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Lipid rafts: contentious only from simplistic standpoints

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Abstract

The hypothesis that lipid rafts exist in plasma membranes and have crucial biological functions remains controversial. The lateral heterogeneity of proteins in the plasma membrane is undisputed, but the contribution of cholesterol-dependent lipid assemblies to this complex, non-random organization promotes vigorous debate. In the light of recent studies with model membranes, computational modelling and innovative cell biology, I propose an updated model of lipid rafts that readily accommodates diverse views on plasma-membrane micro-organization.

A widely accepted hypothesis in contemporary cell biology is that freely diffusing, stable, lateral assemblies of sphingolipids and cholesterol, which are termed lipid rafts[1-3], constitute an important organizing principle for the plasma membrane. The basic concept is that lipid rafts can facilitate selective protein–protein interactions by selectively excluding or including proteins. This lipid-based sorting mechanism has been widely implicated in the assembly of transient signalling platforms and more permanent structures such as the immunological synapse, as well as in the sorting of proteins for entry into specific exocytic and endocytic trafficking pathways[1-3]. Despite the undoubted theoretical utility of lipid rafts to many cell biological processes, the basic hypothesis that stable lipid rafts exist at all in biological membranes is under intense scrutiny[4,5]. This is partly because lipid rafts, if they exist in resting cell membranes, are too small to be resolved by fluorescent microscopy and have no defined ultrastructure; therefore, proving their existence is problematic.

This article will consider the biophysical properties of model membranes that underpin the lipid raft hypothesis, and the limitations of the biochemical approaches that have been used to study rafts in biological membranes that largely account for the current debate on the hypothesis mentioned above. After examining the challenges that are involved in correlating observations, which have been made in model and cellular membranes, I focus on synthesizing recent data on the size of lipid domains in model membranes with observations that have been obtained by imaging intact plasma membranes. These imaging approaches include single particle tracking (SPT), single fluorophore video tracking (SFVT), fluorescence resonance energy transfer (FRET), homo-FRET and electron microscopy (EM). On the one hand, these studies challenge the simplistic null hypothesis that lipid-based assemblies, such as lipid rafts, do not exist in biological membranes. On the other hand, it is timely to reconsider the raft hypothesis in the light of these new data because the consensus model that emerges is more complex than a simplistic notion of stable, freely diffusing lipid rafts. Some intriguing new questions about

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DATABASES

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the structure and function of lipid rafts in the plasma membrane, which are posed by this revised raft model, will be discussed.

Biophysics and model membranes

Although the plasma membrane is a complex organelle, its basic structure consists of a phospholipid bilayer. Some insights into the behaviour of this basic structure might then be anticipated by analysing model membranes that consist of simple, hydrated phospholipid bilayers. An important property of such a model membrane is revealed as the temperature of the bilayer is progressively increased. At a temperature that is characteristic of the particular lipid species, the phospholipids undergo a phase transition from a solid ordered, or gel, phase (S_o) to a liquid disordered phase (L_d). The lateral mobility of the lipids, which is highly restricted in the S_o phase, increases, and the acyl-side chains become disordered and no longer pack together tightly in rigid straight conformations. If the membrane also contains sufficient cholesterol, a third phase (liquid ordered (L_o)) is possible. The L_o phase is characterized by a high degree of acyl-chain ordering, which is typical of the S_o phase, but with the translational disorder (or increased lateral mobility) that is characteristic of the L_d phase, such that lipid diffusion in the L_o phase is only two- to three-fold slower than in the L_d phase. Precisely how cholesterol drives the formation of the L_o phase remains unclear (BOX 1). Importantly, in membranes that comprise appropriate mixtures of sphingomyelin, unsaturated phospholipids and cholesterol, L_o and L_d phases can co-exist[6,7] (FIG. 1).

Box 1 How does cholesterol drive domain formation in membranes?

The presence of cholesterol and saturated phospholipids, such as sphingomyelin, is crucial to observe phase co-existence. However, what does the cholesterol actually do, and what is the structure and size of the two co-existing phases? Cholesterol has a condensing effect on phospholipids. Therefore, a mixture of cholesterol and phospholipid occupies a smaller area than expected from the sum of the constituents. One explanation is that cholesterol forms reversible, condensed complexes of defined stoichiometry with phospholipids[50-51]. Another possibility is that the head groups of the phospholipids shield hydrophobic cholesterol from contact with the membrane–water interface and therefore function as umbrellas[52]. In performing this umbrella function, the side chains of the phospholipids need to become more ordered to allow closer packing and crowding of the lipid head groups. A third and particularly interesting possibility is shown by computational modelling of the molecular interactions in a ternary mixture of cholesterol, 1,2-dioleoylphosphatidylcholine (DOPC) and sphingomyelin[53-54]. As the simulations evolve, cholesterol preferentially localizes at the interface between sphingomyelin-enriched and DOPC-enriched regions, with the saturated sphingomyelin acyl chains packing against the smooth α-face of cholesterol, and the disordered acyl chains of DOPC packing more easily against the opposite β-face, which is rougher because of protruding methyl groups[53]. The intriguing aspect of these simulations is the potential insight that can be given into the very early molecular interactions that lead to domains. Although the simulations were time-limited, formation of small nanoscale domains was observed. Moreover, the domains remained small and were not increasing in size at the end of the simulation (currently to 200 ns)[53]. These results show that the domains that form spontaneously are small and curvilinear, and that cholesterol might have an important role in reducing the line tension between liquid ordered (L_o) and liquid disordered (L_d) domains[53].

Lipid rafts in biological membranes are postulated to be microdomains with a lipid structure that is equivalent to the L_o phase of model membranes, and to be surrounded by a contiguous ‘sea’ of membrane with a lipid structure that is equivalent to the L_d phase of model membranes. The abbreviated term ‘L_o domain’ captures these concepts and will be used hereafter in this

article. The crucial role of cholesterol in allowing L_o phase formation therefore provides the theoretical basis for cholesterol-depletion experiments that are extensively used to disrupt the L_o structure of lipid rafts in living cells. However, it is important to realize that drugs such as β-methyl-cyclodextrin that are used to extract cholesterol from the plasma membrane also have other effects (BOX 2). Furthermore, a comment is needed here on another biochemical technique that is widely used to study lipid rafts. Detergent-resistant membrane (DRM) fractions that are prepared from model membranes and cell membranes are enriched in cholesterol and sphingomyelin, and contain a subset of lipid-anchored and integral plasma-membrane proteins[8-10]. These observations, among other things, have led to the assumption that L_o domains, lipid rafts and DRMs can be considered as synonymous terms (BOX 2). This assumption has led to an ever-increasing number of proteins being assigned to lipid rafts based solely on their DRM association, and has prompted a series of valid, crucial questions to be asked of the lipid-raft hypothesis[4,5] (BOX 2).

Box 2 Limitations of biochemical approaches to study lipid rafts in biological membranes

A detergent-resistant membrane (DRM) fraction is prepared by solubilizing cell membranes in Triton-X100 at 4°C and purifying an insoluble fraction by flotation on a density gradient. A DRM fraction is enriched in cholesterol and sphingomyelin and contains >240 proteins, including glycosylphosphatidylinositol (GPI)-anchored proteins, caveolin and a subset of transmembrane proteins[8-10,55]. The interpretation of DRM experiments is predicated on the assumption that liquid ordered (L_o) domains that exist in intact membranes at 37°C and their associated proteins are faithfully purified by cold-detergent extraction[8-10]. It is clear, however, that the association of a protein with a DRM fraction should not be considered sole evidence that a protein is associated with L_o domains in intact cells. The effects of detergents on membranes are much too complex to draw substantive conclusions about the nanoscale localization of a given protein on native membrane[56]. For example, detergents can create domains, cause mixing of domains and solubilize proteins and lipids irrespective of their intrinsic affinity for L_o domains[56-58]. As one of many examples, consider that 30% of GPI–PLAP (placental alkaline phosphatase) partitions into the L_o phase of a unilamellar model membrane as detected by direct imaging of intact vesicles, whereas >95% is recovered in a DRM fraction prepared from the same vesicles[20]. This topic has been the subject of an excellent recent review[56] and will not be considered further. Precisely what association with a DRM fraction might mean, and whether it is a useful parameter in an experimental context for any given protein in which mutations or activation status alter the extent of DRM association, is beyond the scope of this article, but has been considered elsewhere[23-56]. The bottom line is that there is no substitute for using an imaging technique with nanoscale resolution to visualize intact plasma membrane to provide compelling evidence for non-random protein clustering that is consistent with raft localization.

Cholesterol depletion using agents such as β-methyl-cyclodextrin has been used extensively in model systems. When applied to cells, these cholesterol-binding drugs have effects other than their ability to extract cholesterol from membranes and disrupt L_o domains. Perturbation of the actin cytoskeleton[59] and inhibition of clathrin-mediated endocytosis are probably directly related to cholesterol depletion *per se*[60-62], but β-methyl-cyclodextrin has other effects that are unrelated to cholesterol depletion, such as global inhibition of the lateral mobility of plasma-membrane proteins, irrespective of their putative association with L_o domains[63]. The latter effects are not seen with cholesterol depletion using statins[47,63,64]. Failure to appreciate such phenomena can lead to over-interpretation or misinterpretation of experimental results.

L_o domains in model membranes

In model membranes, macroscopic separation of L_o and L_d phases into large (>200 nm) domains is clearly visible using fluorescent dyes that partition differentially into the two phases (FIG. 1). These large L_o domains are not seen in native cell membranes. However, the use of techniques other than light microscopy[11] (which is limited by diffraction and has a spatial resolution of >200 nm) to probe shorter length scales that are biologically relevant provides ample evidence for much smaller L_o domains. For example, donor-quenching FRET analysis shows nanoscale domain formation (~10–40 nm) in lipid bilayers with a similar composition to that of the outer plasma membrane, at the physiologically relevant temperature of 37°C, when macroscopic phase separation is not evident[12]. Similarly, L_o nanodomains can be detected by FRET in regions of a phase diagram in which confocal microscopy indicates only the presence of a single homogenous phase[13] (FIG. 1). Domains on length scales of 30–80 nm in a region of L_o and L_d co-existence have also been detected using atomic-force microscopy (AFM)[14], deuterium-based nuclear magnetic resonance (^{2}H-NMR) and differential scanning calorimetry[15-17], although there is some debate here as to whether these small domains represent true thermodynamic phase separation[16,17]. The effective local cholesterol concentration might determine L_o domain size; in membranes that show coexistence of L_o and L_d, varying the cholesterol content from ~10% to 35% progressively increases the size of L_o domains from small (<20 nm), through intermediate sizes (20–75 nm) detected by FRET imaging, to larger domains (>100 nm) that are visible by standard microscopy[7] (FIG. 1).

A crucial requirement of the raft hypothesis in biological membranes is that these domains can laterally segregate proteins. There is clear evidence for this function in model membranes. L_o–L_d phase separation in model membranes laterally segregates glycosylphosphatidylinositol (GPI)-anchored proteins, although the segregation is usually incomplete. For example, ~40% of the GPI-anchored protein THY1 partitions into L_o domains of supported monolayers and unsupported bilayers[18,19]. Similarly, ~30% of GPI–PLAP (placental alkaline phosphatase) partitions into L_o domains of giant unilamellar vesicles that are imaged using fluorescence correlation spectroscopy (FCS), although this fraction increases if the GPI–PLAP is crosslinked[20]. The lateral mobility of GPI–PLAP is 1.4-fold slower than the mobility of free lipids in the model membrane[20] — this is an important result because the formation of simple protein clusters would not increase the hydronamic radii of GPI–PLAP complexes sufficiently to retard diffusion. This result therefore implies either nanoscale lipid-domain organization around the GPI–PLAP proteins, or transient interactions with larger L_o domains. In summary, complex lipid–lipid–cholesterol interactions in model membranes with a lipid composition approximating that of the outer plasma membrane spontaneously generate lateral heterogeneity on multiple length scales. The plasma membrane is a much more complex structure than these simple model membranes (BOX 3). However, there is no *a priori* reason to assume that the basic lipid biochemistry and thermodynamics that operate in model systems are not the same as those that operate in the plasma membrane.

Box 3 Extrapolating from model membranes to the plasma membrane

The plasma membrane is a much more complex structure than a model membrane. Extrapolations of observations made in model systems must take account of some of the important differences between a simple bilayer with a defined lipid composition and the plasma membrane. For example:

- The plasma membrane has a much more complex mixture of lipids[65], although the general class mixture (unsaturated phospholipid–sphingomyelin–cholesterol) is matched by the more relevant model membranes.

- The plasma membrane contains a plethora of lipid-anchored proteins, transmembrane proteins and peripheral membrane proteins.
- The plasma membrane is not a static platform and cannot be considered an equilibrated membrane, not least because there is constant internalization and recycling of vesicles through multiple endocytic pathways[66].
- Kusumi and colleagues have shown that the plasma membrane is compartmentalized by a picket fence of transmembrane proteins that are anchored to the submembrane actin cytoskeleton[26,67-69]. The fence allows relative free lateral diffusion of lipids within compartments but restricts movement between compartments. One important consequence of the picket fence is that it slows long-range lipid diffusion, because lipids can only undergo hop diffusion between neighbouring compartments as the fence fluctuates. The restriction on lateral diffusion between compartments means that the lipid bilayer is not well-mixed on long-length scales.
- Cholesterol is excluded from the immediate vicinity of transmembrane proteins; therefore the local concentration of cholesterol will vary on a nanometer-length scale[26,70].
- Cells are not simple spheres, and local differences in membrane curvature can have profound effects on lipid sorting[71].
- Most model membranes have been studied at 23°C rather than 37 °C.
- The plasma membrane is an asymmetric bilayer, the composition of the inner membrane is unclear, and the degree to which the two leaflets are coupled remains unknown.

However, despite these complexities the basic matrix remains a lipid bilayer, with the outer leaflet, at least, similar to lipid cholesterol mixtures that have the intrinsic property of self-organization into domains or lipid assemblies on multiple length scales.

Lipid rafts in the intact plasma membrane?

The classic lipid-raft hypothesis postulates that rafts pre-exist in the plasma membrane[1,3]. Proteins with lipid anchors or transmembrane domains that are able to partition into an L_o environment are sequestered or concentrated in these rafts. A generalization of this hypothesis indicates that proteins dynamically partition into and out of rafts such that only a fraction of proteins are clustered in the rafts. Implicit in this hypothesis is the concept that rafts diffuse as stable entities that impose lateral heterogeneity on plasma-membrane proteins by virtue of the ability of the proteins to partition into the domains or not. These concepts flow directly from the biophysical studies that have already been outlined.

However, the surface distribution of green fluorescent protein (GFP)-labelled GPI (GFP–GPI) on the outer plasma membrane, as determined by a combination of homo-FRET imaging and mathematical modelling[21,22], cannot be reconciled with this classic raft hypothesis[22,23]. Twenty to forty percent of GFP–GPI proteins are present as small clusters on length scales of 5–10 nm that comprise 3–4 proteins on average, with the remainder of the proteins distributed as monomers — however, this clustering is sensitive to cholesterol depletion[22]. Most interestingly, the ratio of monomers to clusters is not altered with increasing expression of GFP–GPI proteins[22]. A similar distribution, determined by a combination of EM spatial-point-pattern analysis and mathematical modelling, is seen for GFP-tH (which is GFP that is targeted to the plasma membrane by the dual palmitoylation and farnesylation anchoring of H-Ras). Forty percent of GFP-tH is present in clusters with a radius of 12–20 nm that comprise 6–7

proteins on average, with the remainder distributed randomly as monomers[24]. Clustering of GFP-tH is abrogated by cholesterol depletion or disassembly of the actin cytoskeleton and, as with GFP–GPI, the ratio of GFP-tH clusters to monomers does not change as a function of GFP-tH expression[24,25]. Computational modelling shows that a lack of dependence of the extent of clustering on expression level essentially excludes partitioning of GFP-tH into a fixed number of stable pre-existing raft domains[24].

Single-particle tracking experiments at high temporal and moderate spatial resolution also indicate that single GPI-anchored proteins are associated with very small (<10 nm) domains with short lifetimes (<0.1 ms), although the size and stability of these domains increase if GPI-anchored proteins are crosslinked[26]. Moreover, the results of earlier FRET and electron paramagnetic resonance (EPR)-spin labelling experiments can only be rationalized if rafts are small and unstable, or raft proteins exchange rapidly with non-raft membrane on a timescale of <0.1 ms (REFS [27,28]). Other FRET studies have shown cholesterol-dependent clustering of certain lipid-anchored proteins on the inner leaflet, but did not estimate domain lifetime or an upper limit of domain size[29].

Starting from these data, consider the proposal that small, laterally mobile L_o domains form spontaneously in the plasma membrane as in model membranes (FIG. 2). The domains exist on short lengths and timescales (<10 nm and <0.1 ms, respectively) and are transient or unstable for several reasons, including physiological temperature and nanoscale heterogeneity in cholesterol distribution (BOX 3). These unstable rafts can however be captured and stabilized by lipid-anchored or transmembrane proteins. If the initial site for protein interaction is the boundary between the L_o domain and the adjacent L_d membrane, then proteins could function as surfactants, reducing line tension and therefore generating increased stability. In fact, preferential localization of lipid-anchored N-Ras to L_o–L_d domain boundaries has recently been shown *in vitro* using AFM[30]. Collision with other protein or lipid domains leads to the fusion of small domains into larger lipid-based protein assemblies and, therefore, the formation of protein clusters (FIG. 2). These basic properties of the model predict that cluster formation is cholesterol-dependent, but do not predict or require the existence of stable, pre-existing, large L_o domains. Larger, even more stable domains can be formed, but these require protein–protein interactions and/or crosslinking — as with the creation of T-cell-signalling domains[31]. Note that this model is different from the previously advanced lipid-shell hypothesis[32], which suggests that lipid shells that are formed around lipid anchors operate as targeting motifs that allow interaction of the shelled protein with pre-existing lipid rafts or caveolae[32].

Can the small, transient structures of the revised model be called rafts? This is almost a semantic question, but the answer is yes because there is a cholesterol-dependent lipid assembly present, even though longer-term stability requires the presence of protein. In the context of this model, two general classes of laterally diffusing plasma membrane proteins can be defined: those that are able to capture and stabilize small L_o domains (raft proteins), and those that cannot (raft excluded). It is possible that these classes represent two ends of a continuous spectrum in which proteins can show intermediate probabilities of capturing and stabilizing small L_o domains; in which case the likelihood of cluster formation and of finding a given protein in a raft cluster will correlate with the protein's ability to stabilize an L_o domain.

Determinants of raft stability

The mobility and localization of a protein that is tethered by a lipid anchor is determined to some extent by the composition of the bilayer, as different lipid anchors preferentially associate with specific lipid assemblies; this is a mainstay of the raft hypothesis for lipid-based protein sorting. However, it is important to appreciate that the lipid anchor also has the capacity to

modify its immediate lipid environment — for example, to facilitate or inhibit the chain and translational ordering of adjacent phospholipids. These phenomena have been clearly shown for lipidated peptides that interact with model membranes[33-37]. So, saturated lipid anchors might not only preferentially segregate to L_o domains, but also increase their stability by virtue of interactions with membrane-anchor phospholipids. Integral membrane proteins also organize lipids, as the intramembrane protein needs to be solvated by the flexible disordered chains of phospholipids. Indeed, Mouritsen's hydrophobic matching hypothesis proposes that integral membrane proteins perturb surrounding lipids so that bilayer thickness matches the length of the transmembrane domain[38]. Consistent with this hypothesis, recent work indicates that proteins might be the most important determinant of membrane thickness, at least in the exocytic pathway[39].

It remains an open question whether lipid-anchored and transmembrane proteins show differential abilities to function as surfactants at L_o–L_d boundaries, given their different mechanisms of organizing membrane lipids. Either way, as diameter increases, the stability of an L_o domain increases because of decreasing line tension with the surrounding L_d membrane. Extra stability will also be provided by protein–protein interactions between co-resident proteins that have an interaction with the L_o platform. In a general sense, this concept of proteins stabilizing a raft domain is simply another example of proteins organizing lipids, as with caveolin, sterol-sensing domain proteins, BAR (Bin, amphiphysin, Rvs)-domain proteins and myristoylated alanine-rich protein kinase C (PKC) substrate (MARCKS) protein. Reciprocally, lipids organize proteins, as with pleckstrin homology and FYVE domain proteins, annexins, PKC and C-Raf. Continuing the analogy with lipid-raft–protein interactions, many of these other protein–lipid interactions are very dynamic, with fast on and off rates and short lifetimes[40]. It is also worth considering whether the plasma-membrane picket fence (BOX 3) is actually required for the formation of transient L_o domains, as disassembling the actin cytoskeleton with latrunculin declusters GFP-tH as effectively as cholesterol depletion[24].

A crucial feature of the revised model is the proposal that proteins have an active role in raft organization; two recent studies are directly relevant to this prediction. In an elegant set of SFVT experiments examining formation of T-cell receptor (TCR) signalling platforms, Douglass and Vale (REF. [31]) showed that full-length lymphocyte-specific protein tyrosine kinase (LCK) stably interacts with CD2-positive and linker for activation of T cells (LAT)-positive TCR-signalling domains, but that the minimal lipid anchor of LCK coupled to GFP does not. Similarly, Larson *et al*.[41] showed that full length Lyn co-localizes (actually co-diffuses as this is an FCS analysis) with crosslinked immunoglobulin E (IgE) receptor, but again the minimal lipid anchor of Lyn coupled to GFP does not. In parallel, Gaus *et al.*[42] have shown, using Laurdan imaging, that the lipids in and around the TCR–protein complex are ordered, which shows with some certainty that these signalling platforms are indeed large, stable L_o domains. Together, these observations raise an important question: why do a subset of lipid-modified GFP probes that putatively associate with L_o domains (raft markers) not associate with the large putative L_o domains that constitute the TCR and IgE receptor signalling platforms? The conclusion of Larson *et al.*[41] is that a protein-interaction domain, provided by the activated kinase domain of Lyn, is required for stable association with the crosslinked-IgE receptor. A similar conclusion is equally plausible in the case of interactions between activated LCK with the TCR-signalling domain[31]. Analysis of the microlocalization of the minimal anchors of Lyn and LCK and the cognate full-length proteins has largely relied on detergent insolubility with all its inherent problems (BOX 2), except for a FRET study that showed cholesterol-dependent clustering of the minimal Lyn anchor[29]. The new studies[31,41] show that the surface distribution of the minimal anchor and the full-length proteins are different, and therefore illustrate the importance of protein–protein as well as anchor–lipid-bilayer interactions in controlling the stable association of proteins with L_o domains.

There is an interesting analogy here with Ras. The minimal anchor of H-Ras attached to GFP (GFP-tH) localizes to L_o domains, whereas full-length activated H-Ras does not. Full-length Ras has a protein domain, adjacent to the anchor, that is required for the formation of non-raft microdomains that constitute the actual Ras signalling platform. Therefore, it is a combination of lipid anchor and protein-based sorting mechanisms that determines the final composition of an H-Ras-signalling complex[24,43,44].

Limiting L_o domain size

The small, transient L_o domains might be expected to progressively coalesce into ever larger, and eventually macroscopic, domains once they are captured by proteins. This clearly does not occur because such domains are not detected in native cell membranes. Furthermore, at shorter-length scales significant aggregation does not occur, as evidenced by the clustering of GFP–GPI and GFP-tH in small nanodomains with a fixed monomer to cluster ratio at all expression levels[22,24]. The simplest explanation to account for these results is that there is an active, energy-dependent process that limits raft-domain size. A recent computational analysis of this problem considered small, spontaneously forming L_o domains that diffuse laterally and fuse on collision, and showed that endocytosis might be the process that limits raft-domain size[45]. This study showed that small domains in the modelled membrane would eventually fuse to one large raft, driven by the reduction of line tension discussed above. By contrast, active, selective removal of large rafts by endocytosis and recycling of disassembled, smaller raft units back to the model membrane limited raft size to a nanometer length scale[45]. In this context, it is worth noting that there are well-defined endocytic pathways that selectively internalize lipid-raft-associated proteins, and traffic through these pathways is increased after crosslinking the rafts into larger domains[22,46]. Intriguingly, a similar limitation in raft size was achieved by randomly recycling areas of membrane without any selection for large rafts[45]. This indicates that all endocytic processes might contribute to the active maintenance of small rafts on the plasma membrane.

Are small, short lifetime rafts useful?

It is also important to consider what small, unstable raft domains might be able to do. Is there actually an advantage to having multiple small domains? This is a difficult question to tackle experimentally, but it is amenable to computational approaches. For example, recent work with Ras signalling platforms indicates that compartmentalizing switch-like activation of the Raf–MEK (mitogen- activated protein kinase (MAPK) and extracellular signal-regulated kinase)–MAPK cascade across multiple nanoscale plasma-membrane domains allows high-fidelity transmembrane signal transmission from growth-factor receptors to MAPK activation (T. Tian, A. Harding and J.F.H.; personal communication). Furthermore, disrupting Ras nanoclustering using multiple different conditions significantly impairs Ras signalling[24,44,47,48], which emphasizes the importance of nanocluster formation. It is tempting to speculate that lipid rafts might have similar roles in other signalling cascades. If this is the case, much larger and more stable rafts, or crosslinked rafts, might predominantly be required for protein trafficking and endocytosis, and for highly specialized functions such as those observed in caveolae or the T-cell synapse.

However, what if we consider that a crucial role of raft domains is to promote reactions between raft-partitioning proteins? Could the type of nanoscale domains proposed here realize this function? A recent computational modelling study of raft–protein dynamics provides some preliminary answers to these questions[49]. This study considered a classic raft model in which proteins dynamically partition into rafts. Inter-protein collisions were observed to be maximal when rafts were small (6–14 nm), mobile and when the diffusion rate of proteins in rafts was two-fold slower than the diffusion rate of proteins outside of rafts. Although the effect of raft

instability *per se* was not investigated, the average residence time of a protein in a raft was <0.1 ms. So, although the simulations of protein dynamics were based on a model of stable raft domains, the conclusion is that transient, short-lifetime protein interactions with small lipid-raft domains might still facilitate useful biochemical reactions[49].

Conclusions

An analysis of recent work with model membranes and intact plasma membranes, using experimental techniques that explore short length and timescales, together with computational modelling, indicate that a revised hypothesis for the structure and function of lipid-raft domains in the plasma membrane is warranted. This revised model needs to take into account a more dominant role for plasma-membrane proteins in capturing and stabilizing intrinsically unstable L_o domains. Importantly, this revised hypothesis indicates a series of testable predictions and offers what might be elusive common ground between two polarized standpoints: the idea that stable pre-existing rafts are the dominant organizing principle in the plasma membrane, versus the idea that rafts do not exist and therefore have no role in plasma membrane function. In summary, rafts exist, but their length and timescale specifications are crucially important characteristics that must be included in any definition.

To rigorously test the hypothesis, a wider application of imaging techniques that can explore short distances and timescales in intact membranes is needed to build a more extensive spatio–temporal map of proteins on the plasma membrane. Such studies will give deeper insights into the diverse mechanisms that drive plasma-membrane compartmentalization and show the overall significance of lipid–protein interactions in generating distinct membrane microdomains. Integrating different data sets will also require more *in silico* modelling of protein dynamics on the plasma membrane. Finally, the extent to which the inner and outer leaflets of the plasma membrane are coupled remains unknown. Progress in solving this complex problem will no doubt reveal new insights into plasma-membrane micro-organization and lipid-raft function.

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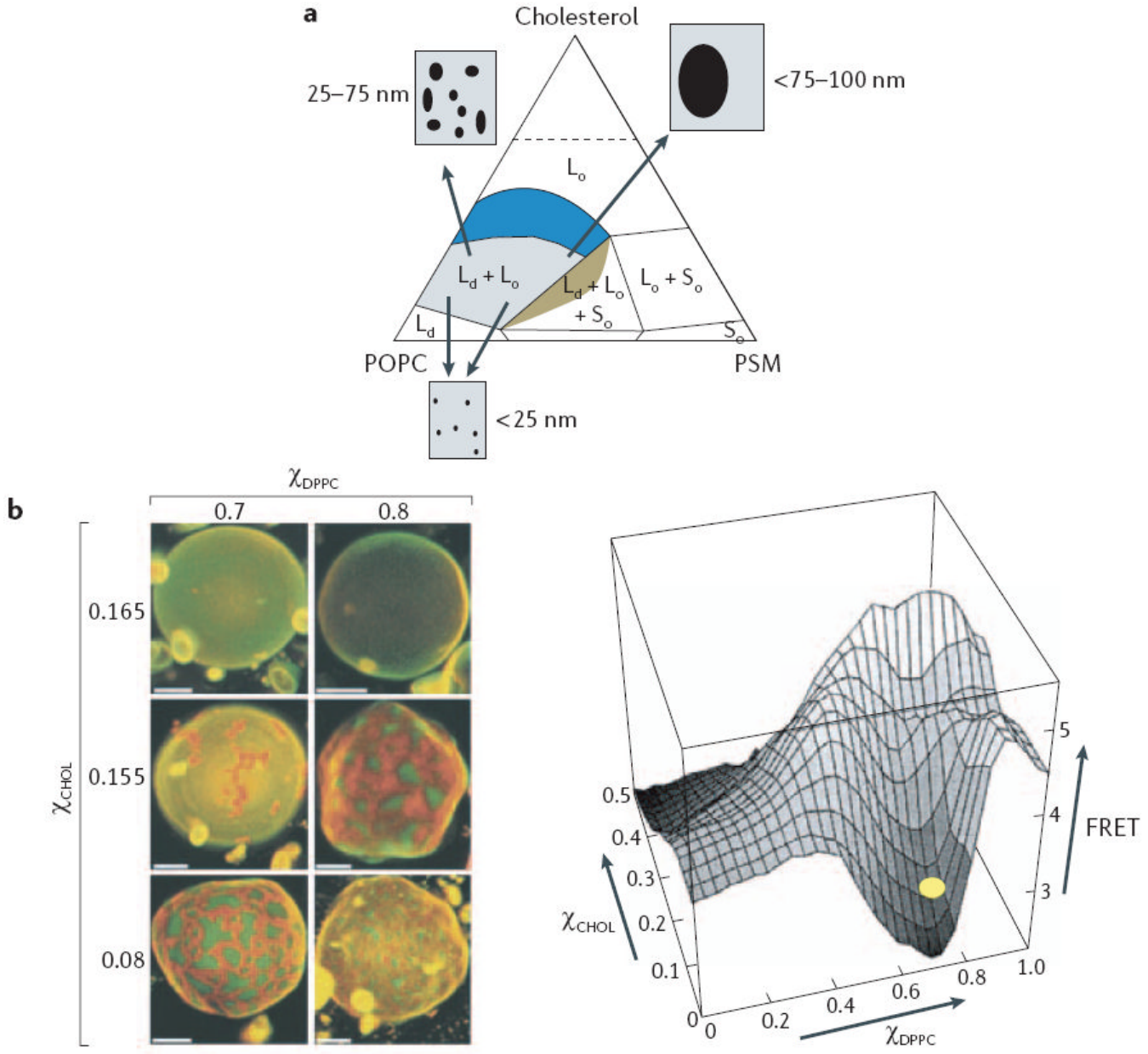

Figure 1. Liquid-ordered domains in model membranes

a Phase diagram at 23°C for ternary mixtures of cholesterol, sphingomyelin (PSM) and l-palmitoyl-2-oleoyl-phosphatidylcholine (POPC). Each vertex of the diagram corresponds to a 100% content of each lipid. Coloured regions represent membrane compositions that can form a liquid disordered (L_d) phase. Within the region of liquid ordered (L_o)–L_d co-existence (blue), varying the cholesterol percentages from ~10% to 35% progressively increases the size of L_o domains that are detected by fluorescence resonance energy transfer (FRET) imaging or standard microscopy[7]. It is important to note that phase diagrams such as this are crucially dependent on temperature, although an extensive area of L_o–L_d phase co-existence is present at 37°C(see REFS [3,6]). **b** Macroscopic domain formation in giant unilamellar vesicles that are composed of cholesterol, 1,2-dipalmitoylphosphatidylcholine (DPPC) and 1,2-dioleoylphosphatidylcholine (DOPC) and visualized using dyes that preferentially partition into L_o domains (orange) and L_d domains (green). When the cholesterol percentage (χ_{CHOL})

is >16%, macroscopic domain formation is no longer seen, but nanodomain formation is still detectable as a loss of a FRET signal between two probes that preferentially partition into L_o and L_d domains. The yellow oval marks the approximate composition of the top row of vesicles. Scale bar = 5μm. χ_{DPPC} = DPPC content as a fraction of DPPC and DOPC. Part **a** is reproduced with permission from REF. [7] © (2005) Elsevier; part **b** is reproduced with permission from REF. [13] © (2001) The Biophysical Society.

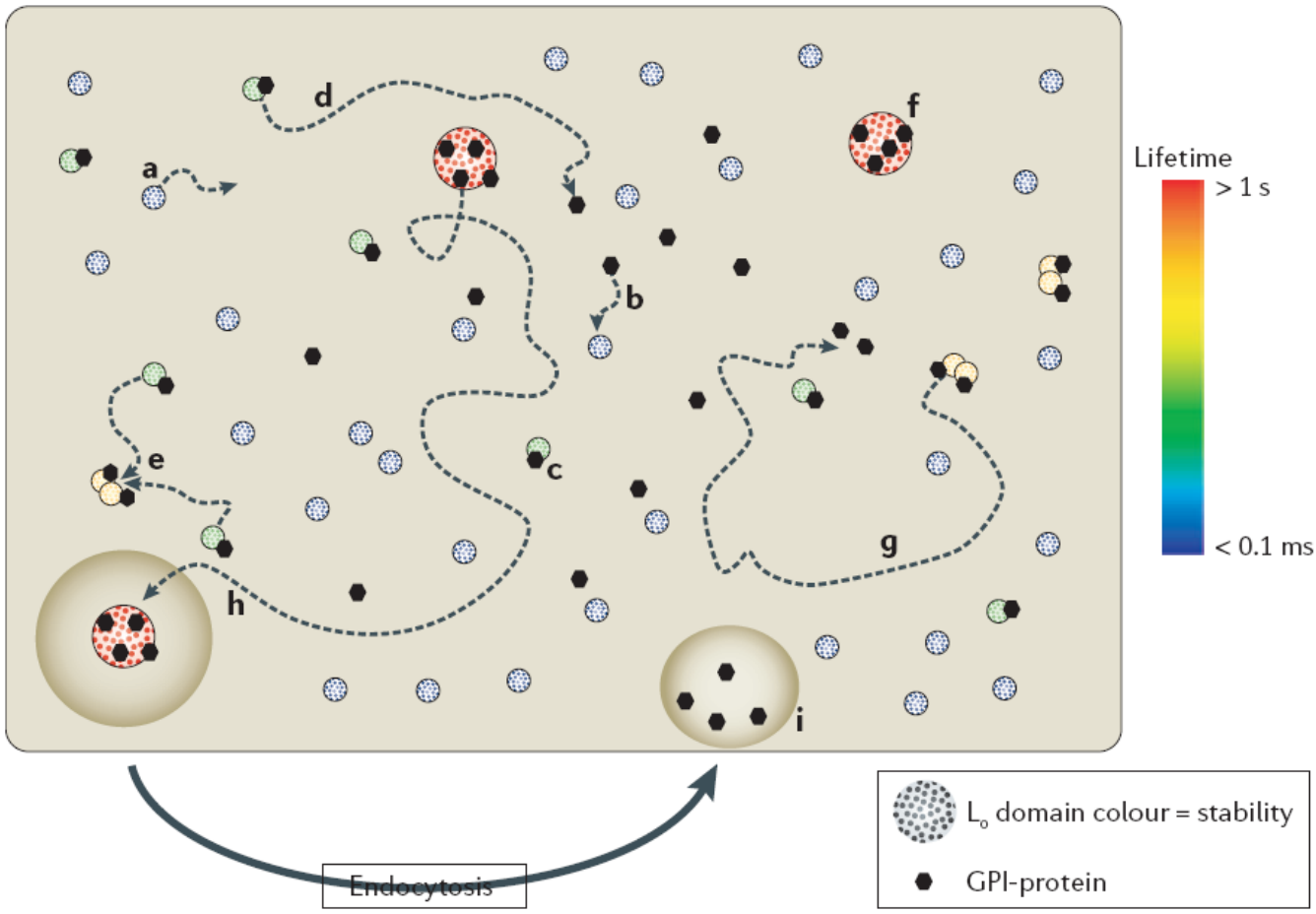

Figure 2. Revised raft model

Liquid ordered (L_o) domains in the plasma membrane are heterogeneous in size and lifetime (from >1 s to <0.1 ms, as indicated by colour). L_o domain stability or lifetime is a function of size, capture by a raft-stabilizing protein and protein–protein interactions of constituent proteins. The length of trajectories (dotted arrowed lines) and, therefore, the probability of collision with proteins or other L_o domains are proportional to lifetime. For simplicity, only sample trajectories are shown — however, all of the domains and proteins that are shown should be envisaged to diffuse laterally. The diagram illustrates the fate of different classes of L_o domain. Small, unstable L_o domains form spontaneously, diffuse laterally in the plasma membrane, but have a limited lifetime (**a**). If captured by a glycosylphosphatidylinisotol (GPI)-anchored protein (or other raft-stabilizing proteins) (**b**) the stability of the L_o domain is increased with the formation of a complex (**c**), which has two possible outcomes: the complex diffuses laterally but the L_o domain disassembles (**d**), or there is a collision with other protein-stabilized L_o domains that creates protein clusters (**e**). Further collisions will generate larger, more stable L_o–protein complexes (**f**) that are further stabilized by protein–protein interactions, or in the absence of collisions (**g**) the L_o–protein complex disassembles. The fate of larger, more stable L_o–protein complexes (**h**) is capture by endocytic pathways that disassemble the complexes and return lipid and protein constituents back to the plasma membrane (**i**). Intuitively, the model predicts the generation of protein clusters that is dependent on their interaction with L_o domains. Endocytosis indicates endocytosis that is specific for larger raft complexes (as shown), possibly all endocytic processes contribute to limiting the size of raft domains. Note that the larger, more stable lipid–protein complexes (**f**) could operate the way

rafts are proposed to in the classic model, with dynamic partitioning of proteins between the domain and the surrounding disordered membrane (not shown).