

The challenge of lipid rafts

Linda J. Pike¹

Washington University School of Medicine Department of Biochemistry and Molecular Biophysics,
St. Louis, MO 63110

Abstract The Singer-Nicolson model of membranes postulated a uniform lipid bilayer randomly studded with floating proteins. However, it became clear almost immediately that membranes were not uniform and that clusters of lipids in a more ordered state existed within the generally disordered lipid milieu of the membrane. These clusters of ordered lipids are now referred to as lipid rafts. This review summarizes current thinking on the nature of lipid rafts focusing on the role of proteomics and lipidomics in understanding the structure of these domains. It also outlines the contribution of single-molecule methods in defining the forces that drive the formation and dynamics of these membrane domains.—Pike, L. J. The challenge of lipid rafts. *J. Lipid Res.* 2009. 50: S323–S328.

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A major step forward in our understanding of the structure of biological membranes was the publication by Singer and Nicolson (1) in 1972 of the fluid mosaic model of membranes. The model described the membrane as a primarily lipid matrix with randomly distributed proteins. The ink had barely dried on this landmark paper before experimental evidence was obtained that suggested that the uniformly random distribution of proteins and lipids envisioned by Singer and Nicolson was probably inaccurate. By 1974, studies on the effects of temperature on membrane behavior had led investigators to propose the presence of “clusters of lipids” in membranes (2), and by the following year data were obtained that suggested that these clusters might be “quasicrystalline” regions surrounded by more freely dispersed liquid crystalline lipid molecules (3). By 1978, this idea had been refined from “rigid liquid crystalline” clusters to “lipids in a more ordered state” (4).

The concept of lipid domains in membranes was formalized in 1982 by Karnovsky et al. (5), who observed heterogeneity in the lifetime decay of 1,6-diphenyl-1,3,5-hexatriene, indicating multiple phases in the lipid envi-

ronment of the membrane. These workers also investigated the functional effect of altering membrane structure by the addition of specific fatty acids, and presciently, by the depletion of cholesterol. They closed their manuscript with a series of questions that were raised by the “concept of the organization of the lipid components of membranes into domains.” Their questions are worth reiterating because almost 3 decades later they remain major challenges in the study of the structures that we have come to call lipid rafts. 1) Do specific membrane proteins reside in specific lipid domains, and can perturbation of the specific domain structure affect protein structure and function? 2) Do lipophilic molecules and drugs preferentially partition and segregate into specific domains rather than into a bulk lipid phase, and may such unique partitioning predicate specific functional effects? 3) What forces underlie the formation, maintenance, and fluctuation of lipid domains? 4) Because the very concept of domains implies domain boundaries or interfaces, what is the possible biological significance of such interfaces?

In this review, I will summarize recent findings on lipid rafts that outline the progress that has been made in addressing these questions, posed nearly 30 years ago. Emphasis will be placed on the application of new technologies to answer these old questions.

A DEFINITION OF LIPID RAFTS

Early descriptions of lipid rafts noted their enrichment in cholesterol and glycosphingolipids and focused on their ability to resist extraction by nonionic detergents (6). The initial vision of a lipid raft was therefore of a sizable structure, perhaps 100–500 nm in diameter, that was stable and held together by lipid-lipid interactions. Proteins could partition into these domains if they had the appropriate affinity for the unusual lipid composition. Experiments ensued and it has become clear that lipid rafts are not a single monolithic structure. They are a heterogeneous collection of domains that differ in protein and lipid composition as well as in temporal stability. A role for the protein com-

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¹To whom correspondence should be addressed.
e-mail: pike@biochem.wustl.edu

ponents of rafts in their organization has also become apparent. This new concept is embodied in the consensus definition of a lipid raft developed at the 2006 Keystone Symposium of Lipid Rafts and Cell Function: "Lipid rafts are small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions." (7). This definition is probably closer to what Karnovsky et al. (5) had in mind when they originally proposed the idea of lipid domains than what the concept of lipid rafts had become in the early 1990s.

DO SPECIFIC MEMBRANE PROTEINS RESIDE IN SPECIFIC LIPID DOMAINS?

The classic observation regarding the localization of specific proteins to lipid rafts was that of Brown and Rose (6) who reported that GPI-anchored proteins selectively partitioned into a Triton-insoluble membrane fraction that was enriched in cholesterol and glycosphingolipids. Subsequently, a plethora of proteins were reported to be recovered in detergent-resistant lipid rafts and their caveolin-containing cousins, caveolae (for review see Refs 8–10).

A large number of studies suggest that lipid modifications such as GPI anchors, palmitoylation, or myristoylation can target proteins to lipid rafts (11, 12). By contrast, proteins with transmembrane segments have been shown to be targeted to rafts by amino acid sequences in their extracellular (13), transmembrane (14), or intracellular domains (15). It has also been hypothesized that "lipid shells" surrounding transmembrane segments of proteins give them an enhanced affinity for cholesterol-enriched lipid rafts allowing them to preferentially partition into these domains (16). Unfortunately, little progress has been made in determining the nature of protein-based raft targeting sequences, so it is difficult to predict, on the basis of sequence, whether a protein is likely to be localized to lipid rafts.

In the absence of such information, broad-based proteomic strategies have been used to identify the protein composition of lipid rafts (i.e., the raft proteome). By one count, lipid rafts rank as the "most popular organelle for proteomic studies" (17). But several important caveats must be considered when interpreting the results of proteomic analyses of lipid rafts. First, the analysis is dependent on the starting material. To the extent that different preparations (i.e., detergent vs. nondetergent methods) will be contaminated with different proteins and membranes, the analyses will provide variable answers to the question of what is the raft proteome. Second, given the problems with isolating lipid rafts, additional evidence for raft association, such as cholesterol-dependence or response to a biological stimulus that involves rafts, is extremely helpful in confirming/interpreting the results of proteomic analyses of rafts. Finally, membrane proteins are notoriously difficult to isolate by some of the methods used for proteomic analysis (20, 21). Thus, the analyses may be skewed away from proteins with transmembrane domains and toward those that

are acylated or simply associated with intrinsic raft proteins. Therefore, absence of a particular protein from an analysis is not necessarily evidence of absence from rafts (or caveolae).

Proteomics analyses have been done on detergent-resistant membranes (18, 19, 23–26), nondetergent membranes (18, 22), and membranes from the cationic silica procedure for in situ isolation of luminal caveolae in endothelial cells (19). In general, it has been found that detergent-resistant membrane preparations provide a cleaner starting material for proteomic analysis than other methods, having a higher ratio of true positives to false positives with respect to raft proteins (18, 19). True positives are perhaps best defined as those proteins whose presence in rafts is dependent on cholesterol (18). However, showing a significant change in the level of a protein in the raft preparation following treatment of cells with a physiological stimulus (24–26) is an alternative that allows selective identification of raft proteins related to a specific biological process.

Despite the differences in approach, there is significant overlap in the proteins identified in the various raft preparations. Lipid rafts have for a long time been associated with cell signaling (9, 10). Thus, it was not surprising to find signaling proteins present in the raft proteome. Included among raft proteins were low molecular weight and heterotrimeric G proteins, nonreceptor tyrosine kinases, and protein phosphatases (18, 19, 22–25). The absence of G protein-coupled receptors as well as tyrosine kinases from these analyses may reflect their low abundance levels as well as their high hydrophobicity that, as noted above, makes their recovery difficult.

Like signaling proteins, cytoskeletal and adhesion proteins are routinely identified in lipid raft preparations. Included in this group of proteins are actin, myosin, vinculin, cofilin, cadherin, filamin, and ezrin (18, 19, 22, 24–26). The presence of cytoskeletal proteins in the raft proteome is not an indication that these are integral raft proteins but rather that rafts interact with the cytoskeleton, and therefore, when isolated, the rafts retain some of their associated cytoskeletal proteins. In this regard, the findings with respect to ezrin are instructive. The association of ezrin with lipid rafts was significantly decreased after engagement of the B cell receptor and this was associated with the ability of lipid rafts to coalesce into a larger signaling platform (25). The data suggest that in B cells, lipid rafts are held apart by the cortical actin cytoskeleton and that ezrin releases rafts from these constraints allowing their aggregation into larger, more stable structures. Thus, proteomics in combination with molecular biology can provide insight into raft mechanics.

GPI-anchored proteins were the original proteins identified as selectively partitioning into detergent-resistant membrane domains based on Western blotting strategies (6). This observation has been confirmed in numerous proteomics analyses in which proteins such as 5'-nucleotidase, Thy-1, DAF, and CD59 (18, 19, 26) have been identified. Similarly, caveolin and flotillin, that were initially reported to be in detergent-resistant membranes, were also identified in proteomics analyses (18, 19, 23, 25). The consistent

identification of caveolin, flotillin, and GPI-anchored proteins in proteomics analyses from lipid rafts prepared by a wide variety of methods suggests that these are true resident raft proteins and hence valid markers for these domains.

Several proteomic analyses identified a large number of ER and mitochondrial proteins in rafts (19, 22–24). These include ATP synthase, prohibitin, VDAC 1 and 2, isocitrate dehydrogenase and calreticulin. Based on these findings, it was proposed that mitochondria contain rafts (24) or that caveolae and the endoplasmic reticulum (ER) interact with each other (22). The association of many of these proteins with detergent-resistant membrane fractions was shown not to be cholesterol-dependent calling into question the legitimacy of their designation as raft proteins. It seems most likely that contamination of the membrane preparations with ER and mitochondrial membranes accounts for the presence of many of these proteins in raft proteomes, though the existence of raft-like domains in mitochondria cannot be excluded (27).

In summary, proteomics analyses have provided confirmation of the raft localization of many proteins previously shown to partition into lipid rafts using other methods. These studies have also identified novel proteins in rafts and led to insights into the physiological regulation of rafts. However, the identification of proteins from mitochondria and the ER, two membranes known to be low in cholesterol, suggests that such unexpected results from proteomics analyses must be viewed with caution unless parallel studies are undertaken to validate the localization of the identified proteins.

DO LIPOPHILIC MOLECULES PREFERENTIALLY PARTITION AND SEGREGATE INTO SPECIFIC DOMAINS?

The distinctive lipid composition of membrane rafts, namely high levels of cholesterol and sphingolipids, was noted early in the study of membrane domains (6, 28). Recent advances in the analysis of lipids by mass spectrometry inaugurated the field of lipidomics and have yielded a clearer picture of the lipid composition of membrane rafts.

Cholesterol levels in rafts are generally double those found in the plasma membranes from which they were derived (29). Likewise, sphingomyelin levels are elevated by approximately 50% compared with plasma membranes (29, 30). The elevated sphingomyelin levels are offset by decreased levels of phosphatidylcholine (29, 30) so the total amount of choline-containing lipids is similar in rafts and plasma membranes.

Most schematic diagrams of lipid rafts show domains in which the component glycerophospholipids contain two saturated acyl chains. This view derives from observations that the lipids in rafts tend to be in a less fluid state than the surrounding membrane. This has been attributed to the tight packing of saturated acyl chains of the phospholipids in rafts (31). However, in many cells, the total amount of phospholipid harboring two saturated fatty acyl groups is generally <10 mol% (29, 30, 32). As rafts may repre-

sent as much as 30% of the plasma membrane surface (33), there simply is not enough disaturated phospholipid available to form the requisite number of rafts. Instead, lipidomics studies have shown that the bulk of the glycerophospholipids present in membrane rafts contain at least one monounsaturated acyl chain (29, 30, 32). Thus, the concept of rafts as domains that contain phospholipids with fully saturated acyl chains needs to be revisited.

Lipidomic analyses of membrane rafts have provided several other unexpected findings. First, phosphatidylserine levels are elevated 2- to 3-fold in rafts as compared with plasma membranes (29, 32). This suggests that rafts may be a source for the rapid externalization of phosphatidylserine during apoptosis or platelet activation. Second, rafts are enriched in ethanolamine plasmalogens, particularly those containing arachidonic acid (29, 32). Plasmalogens can function as antioxidants and the presence of these compounds in rafts may serve to detoxify molecules that are internalized via lipid rafts or caveolae. It is also possible that rafts serve as an enriched source of arachidonic acid-containing phospholipids for hydrolysis by phospholipase A2 enzymes.

As with proteomic studies of lipid rafts, lipidomic studies of these domains have been done using rafts prepared by both detergent-free and detergent-containing protocols. When direct comparisons of the various preparations have been done, significant differences in lipid composition have been identified (32). Furthermore, comparison of the lipid composition of rafts generated by extraction with different detergents showed substantial differences in the enrichment of cholesterol and sphingolipids in the resulting membrane fractions (34). Thus, caution is warranted when assessing the results of individual raft lipidomics studies.

It could be argued that the simple act of isolating lipid rafts by whatever method introduces artifacts into the system and that the results therefore do not provide an accurate picture of the composition of lipid rafts *in vivo*. This view is challenged by the findings of Brugger et al. (35) who reported the HIV lipidome. The HIV virus is an enveloped retrovirus that buds from the membrane of infected cells. Based on the presence of raft marker proteins in the envelope of HIV, it has been proposed that budding occurs from lipid rafts (36). Brugger et al. (35) isolated budded HIV virus and demonstrated that it was enriched in cholesterol, sphingolipids, phosphatidylserine, and plasmalogen ethanolamine. Thus, the HIV membrane exhibited characteristics similar to those of lipid rafts isolated from the cells from which it budded. The fact that this lipid composition was present in the isolated virus suggests that a membrane domain of this distinct composition must have existed in the cells at the location from which the virus budded. This provides strong evidence for the existence of membrane rafts in intact cells.

Most lipidomic studies of rafts have been done on the total raft population, which as noted above is known to be heterogeneous. Using immunoaffinity purification, Brugger, Graham, and Leibrecht (37) isolated rafts enriched in the GPI-anchored prion protein or the GPI-anchored Thy-1 protein. Their analyses demonstrated significant differences in

the levels of cholesterol, phosphatidylcholine, hexosylceramide, and N-stearoylceramide between Thy-1-containing rafts and PrP-containing rafts. These findings confirm the view that rafts are heterogeneous in protein and lipid composition and also suggest that rafts retain at least some of their biological differences after isolation.

WHAT FORCES UNDERLIE THE FORMATION, MAINTENANCE, AND FLUCTUATION OF LIPID DOMAINS?

Lipid rafts were so named because it was originally thought that they represented pre-existing domains in membranes into which different proteins partitioned. In this view, rafts represented small areas of phase separation in biological membranes. Phase separation in model membrane systems has been well-studied. However, as has been pointed out by Mayor and Rao (38), biological membranes are held in a state far from equilibrium. Therefore, extrapolation of results from model membrane systems to cell membranes is fraught with difficulties. Nonetheless, if due caution is exercised, information can be gained from such studies that provides insight into the formation and maintenance of lipid rafts. Furthermore, recent studies using cell-derived vesicle systems suggest that these compositionally complex membranes behave similarly to model membranes.

From studies in model membranes, it appears that the key (though not only) driving force in domain formation is line tension (39). Line tension refers to the energy required to create the boundary between a domain (referred to hereafter as a raft) and the surrounding membrane. In practice, rafts are thicker than the surrounding membrane, and this hydrophobic mismatch contributes to the energy required to maintain rafts as a separate phase. Studies have shown that the greater the difference in thickness between the two phases, the higher the line tension and this is associated, in turn, with the formation of larger rafts (40). Deformation of the lipids at the boundary of rafts and the surround, including changes in tilt and splay, help to reduce the line tension (39). The presence of a wide variety of lipid species with different chain lengths and degrees of saturation *in vivo* probably makes it easier to compensate for differences in membrane thickness and serves to limit raft size in cells.

Spontaneous curvature of the membrane can also reduce line tension (41). If the hydrophobic mismatch between phases is sufficient, budding from lipid vesicles can occur (40). This observation is of particular interest in light of the findings of Le et al. (42) who reported that lipid rafts containing the autocrine motility factor receptor rapidly bud and detach from the plasma membrane. Caveolin-1 stabilized the invaginated form of these rafts, reducing the rate of endocytosis of the receptor. These data suggest that hydrophobic mismatch in rafts and the negative curvature that it promotes may be important contributors to the physiological function of these domains.

Line tension can also be minimized by fusing small rafts into larger rafts. However, this tendency is balanced by the

decrease in entropy resulting from the generation of fewer, larger domains. The tendency of phases to separate into large domains has long been noted in model membrane systems. However, given the difference in complexity between the ternary lipid mixtures used in model systems and the collection of lipids and proteins present in cell membranes, the applicability of results from model membranes to the physiological situation has been challenged.

Recently, however, several groups have used giant vesicles blebbed from cells to study fluid phase separation of proteins and lipids. Baumgart, Hammond, and Sengupta (43) used giant plasma membrane vesicles derived from mast cells and fibroblasts to show that these cell-based membranes underwent phase separation at temperatures between 10°C and 25°C. They also showed preferential partitioning of different proteins into the liquid-ordered or liquid-disordered phases that were consistent with previous reports on the raft (or nonraft) localization of these proteins. Using plasma membrane spheres from A431 cells, Lingwood et al. (44) showed that cholera toxin-mediated cross-linking of the raft lipid, GM1, resulted in the coalescence of small rafts into micrometer-sized domains and the reorganization of known raft proteins into the GM1 phase. These experiments were carried out at 37°C demonstrating that phase separation can occur in biological membranes at physiological temperature. Ayuyan and Cohen (45) showed that increased lateral tension generated by osmotic swelling of intact cells at 37°C induced the formation of GM1-enriched domains that could be labeled with fluorescent cholera toxin. And Hofman et al. (46) showed that epidermal growth factor induced the merger of two types of GM1-containing microdomains. Together, these studies demonstrate that raft coalescence (and hence phase separation) can occur in complex biological membranes at physiological temperatures. More importantly, they show that the fusion of nanoscale domains into larger, phase-separated structures can be induced by physiologically relevant stimuli.

It has been hypothesized that cell membranes stay close to the transition temperature for phase separation to allow better control of raft dynamics (40). However, thermal regulation of raft coalescence would not be conducive to the selective control of domain size for specific cellular functions (38). Thus, in cell membranes, which are dynamic systems not in thermodynamic equilibrium, the underlying propensity of the lipids to phase separate is likely modulated by the presence of proteins and their state of aggregation as well as the continuous trafficking of lipids to and from the plasma membrane.

WHAT IS THE BIOLOGICAL SIGNIFICANCE OF LIPID DOMAIN BOUNDARIES OR INTERFACES?

The interface of a lipid domain can be defined as the region in which line tension occurs as a result of hydrophobic mismatch between the lipids in the raft and those in the surrounding membrane. As noted above, a variety of mechanisms are used to reduce line tension including deformation of lipids, induction of membrane curvature,


and fusion of small domains into larger ones. Not included in this list is the possibility of using proteins to reduce line tension. However, data from model membrane systems suggest that proteins can contribute to a reduction in line tension.

Using confocal and atomic force microscopy on supported bilayers of phosphatidylcholine/sphingomyelin/cholesterol, Garcia-Saez et al. (47) showed that a liquid-ordered phase rich in cholesterol and sphingomyelin and a liquid-disordered phase rich in phosphatidylcholine coexisted. Addition of a peptide derived from the pore-forming protein, Bax, led to morphological changes in the liquid-ordered domains, which became irregular in shape and larger. These data are consistent with the interpretation that the peptide reduced the line tension at the phase boundary.

Nicolini et al. (48) examined the effect of the addition of hexadecylated, farnesylated N-ras on domain formation in giant unilamellar vesicles made from a mixture of phosphatidylcholine, sphingomyelin, and cholesterol. These workers noted no change in the shape of the raft-like domains following addition of N-Ras but atomic force microscopy showed that a large fraction of the N-Ras that inserted into the vesicles did so at the boundary between the liquid-ordered and liquid-disordered phases. These findings suggest that the raft/membrane interface may represent a unique environment in which a subset of proteins becomes locally concentrated. This could serve to enhance biological processes, such as cell signaling, by increasing the likelihood of specific protein-protein interactions. Almost certainly, the inclusion of proteins at the domain boundary would alter line tension, changing the propensity of the domain to bud or fuse. Thus, signals that altered the localization of interfacial proteins might secondarily induce changes in domain size or curvature.

CONCLUSIONS

The introduction of “-omics” and single molecule methods has permitted an analysis of lipid domains at a scale unimaginable 30 years ago. While significant progress has been made in defining the constituents of rafts, both protein and lipid, much work remains to be done to understand how these molecules work together to generate and maintain lipid domains. Work in model membrane systems has provided a theoretical framework for predicting the domain-forming behavior of mixtures of lipids. However, even when applied to cell-derived vesicles, these approaches do not replicate the conditions present in intact cells. Our challenge for the future is to address the issue of how membrane dynamics modifies the processes that to date have been largely studied under equilibrium conditions. In view of the progress made not only in raft biology, but in cell biology in general, the original questions posed by Karnofsky et al. (5) might be updated to: 1) How do changes in membrane protein levels in response to environmental signals affect the composition and behavior of lipid rafts? 2) What is the physiological function of lipid rafts? 3) How does the continuous flux of membrane lipids into and out of the

plasma membrane affect domain formation, and can dietary or drug-induced changes in membrane lipid composition alter the function of lipid domains? 4) What kinds of proteins are found at domain boundaries, and do changes in their localization or level alter the physicochemical characteristics of the lipid domains? Maybe the *Journal of Lipid Research* will revisit these questions on their 75th anniversary. 

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