlighted the interplay between cytoskeleton and lipid rafts in guiding cellular control of membrane species distribution[83]. Because of the tight coupling between

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rafts and the cytoskeleton, separating the impact of these two structures in whole cells is difficult[84], [85].

Several models for the complex diffusion of membrane proteins among domains and cytoskeletal corrals have been developed and evaluated using single molecule tracking (SMT) techniques in cells. Kusumi's hop-diffusion compartmentalization model claims that molecular "fences" formed by cytoskeletal elements and bound membrane protein "pickets" help to organize the plasma membrane into patches with free diffusion within a confinement patch, and occasional hops between patches[86], [87]. Weigel et al. analyzed Kv2.1 protein tracking, with green fluorescent protein (GFP) and quantum dot labeling, to determine the diffusion process is non-random such that certain regions of the membrane are more likely to be sampled by the membrane protein and proposed a model of a random walk on a fractal[88]. By disrupting actin with depolymerization drugs, they were able to see random diffusion, indicating Kv2.1 binding to actin plays a key role in its diffusional behavior. In another SMT study, Türkcan et al. used a Bayesian inference scheme to characterize hopping events of ε-toxin receptor (labeled with fluorescent nanoparticles) between raft domains, where they found that hopping between raft domains was limited by the proximity of adjacent domains[89]. After destabilization of rafts with sphingomyelinase or cholesterol oxidase, diffusivity of ε-toxin receptor increases and confinement decreases, indicating the influence of rafts on ε-toxin receptor diffusion.

While SMT studies have provided a wealth of new knowledge about protein behavior in cell membranes, it is important to recognize that not all protein clustering and domain formation involves lipid rafts. Other domains can form as a result of protein-protein interactions without typical raft constituents. Douglass & Vale used SMT of membrane proteins to investigate the mechanisms behind CD2 cluster formation in T cell receptor signaling[90]. LAT and Lck were

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found to transiently interact with CD2 to form clustered microdomains. Mutating LAT to prevent its signaling through a protein-protein interaction domain stopped cluster formation. They found that CD2 cluster domains were not enriched or depleted in raft marker proteins and that cholesterol depletion with methyl-β-cyclodextrin did not affect clustering. This indicates that this protein-protein interaction is the major mechanism by which CD2 clustering occurs. Magenau et al. used PALM to study clustering of five membrane proteins fused to two different photo-switchable proteins[91]. While the type of membrane anchor and positioning on the inner or outer leaflet played a role in clustering via rafts, this effect could be overridden when the fusion protein was swapped with one that had a tendency to oligomerize. The authors proposed that protein-protein interaction was stronger than the protein-lipid interactions of raft partitioning in some of the cases studied.

All of this work suggests a complex interplay of lipid-protein and protein-protein driving forces for lateral membrane organization. Decoupling this complexity to conclusively identify and distinguish protein-protein from lipid-protein interactions and characterize their mechanisms of action is an impetus to use simplified model membrane platforms.

3.3. Traditional and emerging model membrane techniques to study lipid rafts

Model membrane methods for probing membrane protein activity in lipid environments aim to bridge the approaches of traditional proteomics and cell based assays. Cellular processes are entangled and individual factor effects are difficult to isolate. This cell complexity is the motivation for the development and use of biomimetic membrane strategies to model and deconstruct cell membrane processes. Model systems are simplified and tunable, helping to visualize organization and dynamics of membrane species and assay activities of individual components, oftentimes using many of the aforementioned microscopy techniques. In the

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minimal model approach, these systems contain known lipid and protein components and can be used to isolate and assay behavior of individual species. At the other end of the spectrum, incorporation of cell extracts, including cell membrane-derived vesicles (blebs), combines the beneficial techniques for imaging and assaying model membrane systems with the increased complexity and biological relevance of species studied. The two most commonly used model membrane systems that will be reviewed here are vesicles and solid supported lipid bilayers (SLBs) (Table 1). Less common systems, such as nanodiscs, fluorinated surfactants, and amphiphols are reviewed by Popot[92].

3.3.1. Vesicle-based model membrane studies: Giant unilamellar vesicles (GUVs) and giant plasma membrane vesicles (GPMVs) are the major vesicle-based model membrane systems. A range of biomolecule complexity can be spanned by these systems from simple, few-component lipid GUVs, to reconstituted proteoliposomes[93], to cell-extracted GPMVs[94]. GUVs are reconstituted lipid systems formed from minimal components. These reconstituted vesicle systems have been crucial for the characterization of phase behavior of lipid mixtures and phase segregation of probes and membrane protein components[76], [95]–[100]. GUVs were used to show that cross-linking of GM_1 glycolipids in membranes led to large-scale membrane phase separation[101], for example.

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Model Membrane	Features	Selected References
GUV	1. Easy to control lipid/protein composition 2. Easily reconstitute membrane proteins in GUV 3. System is fragile due to the lack of support and large size 4. Requires sophisticated microscopy for analysis	[76] [91] [96-101] [104-113] [125] [152-153] [155]
GPMV	1. Representative of cell plasma membrane 2. Does not require protein purification and reconstitution processes to incorporate proteins 3. Increased complexity, but no cytoskeleton 4. Requires sophisticated microscopy for analysis	[22-23] [94-96] [102-103]
Support Supported Lipid Bilayer (SLB)	1. Bilayer is robust due to solid support 2. Amenable to quantitative surface characterization techniques 3. Planar geometry compatible with simple microscopy techniques 4. Membrane protein mobility is restricted due to interaction with support 5. Bilayer cushion can be used as cytoskeleton mimic 6. Can be spatially patterned	[77] [114-122][126-137] [139-140] [142-146] [153-154] [156-169] [171-179]
Support Supported Plasma Membrane Bilayer (SPMB)	1. Representative of cell plasma membrane 2. Does not require protein purification and reconstitution processes 3. Bilayer is robust due to solid support 4. Amenable to quantitative surface characterization techniques 5. Membrane protein mobility restricted due to interaction with support	[147-148] [150-151]

Table 1. Types of model membrane systems and their respective features.

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GPMVs formed from cell plasma membranes contain native lipids and proteins and therefore capture more biological complexity. GPMVs generally display a single lipid phase until temperatures are lowered to where two phases form and protein partitioning occurs[95], [102]. These studies reveal that the cell membrane is near a miscibility critical point. Thus perturbations, such as local composition fluctuations, can cause changes in miscibility and may serve as a means to control raft formation and protein partitioning in the cell membrane. Levental et al. showed that GPMVs of various preparations contained a variety of phases with different properties and compositions reflecting complexity of domains that are possible in cells[103].

Vesicle systems have been used to study protein function in presence of specific lipids and ligands. Two particular studies generated GUVs with a wide variety of lipid compositions to determine which species can alter protein function. In the first study, β-secretase or BACE, a membrane spanning protease, was shown to have its activity most strongly affected by anionic lipids, but also to some extent by glycosphingolipids and cholesterol[104]. The enhancement of activity by classical raft lipids fits the idea that BACE activity is enhanced in rafts where it is able to interact with its substrate, the amyloid precursor protein[105]. In a second study, the activity of placental alkaline phosphatase (PLAP), a GPI-linked protein, was shown to decrease in the presence of cholesterol and lipid rafts[106]. Since PLAP, like many GPI proteins, is known to be associated with rafts[107], this response follows a repressive regulation of activity in the presence of rafts, as has also been observed for some other GPI proteins[108].

Vesicle systems are advantageous because they can easily incorporate mobile integral membrane proteins[109], [110]. Because of their spherical geometry, vesicles contain an isolated lumen making them great systems for also evaluating function of transport proteins like ion channels[111]–[113]. However, because vesicles have a fragile, three-dimensional structure,

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many surface techniques cannot be used and quantitative imaging often requires confocal or other more sophisticated techniques. In these systems, phase separation and protein partitioning occur at the same time, so the kinetics of protein partitioning cannot be monitored easily; usually these systems are used to monitor distributions at equilibrium conditions. However, phase separation in vesicles cannot be spatially controlled, so labels are needed to indicate phase and protein locations.

3.3.2. Supported lipid bilayer-based model membrane studies: Supported lipid bilayers consist of a solid surface onto which a lipid bilayer is adsorbed typically via a vesicle fusion[114] or Langmuir-Blodgett-Schaeffer transfer technique[115]. The SLB provides a chemically tunable, planar platform that is compatible with a vast array of surface characterization tools, such as total internal reflection fluorescence microscopy (TIRFM)[116], atomic force microscopy (AFM)[117], quartz crystal microbalance (QCM)[118], [119], and surface plasmon resonance (SPR)[120]–[122], among many others. Like vesicle systems, SLBs span the full range of complexity from simple lipid-only platforms to the complex chemistries of cell plasma membrane bilayers.

One drawback of SLB systems is the close proximity of the bilayer to the support that effectively reduces the diffusion of membrane species [123]–[125], relative to free-standing or vesicle bilayers. This drawback becomes more pronounced when attempting to reconstitute fully functional mobile membrane proteins in SLBs. In a typical phosphatidylcholine (PC) SLB on glass there is a ~1 nm water gap between the bottom leaflet and the glass surface[126] that is too small to accommodate most soluble domains of membrane proteins, which can extend up to 10 nm[127]. Strong interaction with the solid support often leads to immobilization of membrane proteins. To solve these problems, various bilayer cushioning or tethering strategies have been

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proposed that extend the distance between the bilayer and the support[128]–[131]. One particular strategy that is relatively easy to incorporate and shows improved membrane protein mobility is the double cushion strategy[132][133]. The first cushion is created by adsorbing a passivating layer of proteins (typically bovine serum albumin) to the glass support to reduce non-specific binding. The second cushion is composed of polyethylene glycol (PEG) polymer functionalized lipids interspersed in the bilayer[134]–[136]. The extension length of the polymer cushion can be controlled by selecting the PEG chain length and the concentration in the bilayer[137][138]. Unfortunately, although cushioning and tethering techniques show improvement over uncushioned systems, many of these strategies still result in less than half of proteins showing any significant mobility, necessitating further investigations of cushioning and fluidization of membrane proteins by these polymers and integrating what is learned into next generation spacing strategies. In particular, it is unclear if the PEG polymers can provide a uniformly cushioned bilayer that can protect the incorporated membrane proteins or if cushioned and uncushioned domains will form [132][139]. An additional concern is that at high grafting densities, PEG cushioning can provide a steric barrier preventing access for ligands to the membrane proteins[140]–[142]. Because of these limitations, most SLB investigations have been restricted to peripheral proteins, small self-inserting proteins, or proteins without domains that extend towards the support.

The flat geometry of a SLB favors using quantitative techniques to characterize important aspects of lipid-protein and protein-protein interactions. For instance, a SLB-based single molecule tracking study tracked 17 different peripheral protein-lipid complexes to find that the drag effects on peripheral membrane proteins depended strongly on bound lipids and extent of penetration of protein domains into the bilayer[143]. Another study used high-speed AFM to

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track OmpF protein trimers in a supported lipid bilayer at sub-500 ms resolution[144]. With this method it was possible to determine the orientation of trimers and show that interactions between proteins are crucial to the formation of slow, stable assemblies. Deverall used SMT in a tethered SLB to study effects of obstacles on bacteriorhodopsin protein diffusion[145]. Tethers consisted of individual lipids attached to underlying polymer, effectively immobilizing lipid molecules in the bilayer. The impact of tether density on protein diffusion was investigated, showing similarities to the observed effect of the cytoskeleton in cells on protein diffusion, and followed a model of obstructed diffusion.

While these studies show that SLBs are promising mimics for cell membranes, two major challenges are associated with extending their usefulness to studying myriad membrane proteins: 1) incorporating membrane proteins into the SLBs with their native cell membrane lipid associations, and 2) minimizing interactions between the extramembranous regions of the proteins and the underlying glass support. Detergent mediated methods exist for protein reconstitution into GUVs and SLBs, but they require careful optimization of conditions for the protein of interest and may cause changes to the protein conformation[146]. Some creative methods have been proposed that do not require use of detergents. One example is the use of whole-cell membrane vesicles ruptured into bilayers using the rolling motion of a lipid bilayer under shear to catalyze the rupture process[147]. The process used for generating the cell membrane vesicles is to extrude the host cells, but it is important to note that this may result in unwanted scrambling between leaflets and between various membranes of the cells. Another approach is to solubilize/extract membrane components from cells and use the extracted material to form supported bilayers[148]. Again this method certainly results in mixed orientations of components between the two leaflets and possible contamination from other membranes in the

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cells.

In light of these challenges, we developed an alternative method that tackles both the protein reconstitution and cushioning problems. Delivery of membrane proteins to the supported bilayer platform is via cell bleb fusion. Cell blebs are similar to GPMVs but distinguished by their size; blebs are typically 500 nm diameter or smaller. Cell blebs are parts of the cell membrane that bud off into a proteoliposome when local detachment of the membrane from the actin cystoskeleton occurs[149]. We recently developed a process to generate SLBs using blebs[150], [151]. Mammalian cells expressing target membrane proteins are either chemically induced or naturally bleb off membrane vesicles, depending on the cell type. Blebs are separated from the cells and collected, then adsorbed to a glass surface. Adsorbed blebs rupture forming a bilayer when additional fusogenic lipid vesicles are added, as shown in Fig. 2. This platform has enabled us to study viral fusion, which requires membrane protein receptors in a mobile lipid bilayer.

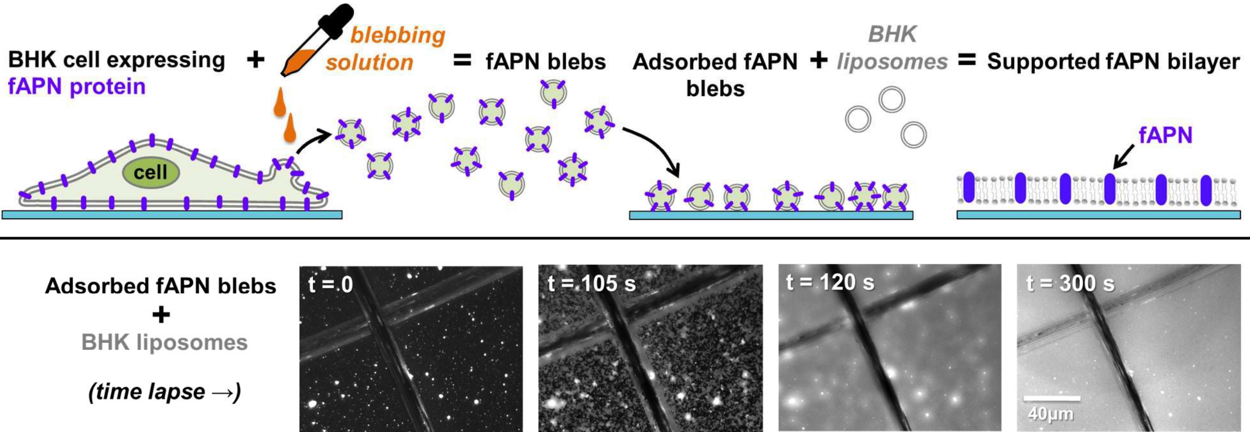

Figure 2. (Top) Illustration of the formation of a feline aminopeptidase (fAPN)-bleb supported bilayer from cell blebs derived from baby hamster kidney (BHK) cells. fAPN is a proteinaceous receptor for feline coronavirus. (Bottom) Fluorescence images of fAPN-SB formation, corresponding to the above cartoon. (Left, t = 0) fAPN-blebs containing a membrane labeling fluorophore, R18, adsorbed to glass substrate. Note that some larger blebs dominate the signal, but many smaller blebs are adsorbed as well. (Middle images) ~ 100 seconds after the addition of BHK-liposome solution to adsorbed blebs. Note that the BHK-liposome solution is devoid of fluorescent label, thus all signal comes from release of R18 initially confined to the bleb vesicle before rupture. (Right, t = 300 s) Continuous supported bilayer observed 300 seconds after the addition of liposomes. These images are all taken under 40x magnification. The dark lines in

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each image are scratches intentionally made with a dissection tool to find the focal plane of the bilayer. The continuous focus of this line throughout the rupture process indicates that the focal plane did not change and that the uniform distribution of fluorescence at t = 300 s is due to mobility of fluorophores redistributed throughout the newly-formed planar bilayer. (*Reprinted from Costello et al, Biomaterials, 2013*)

To use this system to study protein partitioning into lipid rafts and other lipid-protein interactions, we require mobile membrane proteins that do not interact with the support. By incubating blebs with PEGylated liposomes, a cushioned bleb bilayer can be generated (Fig. 3). This approach results in more membrane protein mobility, while addressing both protein delivery and cushioning problems in one step (unpublished work). The PEG cushion increases the distance between the bilayer and the substrate, providing space for the extracellular parts of the membrane proteins. Additionally, using blebs means native plasma membrane travels with the membrane proteins all the way to the SLB platform, so any crucial lipid-protein interactions can be preserved. From here, various operations probing lipid-protein and protein-protein interactions can be carried out on chip including partitioning, probing binding affinity, and other assays with the ability to alter lipid compositions, buffer conditions, and the physical environment (e.g., temperature, pressure, shear flow, etc.).

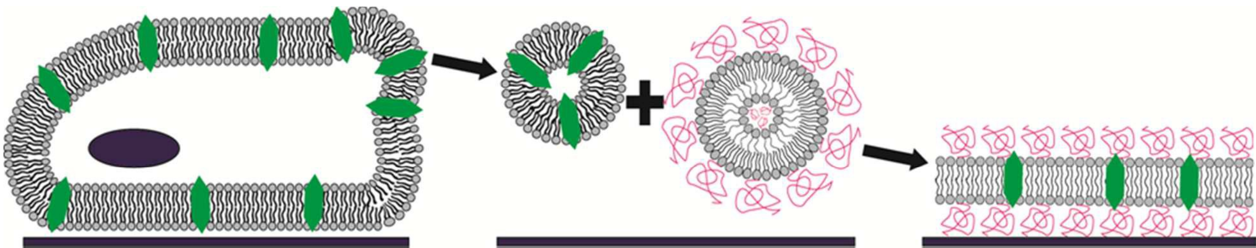

Figure 3. Formation of cushioned bleb SLB follows a similar process in Figure 2. Cells expressing membrane proteins of interest (green) are cultured and blebs are collected. Blebs are incubated with PEGylated liposomes (red) in a PDMS well or microfluidic device. Rupture and fusion of blebs/vesicles results in a cushioned bleb SLB.

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3.3.3. Measuring biomolecule partitioning in model membrane systems: Existing methods to probe raft partitioning in intact cells (DRMs and cholesterol depletion with cyclodextrins) described above are fraught with complications and possible artifacts. Additionally, many methods, including GUV partitioning studies[152] are equilibrium measurements and cannot provide information regarding the dynamics of partitioning. The standard approach for studying rafts in GUVs and SLBs has been to use the canonical raft mixture, a composition of lipids that will spontaneously separate into raft-like ordered and non-raft disordered lipid phases[153]–[155], e.g., point 0 on the tie line in Figure 4A. This approach results in random distribution of phases in the bilayer and requires tags to locate phases (Fig. 4B). Due to the nature of how GUVs are formed, once phase separation occurs, species have already distributed between the phases so kinetic information about the partitioning process cannot be obtained.

To circumvent some of these complications, we designed an SLB platform to investigate raft partitioning of membrane species that takes advantage of the unique ability to selectively pattern SLBs.

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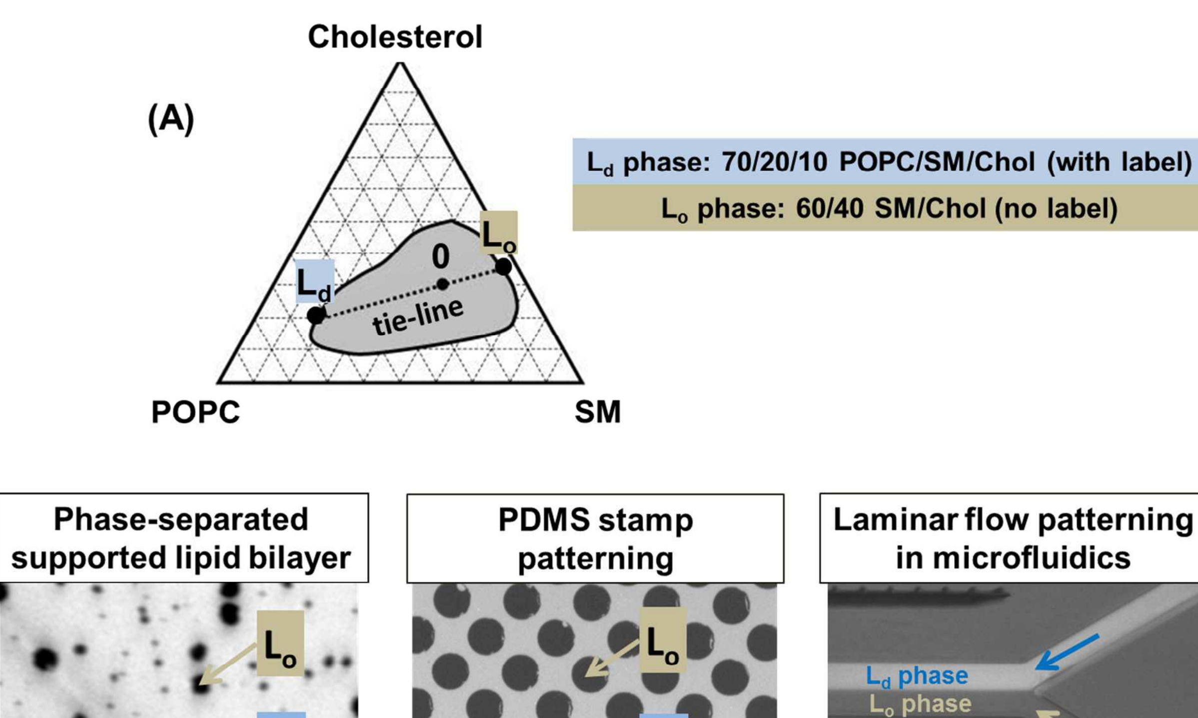

Figure 4. (A) Ternary phase diagram for the 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine POPC, sphingomyelin (SM), and cholesterol system. The gray area represents the two-phase coexistence zone. The tie line (dashed) runs through point 0, which spontaneously phase separates into two phases, each given by the compositions at the end of the tie line and denoted as l_d (liquid-disordered) and l_o (liquid-ordered). (B) A lipid mixture of composition 0 phase separates into a random pattern after formed into a supported bilayer. Both position and size of the domains are variable. (C) Patterning a SLB using PDMS stamping where two separate lipid phases are used (each at the ends of the tie line) to create regularly patterned domains of l_o phase within the continuous l_d phase. (D) Patterning a SLB using laminar flow in a microfluidic device results in a striped pattern of l_o / l_d phases along the channel.

3.3.4. Emergence of patterned SLBs to model raft partitioning: An advantage of supported lipid bilayers is that they can be patterned to create arrays of bilayer domains of varied composition or separate bilayers into distinct isolated patches[156]–[159]. The four main patterning techniques are polymer mold based stamping or blotting[158], [160], [161], polymer dry lift-off stenciling[162], [163], UV-photopatterning[164] and laminar flow patterning[77],

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[165], [166]. To generate different geometries of liquid-ordered/liquid-disordered bilayers, we have used the blotting method (Fig. 4C) and laminar flow patterning (Fig. 4D). These patterned bilayers serve as model raft membranes that can be used to study the partitioning of biomolecules to/from these domains.

In our first study we aimed to quantify partitioning kinetics of membrane glycolipids using patterned SLBs[77]. The key design point for creating a heterogeneous SLB of pre-determined bilayer phase locations and compositions, e.g., those at the end of the tie line in Figure 4A, is to create two separate lipid solutions already at these precise co-existent compositions rather than one solution that later phase separates on its own at random locations. These two lipid solutions combined with laminar flow were used in a microfluidic channel to constrain vesicles to specific areas of the platform such that via vesicle fusion they would only form bilayers of a distinct lipid phase in a prescribed area. Our experimental design generated two parallel bilayers of co-existent phases that meet along a line interface (Fig. 5A). One side contained lipids in the liquid-ordered phase (l_o) (raft-like) and the other contained lipids in the liquid-disordered phase (l_d) and thus eliminated the need for additional components to label the phases. Using a hydrodynamic force provided by the bulk flow in the microchannel, target membrane-bound species to be assayed can be transported in the bilayers (Fig. 5B). The pre-defined location of stably coexistent phases, in addition to the controllable movement of the target species allowed us to control and monitor when and where the target molecules approach or leave different lipid phases. Using this approach with appropriate experimental designs, we obtain the association and dissociation kinetic parameters for three membrane-bound species, including the glycolipid, GM_1, an important cell signaling molecule and raft domain marker. We examined two different versions of GM_1 and concluded that structural differences between them impact the kinetics of association

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of these molecules to raft-like phases. Extensions of this approach that we are currently working on include measuring the partitioning kinetics of other glycolipids; lipid-linked proteins with posttranslational modifications; and transmembrane proteins introduced to the bilayer, enabled by the bleb-SLB approach. We believe this platform will provide insight into how structural factors, membrane compositions, and environmental factors influence dynamic partitioning.

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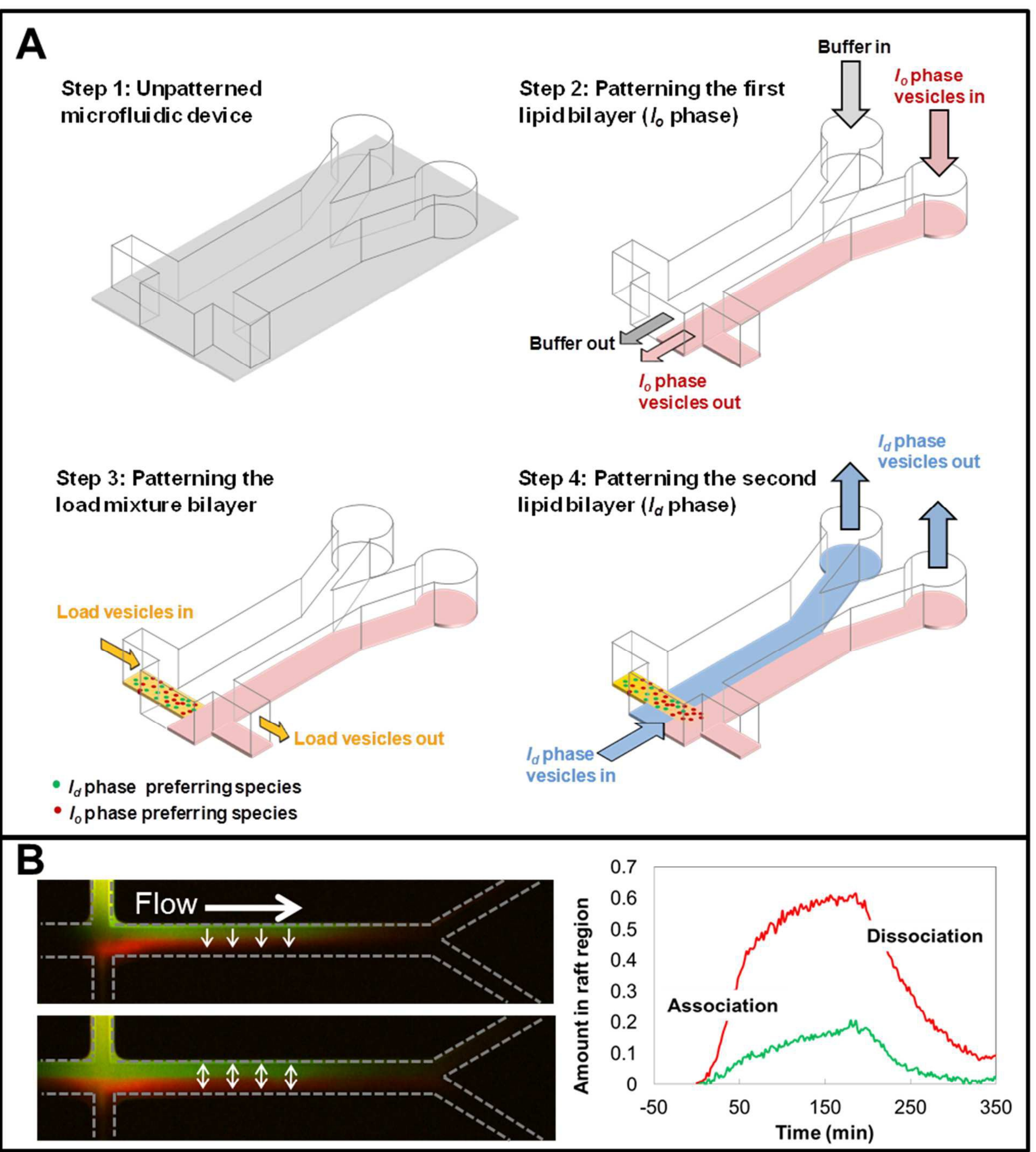

Figure 5. (A) A three dimensional cut-away view illustrating the loading and patterning of bilayers in a microfluidic device via vesicle fusion and laminar flow patterning. The pink color represents lipid phase l_o phase, the lipid-ordered bilayer; the blue color represents lipid l_d phase, the lipid-disordered bilayer; and the orange color represents the load bilayer that is the same composition as l_d phase, except that it contains the biomolecules to be separated and sorted. Green and red circles represent the biomolecule mixture. The arrows show the direction of the flow and streamlines as the pattern is being formed. Step 1: the blank microfluidic device design consisting of a clear PDMS mold bound to a glass support. The glass support is removed in the subsequent illustrations for clarity. Step 2: patterning lipid l_o phase. Step 3: forming the load

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bilayer containing the mixture of membrane-bound biomolecules. Step 4: patterning the l_d phase bilayer. Note that while the l_d phase bilayer is forming, some of the l_o phase-preferring species (red here) begin to partition into the l_o phase bilayer adjacent to the mixture load. (B) Images of partitioning of GM_1 (red fluorophore tag) into the l_o phase and the enrichment of BODIPY DHPE (green tag) in the l_d phase. The plot quantifies the partitioning into the raft phase. (*Figure adapted from Chao & Daniel, JACS, 2011*).

A second application of this basic patterning design was to continuously separate a mixture of membrane glycolipids based on their chemical affinity for raft domains[167]. In this case, mixed glycolipids (a fluorescently-labeled GM_1 derivative of a raft marker and a phospholipid labeled with BODIPY fluorophore) were loaded into the load region of the device and convected down the length of the channel using a hydrodynamic force provided by the bulk flow in the microchannel (Fig. 6). Separation of the species occurs as they travel through the two-phase region. This separation can be predicted by a convection and diffusion model using the kinetic phase partitioning parameters determined previously[77] and calculating the velocity profile for a two-phase bilayer under shear flow. At the end of the two-phase region, separated material is collected in separate collection ports. One future application of this platform that we are currently working on is an alternative approach for identifying and isolating raft species in cell membranes, in contrast to the DRM approach, through the integration of cell bleb SLBs into the device.

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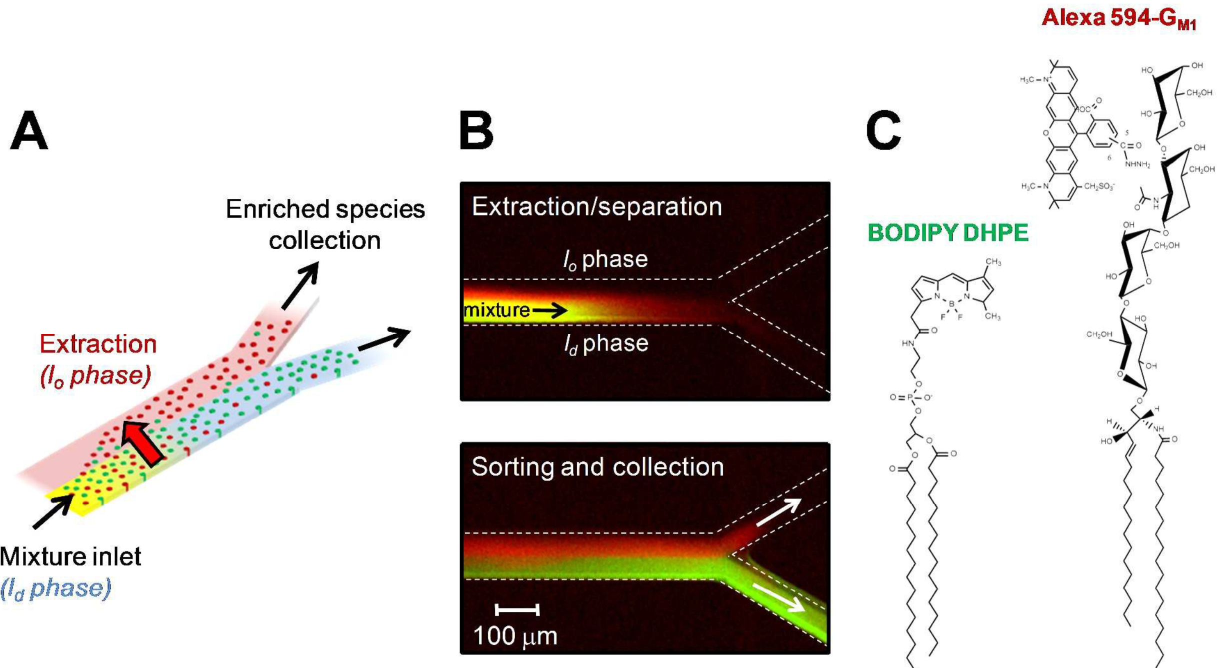

Figure 6. (A) A three-dimensional illustration of the two-phase supported lipid bilayer designed to separate and sort membrane biomolecules. The microfluidic device and glass support have been omitted for clarity. Laminar flow in a microfluidic device is used to create parallel stripes of coexistent lipid phases (l_d phase = blue, l_o phase = pink). The interface between the phases is contiguous, allowing membrane-bound molecules to partition into a preferred phase as they are transported down the main channel. The initial mixture is color-coded as red and green dots and is transported in the l_d phase. Red species are extracted into the l_o phase bilayer, causing the l_d phase to become more enriched in green species. (B) In the experiment, the mixture is BODIPY DHPE (green) and Alexa 594-GM$_1$ (red) and appears yellow in the upper image. In these top-view images, the l_d phase was patterned in the bottom section, where yellow is dominant, while l_o phase is in the top half (initially devoid of any fluorophore). The species are transported to the right in the l_d phase membrane along the main channel. The red color ahead of the yellow plug is a small amount of Alexa 594-GM$_1$ that moves slightly faster under bulk flow than BODIPY DHPE because it has a larger cross section. In the bottom image, the red Alexa 594-GM$_1$ is extracted into the l_o phase, while BODIPY DHPE generally remains in the l_d phase. Separated fractions are split by the “Y” at the end of the channel. (C) The chemical structures of Alexa 594-GM$_1$ and BODIPY DHPE. (*Reprinted from Chao et al, Analytical Chemistry, 2013*)

Recently, other multiphase bilayer patterning strategies have emerged. Roder et al. developed a clever technique for patterning polymer tethered supported lipid bilayers[168]. In their system, the polyethylene glycol (PEG) polymer brushes were functionalized with fatty acid moieties on the ends. The authors found that phase separation could spontaneously occur on the tethered

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bilayer, but that using oleic acid interfered with domain formation, while palmitic acid did not. Exploiting this difference, they patterned PEG brushes with each fatty acid type into predetermined regions. This resulted in controlled phase separation in their tethered supported lipid bilayer. Okada & Morigaki showed that quantitative control of supported lipid bilayer phase separation could be attained using polymerizable lipids to create domains[169]. The authors determined that including a percentage or coverage fraction of polymerized bilayer in patterned regions would prevent raft formation because of the bending energy penalty required between thick rafts and thinner polymerized lipids. Thus when using a raft-forming lipid mixture, rafts spontaneously form preferentially in polymer free areas, effectively patterning the bilayer.

3.3.5. Capturing more complexity of the plasma membrane in model systems: It is important to be mindful that model systems do not represent the full complexity of the cell membrane, and strive to strike a balance between simplicity and necessary complexity. One important and sometimes overlooked aspect is membrane asymmetry. While model systems typically employ symmetric bilayers, i.e. bilayers with the same compositions of lipids in each leaflet, the cell plasma membrane is asymmetric and traditional cholesterol and sphingolipid enriched rafts are only believed to exist in the extracellular leaflet[170][155]. Signal transduction across leaflets through registration of domains is a topic of considerable interest in the community[171]–[173]. Asymmetric leaflets can be constructed in SLBs by using a Langmuir-Blodgett and Langmuir-Schaefer transfer process with different compositions in each leaflet. When a sufficiently long enough polymer tether was used between the lower leaflet and the glass, domain registration would occur since the influence of the substrate could be minimized[174]. Interestingly, compositions that would not normally phase separate can be induced to form registered domains

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by raft-like domains in the other leaflet.

Applying asymmetric raft model membranes to protein partitioning, Hussain et al. investigated integrin partitioning[156]. Integrin partitioning into rafts was detected by colocalization with NBD probes, and exhibited a partitioning preference for asymmetric rafts but not symmetric rafts[175]. This behavior could be due to differences in hydrophobic thickness between asymmetric and symmetric raft domains. This work highlights the need to consider the importance of asymmetry in these SLB systems to better mimic the true cellular physiology. However, care must be taken when working with asymmetric bilayers in model systems as the lipids can flip-flop between leaflets with a half-time of ≈ 15 hr [171], but may be much shorter in the presence of proteins[176], or small defects (holes) in planar bilayers[177] or at temperatures approaching the lipid transition temperature[178].

Another key aspect of cell membranes often overlooked in model systems are the supporting structures adjacent to the cell membrane, like the cytoskeleton and glycocalyx, and the roles they may play on domain formation. Moving toward this direction, patterned glycans were used to influence phase separation in supported bilayers [179]. In this work glycans were patterned on supports uniformly or patch-wise (heterogeneously) upon which multi-component lipid vesicles fused to form supported bilayers. Depending on the underlying glycan pattern (and temperature of the system) multiple lipid phases can form within the SLB. This work highlights the need to not only understand the influence of the components within the membrane on phase separation and raft formation, but also the effect of the glycocalyx and extracellular parts on membrane organization.

Future Outlook

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While proteins have long been the focus of studies aimed at understanding biological function in the cell, lipids are also becoming appreciated for their complex interactions with membrane proteins and their impact on protein activity and function. Novel platforms and approaches are being developed to probe these interactions and determine their regulatory roles in biology, bridging the gaps between structural studies, classical proteomics and cell functional assays. As more membrane species and their analogs are discovered, approaches for investigating lipid-protein interactions will play an increasing role in helping to decode and understand the complexity of membrane biology. Two emerging areas we believe will be especially important to investigate with the approaches outlined in this review are protein posttranslational modifications and lipid glycolysis. Deciphering their effects on protein-lipid interactions and regulating protein function will eventually lead us to a better understanding of how they influence human disease and may be used in novel biotechnology applications.

In our parting thoughts, we leave the reader with what we believe are the most challenging improvements necessary in model systems and opportunities where the greatest development is currently taking place. A lingering challenge is the development of a cushioning system that best mimics and preserves native protein mobility in cell membranes. While some advances have been made, the objectives in designing model systems with embedded proteins, i.e. supported bilayers, to date has been to completely fluidize membrane proteins and minimize the influence of the support. But as observed in cells, continuous protein mobility is not the norm and membrane proteins certainly interact with cytoskeletal supports beneath the membrane. We believe that focusing on understanding the critical interactions of the cell membrane with the cytoskeleton and then recapitulating the essential cytoskeleton features when engineering supports and cushions to capture these native interactions will move the field towards a much

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improved membrane model and tool for understanding lipid-protein interactions.

Along this same theme, inclusion of glycocalyx elements in model membranes is often ignored. However, recent work has shown glycans interacting with membranes can influence membrane organization. In the future it will be essential to understand fundamentally the role of the glycocalyx on membrane organization and then to design platforms that also take care to include these elements into model membranes. We believe incorporating glycocalyx-mimicking polymers into next generation model membrane systems will be another useful design parameter to use to study lipid-protein interactions in these in vitro platforms and presents an opportunity for advancement of this field.

For our final thought, we believe that the biggest opportunity to improve model systems is to incorporate dynamic reorganization through triggered and reversible domain formation. While there have certainly been many studies using temperature to induce domain formation, far fewer examples exist of other stimuli, such as chemical or environmental. However by mimicking the non-equilibrium, dynamic conditions of the cell membrane, such an approach would allow scientists to gain insight on cause-effect relationships of protein regulation by lipids and other membrane-bound species, when combined with functional read-outs of protein function. In the state-of-the-art membrane platforms, such dynamic experiments are currently impossible to conduct.

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