PERSPECTIVE

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Lipid rafts: now you see them, now you don't

Andrey S Shaw

The 'lipid raft' hypothesis has been a contentious topic over the past 5 years, with much of the immunology community divided into 'believers' and 'nonbelievers'. The disagreement is due mainly to the inability to observe these membrane domains directly and to the widespread use of experimental approaches of dubious utility. As a lipid raft 'dilettante' who has dabbled in the area over the years, I view the lipid raft model with some skepticism and disinterest because of that confusion. Although progress in the field has helped clarify some of the issues, more work is still needed to formally confirm the lipid raft hypothesis and to reestablish the scientific credibility of this area.

It has been known for decades that certain membrane proteins are poorly solubilized in common nonionic detergents such as Triton X-100 (ref. 1). Insolubility in nonionic detergents was once widely accepted as indicating that a molecule is associated with the cytoskeleton[2]. However, the realization that some such proteins are attached to the membrane by a glycosylphosphatidylinositol (GPI) lipid 'anchor'[3] led to the hypothesis that the different affinities of membrane proteins for specific lipids might be an important organizational principle in membranes[4]. That idea was advanced by the demonstration that GPI-anchored proteins are sorted to the apical surface of polarized epithelial cells and are associated with detergent-insoluble lipids that float on sucrose gradients[5,6]. It was then shown that those insoluble lipids are enriched not only in GPI-anchored membrane proteins but also in lipids such as cholesterol and sphingomyelin[7]. The function of lipid subdomains in the organization of proteins in the membrane was then transformed into the 'lipid raft' hypothesis[8]. The model proposed that the plasma membrane is organized into lipid raft and non–lipid raft domains and that this segregation of lipids is important for the organization of membranes for critical functions such as vesicular trafficking and signaling.

The raft idea

In its best formulated state, the lipid raft hypothesis proposed that different lipids found in plasma membranes would have different biophysical propensities to associate with each other. That 'phase separation' of lipids would result in the formation of distinct and stable domains by membranes. Underpinning the model is the propensity, *in vitro*, for cholesterol and saturated phospholipids such as sphingomyelin to associate together, away from the more abundant unsaturated phospholipids[9,10]. Because cholesterol is a relatively rigid molecule, it was postulated that those cholesterol-rich domains have a more ordered structure and therefore a slower mobility in the membrane than the disordered phospholipids surrounding them. The model was supported by experiments using model membranes, which confirmed that mixtures of sphingolipids, cholesterol and unsaturated phospholipids 'phase separate' into macroscopic domains of different lipid compositions[10]. Those *in vitro* data were exciting because they confirmed an important tenet of the lipid raft hypothesis, that mixtures of different lipids can drive the segregation of membranes into distinct subdomains.

An important feature of the model was that proteins have different affinities for those different domains, with some proteins 'preferring' lipid rafts and others 'preferring' more disordered lipids. Why some molecules might 'prefer' one domain rather than another is not completely apparent, but lipid modifications have been shown to be important in enhancing the association of specific proteins with the 'raft' fraction[11]. Originally, that separation of proteins into different lipid domains was postulated to be important in the sorting and trafficking of proteins to distinct cellular regions (explaining, for example, why GPI-anchored proteins are apically sorted). However, the finding that many signaling molecules are also found in rafts sparked the generation of models in which lipid rafts were proposed to organize signaling proteins. The likelihood of those models served to recruit many investigators into the area.

Immunological exuberance

The interest of immunologists in lipid rafts began with studies aimed at elucidating how crosslinking of GPI-anchored proteins such as Thy-1 could activate immune cells. As the GPI anchor does not extend through the lipid bilayer, it was inexplicable how and why GPI-anchored proteins could stimulate intracellular signaling. Immunologists were brought into the field with the discovery that immunoprecipitates of GPI-anchored proteins precipitate together with the Src family tyrosine kinase Lck[12]. Initially, that interaction was thought to be mediated by an intermediary protein, but the consensus now is that coprecipitation is due to the presence of both GPI-anchored proteins and certain Src kinases in a detergent-insoluble compartment. The simplicity of that method (detergent insolubility) led to an explosion of papers in which immunologists used those assays to argue that many T cell signaling proteins, including Lck, Fyn and Lat, localize in the lipid raft compartment[11,13]. More interesting was the finding that after crosslinking, some T cell surface proteins, including the T cell receptor, become detergent insoluble[14,15]. Because important phosphatases such as CD45, SHP-1 and SHP-2 are generally detergent soluble, it was proposed that lipid rafts promote signaling by separating positive signaling molecules (kinases) from negative signaling molecules (phosphatases) in the plasma membrane. An especially provocative finding was the report

Andrey S. Shaw is in the Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, Missouri 63110, USA.
e-mail: ashaw@wustl.edu
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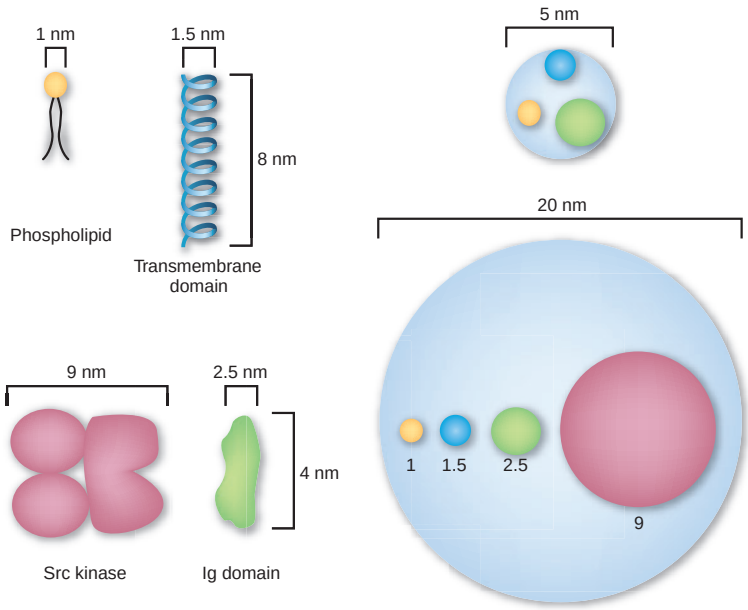

Figure 1 How big is a lipid raft relative to the molecules it comprises? These are the dimensions of typical lipid raft constituents, including a phospholipid, a Src kinase, a typical transmembrane domain and an immunoglobulin (Ig) domain (as they are the most common type of ectodomain). The two blue circles (5 nm and 20 nm in diameter) indicate the lower and upper limits of the range of estimated sizes of lipid rafts. Although the protein constituents are wholly contained in the lipid raft here, it is possible that they are instead associated with the boundaries of the lipid raft. That would allow the association of many more molecules with the lipid raft.

that the recruitment of rafts to the immunological synapse is considerably enhanced by signaling of the costimulatory molecule CD28, which is indispensable for T cell activation[16]. That observation suggested that lipid raft recruitment might be an important component of membrane signaling events and led to the widely accepted (but still controversial) idea that the immunological synapse is a macroscopic coalescence of lipid rafts[17].

Problems

Early on, problems in this field gave birth to a community of lipid raft skeptics. A chief problem was (and still is) the definition of a lipid raft. What is exactly a lipid raft? Although the definition is physical (a membrane domain) and is imbued with specific functions (signaling and intracellular trafficking), the working definition is biochemical. Proteins are deemed to be localized to lipid rafts if they are insoluble in certain detergents and float in various gradients. As the formation of rafts requires the presence of cholesterol, sensitivity to cholesterol chelation with drugs such as β-methyl-cyclodextrin is also widely used as a criterion for lipid raft localization. For example, if the insolubility of a protein changes after treatment with β-methyl-cyclodextrin, that observation is used to support the argument that the protein is in a lipid raft. However, that working definition is still very vague and depends on somewhat dubious methods. Over time, the definition of the raft continued to change as people began to use different detergents and to alter the duration of incubation or the temperature. The results of those experiments suggested that more and more proteins should be considered resident in 'lipid rafts'. An important issue was that no rules were set regarding what proportion of a protein needed to be detergent insoluble for the protein to be labeled as being in lipid rafts. Examples in the literature range from less than 10% to over 50%.

That changing and loose definition of what constitutes lipid raft localization only served to confirm the suspicion of skeptics that almost anything could be shown to be in a lipid raft. More troubling was experimental work suggesting that treatment with detergent might actually induce artifactual changes in protein solubility[18]. It has also become apparent that drugs such as β-methyl-cyclodextrin are potentially toxic and have a range of effects not directly related to cholesterol chelation[19].

Finally, quantitative studies of cholesterol and sphingolipid compartments have shown that the 'raft' component of the membrane might not constitute a 'special' or 'rare' fraction of the plasma membrane but could instead potentially comprise up to 30–50% of the plasma membrane[20]. If the 'raft' compartment constitutes 50% of the plasma membrane, then which is the 'specialized' lipid compartment of the plasma membrane—the raft fraction or the non-raft fraction? Notably, the size of the raft compartment also limits its ability to concentrate signaling proteins, which is a key idea in the model of raft preassembly of signaling components. For example, if the proportion of lipid rafts in the plasma membrane is 50%, and all signaling proteins are found in lipid rafts, the maximum concentration effect achieved can be only about twofold. Even twofold is probably an overestimation, as most raft proteins are only about 50% detergent insoluble. Conversely, using a similar logic, because all of the proteins in the non-raft compartment are by definition completely soluble in Triton X-100, partitioning in the non-raft compartment may actually be a more effective means of concentration.

Better proof for the existence of rafts would require direct visualization of rafts in the cell membrane. Because the phase separation of lipids is easily visualized using model membranes[21], it was initially unexpected that no raft subdomains could be seen by examination of biological membranes[22]. That suggested either that lipid rafts do not exist or that they are of a size below the resolution of the light microscope (about 300 nm). Approaches with higher resolution, such as electron microscopy, have been tried, but they are limited by issues regarding fixation and tissue preparation and the lack of an obvious morphological structure that would define a raft domain. More sophisticated biophysical approaches such as fluorescence recovery after photobleaching (FRAP) and fluorescence resonance energy transfer initially led to mixed results, with only some data supporting the idea of the existence of membrane heterogeneity[23,24].

The present model

The original lipid raft idea envisioned large (micron-sized) stable raft domains that should have been large enough to be visible by light microscopy. However, investigators failed in their attempts to image raft domains in biological membranes, leading to a reevaluation of the original raft idea. That reevaluation has led to the present idea that 'rafts' are extremely small and highly dynamic and involve interactions of both proteins and lipids[25].

How small are the rafts? Measurements using fluorescence resonance energy transfer (which can detect protein interactions less than 30Å apart) with labeled GPI-anchored proteins suggest that about 40% of

GPI-anchored proteins are localized in small clusters (three to four molecules), with the rest existing as monomers 'floating' in the membrane[26,27]. Clusters that small are near the limits of detection and could easily be interpreted as 'stochastic noise'. Mathematical modeling has therefore been used to evaluate both fluorescence resonance energy transfer data and electron microscopy images[26,28]. The modeling studies are consistent with the idea that if rafts do exist, they are between 5 nm and 20 nm in diameter. As an example of how small this is, a fragment of membrane 5 nm in diameter would contain only about 15–20 lipid molecules and would contain at most only 3–5 membrane proteins (**Fig. 1**). However, just because a structure cannot be easily visualized does not mean that it does not influence cellular functions.

How dynamic are the rafts? Data suggest that lipid rafts have half-lives in the range of 100 nanoseconds or less. Electron paramagnetic resonance studies have been used to determine the half-life of lipids in rafts, and single-molecule tracking methods have been used to measure the interactions of proteins with raft domains[29–31]. Both measurements have provided similar results, with half-lives in the 100 nanosecond range. That time scale is faster than the rate of many enzymes and also much shorter than the half-lives of many of the protein-protein interactions underlying signal transduction (which are on the scale of seconds). For example, the turnover rate of the kinase activity of Lck is estimated to be 0.02–2 molecules per second (refs. 32,33). The half-life of the interaction between Zap70 and phosphorylated T cell receptor ζ-chain is about 8 seconds (ref. 34).

Several observations probably explain the very short half-lives of lipid rafts and their components. First, the propensity of different lipids to associate is not so strong as to be entropically stable; thus, mixtures of cholesterol, sphingomyelin and unsaturated phospholipids at physiological temperatures will have a tendency to dissipate and reform[35]. Similarly, proteins associated with those lipid domains can potentially act as detergents to decrease the 'line tension' at boundaries between the different lipid domains, effectively decreasing the stability of those domains[25]. Finally, cell biological processes such as endocytosis and vesicular trafficking may function to keep the membrane 'well mixed', acting to prevent any large-scale coalescence of raft membranes.

Because the dynamic nature of lipid rafts is influenced by proteins contained in them, one implication of that model is that perturbations of membrane proteins that might occur during cell-cell contact or receptor engagement may modulate the size and stability of rafts. Such regulation might allow the formation of larger raft structures with longer half-lives. In fact, a new biophysical approach has suggested that large, ordered lipid domains may be present in the T cell immunological synapse[36].

Although the lipid raft hypothesis proposes that the recruitment and aggregation of lipid rafts is a driving force in organizing signaling in T cells and other immune cells, data have suggested that protein-protein interactions, not lipid raft recruitment, are the main driving force for the assembly of signaling complexes in immune cells. In both T cells and mast cells, it has been shown that a 'generic' GPI-anchored molecule is not 'preferentially' recruited to signaling complexes[37,38]. That suggests that lipid raft recruitment (which should have included the GPI-anchored molecules) does not serve to enrich for proteins enriched in lipid rafts. Instead, the recruitment of signaling molecules requires the interaction of a protein-interaction domain with the signaling complex. For T cells, the recruitment of lipid rafts to the immunological synapse seems to be passive and related to protein engagement in the synapse and is not due to an active process of raft recruitment[39]. Therefore, in the present model, proteins are recruited first to a specific location in the membrane, and lipids are recruited because of their association with that protein. However, it is also possible that because other lipid and protein molecules are associated with the raft, raft recruitment helps to recruit additional proteins and lipids to the signaling complex.

Questions for the future

The lipid raft hypothesis has been a compelling idea since its inception almost 10 years ago. However, the field continues to be plagued by the weak definition of what consitutes a raft. A consensus report has defined rafts as small, dynamic and cholesterol dependent and, in a bow to data on the function of proteins in raft formation, has suggested that they now be referred to as 'membrane rafts' rather than 'lipid rafts'[40]. Still, that definition is conceptual and lends no guidance as to a practical definition. Although it is apparent now that criteria commonly used to define rafts, such as detergent solubility, are potentially misleading, it is still not apparent how newer definitions clarify how rafts will be defined. There is no question that sophisticated biophysical approaches combined with mathematical modeling have helped to regenerate this field and have led to the generation of a new modern model. However, the new model is still problematic, as it envisions rafts to be almost invisible entities with sizes at the limits of detection and with half-lives that are 'blazingly' short.

Whether domains this small and this transient are of biological utility remains to be determined. There is much discussion about raft heterogeneity, but there is little solid evidence that different types of raft exist. Subsets of raft domains, however, would explain how high concentrations of signaling proteins could be achieved. Another important not-yet-understood area is how the inner leaflet of lipid bilayers is organized and how it is coupled to the outer leaflet. Although undoubtedly the idea of lipid rafts will continue to capture the imagination of those interested in the function of membrane organization on signaling and vesicular trafficking, prudence dictates that efforts be made to sort out exactly what lipid rafts are and how they will be defined.

COMPETING INTERESTS STATEMENT
The author declares that he has no competing financial interests.

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