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Review

Thermodynamics of membrane domains

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Abstract

The concept of lipid rafts and the intense work toward their characterization in biological membranes has spurred a renewed interest in the understanding of domain formation, particularly in the case of cholesterol-containing membranes. The thermodynamic principles underlying formation of domains, rafts, or cholesterol/phospholipid complexes are reviewed here, along with recent work in model and biological membranes. A major motivation for this review was to present those concepts in a way appropriate for the broad readership that has been drawn to the field. Evidence from a number of different techniques points to the conclusion that lipid–lipid interactions are generally weak; therefore, in most cases, massive phase separations are not to be expected in membranes. On the contrary, small, dynamic lipid domains, possibly stabilized by proteins are the most likely outcome. The results on mixed lipid bilayers are used to discuss recent experiments in biological membranes. The clear indication is that proteins partition preferentially into fluid, disordered lipid domains, which is contrary to their localization in ordered, cholesterol/sphingomyelin rafts inferred from detergent extraction experiments on cell membranes. Globally, the evidence appears most consistent with a membrane model in which the majority of the lipid is in a liquid-ordered phase, with dispersed, small, liquid-disordered domains, where most proteins reside. Co-clustering of proteins and their concentration in some membrane areas may occur because of similar preferences for a particular domain but also because of simultaneous exclusion from other lipid phases. Specialized structures, such as caveolae, which contain high concentrations of cholesterol and caveolin are not necessarily similar to bulk liquid-ordered phase.

Keywords: Raft; Liquid-ordered; Phase separation; Lipid–lipid interaction; Protein–lipid interaction; Cholesterol complex

Contents

Abbreviations: Chol, cholesterol; SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PG, phosphatidylglycerol; DAG, diacylglycerol; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPS, 1-palmitoyl-2-oleoylphosphatidylserine; DOPC, dioleoylphosphatidylcholine; 12:0, lauroyl; 13:0, tridecanoyl; 14:0, myristoyl; 16:0, palmitoyl; 18:0, stearoyl; 14:1, myristoleoyl; 16:1, palmitoleoyl; 18:1, oleoyl; 24:1, nervonoyl; GPI, glycosylphosphatidylinositol; ER, endoplasmic reticulum; DSC, differential scanning calorimetry; T_m, main transition temperature; ω_{AB}, lipid–lipid, nearest-neighbor interaction free energy; FRET, fluorescence resonance energy transfer; DRM, detergent-resistant membrane; GFP, green fluorescent protein; GUV, giant unilamellar vesicle

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1. Lipid rafts, domains, and inhomogeneous membranes

Recently, domains rich in cholesterol (Chol) and sphingomyelin (SM), commonly known as 'lipid rafts' [1–3], have received enormous attention and rekindled the interest in understanding the formation of domains in model and biological membranes (for reviews see [4–7]). A classical approach to understanding a complex system, such as a biological membrane, is to simplify it by preparing a model system that still contains the properties of interest of the complex system but is simple enough to be amenable to direct experimentation. In this sense, the ancestors of the lipid raft field are the pioneering studies of phase separations in phospholipid bilayers, in the 1970s [8–12]. Those studies were mainly concerned with gel–fluid phase separation, but also included phospholipid/Chol mixtures, which contained fluid–fluid immiscibility regions [9]. These types of lipid systems have also been modeled theoretically by several groups [13–21]. It is presently believed that structural or compositional domain coexistence of fluid states, for example the liquid-ordered (l_o) and liquid-disordered (l_d) states [20–25], is probably more relevant for biological membranes than gel–fluid coexistence [26,27]. Lipid domains are often perceived as static structures, although their dynamic nature [18,19] has been stressed including the case of rafts [2]. The importance of domains in membrane function has been suggested through both experimental and theoretical studies [28–33]. Propagation of a signal transduction event usually involves protein–protein interactions, for example, in phosphorylation of proteins and substrate–enzyme reactions. Such events are highly enhanced in magnitude and specificity if the proteins involved are concentrated in the same domain, rather than distributed over a large number of disconnected domains [26,29,33]. Experiments performed in cells have suggested that many proteins partition into rafts, at least during some cell stages, including glycosylphosphatidylinositol (GPI)-linked proteins, some transmembrane proteins, non-receptor tyrosine kinases, G-proteins, and transporters. Because many of those proteins are involved in signal transduction and appear to co-localize in the rafts, these domains have been suggested to be 'hot spots' for signaling. For example, in lymphoid and endothelial cell rafts, the CD44 receptor associates preferentially with the src-family kinases Lck and Lyn, increasing the probability of interaction [2,34]. In basophils, granulocytes, and mast cells, upon activation by antigen binding, the IgE receptor FcεR1 aggregates and partitions into the rafts, where it interacts with co-localized tyrosine kinase Lyn [34]. These observations are in sharp contrast with determinations of partition coefficients of protein and peptides between l_d and l_o phases, which indicate a general preference of hydrophobic, transmembrane helices [35–39] and also amphipathic helices [37,40–42] to partition into the l_d regions. This is a puzzling issue that begs to be addressed.

The basic structural unit of all biological membranes is the lipid bilayer. This can be viewed as a two-dimensional surface that separates cellular compartments and isolates the cell from the extracellular environment. However, the complex composition of biological membranes [43] clearly indicates that its function is not merely the separation of the cell from the extracellular environment. In fact, a very large number of different protein species are embedded in (integral proteins) or surface-adsorbed onto (peripheral proteins) the lipid bilayer, which is itself a complex mixture of lipids. These components interact differently with each other, by virtue of their specific chemical nature, thus showing mutual 'likes' and 'dislikes'. The result is a membrane that is not uniform at the molecular level. Its structure in the plane of the bilayer is that of a microscopic mosaic of regions that differ in composition, that is, domains [44,45]. Besides composition, differences in the state of the lipids can also lead to domain formation. For example, some lipids can exist in a fluid or in a gel state at physiological temperatures [8,10]; others, in particular in mixtures with cholesterol, will be in the l_o state [20]. These states do not mix ideally, thus leading to the formation of domains that coexist in a single membrane. If the magnitudes of lipid–lipid interactions were large, formation of lipid domains would be irreversible, leading indeed to static structures. However, in model bilayers, differences in interaction Gibbs energies between different lipid species typically vary between −200 and +200 cal/mol [16,17,32,33,46–55], and only in rare cases approach the value of + 400 cal/mol required for complete phase separation [56]. Because of the large number of molecules involved, these interactions are amplified leading to domain formation, but the energy barriers are small and the process remains reversible. On the other hand, the changes in Gibbs free energies involved in protein–membrane interactions are typically much larger (several kcal/mol-protein) and vary considerably with the lipid composition of the membrane [32,33]. Differential lipid–protein interaction energies can thus lead to formation or disruption of domains.

2. Why are domains formed?

Biological membranes are not in equilibrium. (The first step toward that would be cell death.) Domains can therefore form in biological membranes as a purely kinetic process, as some components transiently accumulate in a particular area. This could be the result of the action of an enzyme, for example, the hydrolysis of phosphatidylinositol-4,5-bisphosphate by phospholipase C to produce a local increase of the concentration of

diacylglycerol (DAG). A DAG domain would then form locally at a particular time, but it would dissipate quickly, thus having but a transient existence. The same is true in model membranes, in fact. If an amphiphile is added with a micropipette to a giant unilamellar vesicle (GUV), it will insert in the membrane locally and create a transient (kinetic) domain (which may have important, and not always wanted, consequences for the vesicle morphology). These types of kinetic domains are likely to form in biological membranes all the time, but given diffusion coefficients of lipids in membranes of about 5×10^{-8} cm^2/s, a domain of this kind formed in a small area, say 10,000 Å^2, which corresponds to about 150 lipids, would dissipate in less than 10 μs. This might be a useful transient signal into the cytoplasm. However, if these domains are to persist for long enough to contribute to the structural organization of the membrane, they will have to be significantly more long-lived. That is, they may begin as kinetic domains, but thermodynamic stabilizing interactions must be present to ensure a significant domain lifetime.

In a system in thermodynamical equilibrium, the reason for domain formation is simply the differential interaction between membrane components, that is, their mutual 'likes' and 'dislikes'. In this section, we translate this humanized view of the bilayer into an exact and quantitative form. Consider a lipid molecule in one of the leaflets of the bilayer. In this leaflet, our molecule is surrounded by six nearest-neighbor lipids. (Some approximation is involved here: the acyl chains, not the lipids, are in a regular triangular lattice in the gel state, but the error is minor [17]). If the lipids are of two different species, for example, phosphatidylcholine (PC) and DAG, both in the fluid state, there are three lipid–lipid interactions involved: PC–PC, PC–DAG, and DAG–DAG. However, only one thermodynamic parameter is necessary to describe the mutual interactions of these two types of lipids: the difference between a PC–DAG interaction and the average of the interactions between PC–PC and DAG–DAG. To see this, consider the 'reaction' shown in Fig. 1 where two pairs of like neighbors are exchanged to produce two pairs of unlike neighbors. For simplicity of notation, let us call PC lipid A and DAG lipid B, in this example. The Gibbs free energy change for this reaction per molecule (there are two molecules of each, so we divide by 2) is given by,

$$x_{AB} = g_{AB} - \frac{1}{2}(g_{AA} + g_{BB}), \quad (1)$$

where g_{AA} and g_{BB} are the Gibbs free energies of interaction between two A or two B molecules, and g_{AB} is the Gibbs free energy of interaction between one A and one B molecule. The

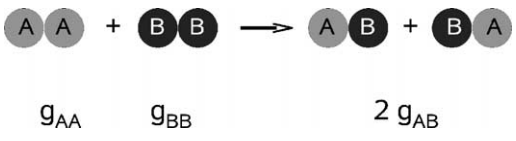

Fig. 1. Exchange reaction of two pairs of like lipids to produce two pairs of unlike lipids. Each circle represents a lipid, not an acyl chain. This reaction defines the Gibbs free energy of interaction, ω_{AB}.

Fig. 2. Exchange between two lipids in one leaflet of a lipid bilayer (top view). The free energy difference between the initial and the final states is a multiple of ω_{AB}.

parameter ω_{AB} is the unlike nearest-neighbor interaction Gibbs free energy, or interaction energy for short, and it is this parameter that determines whether lipids A and B mix well or separate into domains. If we now consider again a lipid molecule in a bilayer (Fig. 2) and calculate the Gibbs free energy change (ΔG^o) for the exchange of positions with one of its neighbors, the result depends only on the value of ω_{AB}. For example, for the reaction shown in Fig. 2, the initial Gibbs energy is $G_i^o = 7g_{AA} + 7g_{BB} + 5g_{AB}$ and the final, after the reaction, is $G_f^o = 4g_{AA} + 4g_{BB} + 11g_{AB}$; but the difference is simply $\Delta G^o = 6g_{AB} - 3g_{AA} - 3g_{BB} = 6\omega_{AB}$.

Simple mixing entropy favors the right-side of the reaction of Fig. 2 and further mixing thereafter. On the other hand, if $\omega_{AB} > 0$ the left side is favored because the reaction probability is proportional to $e^{-\Delta G^o/RT}$. Whether separate domains of A and B will form (left side of Fig. 2) depends on the sign and value of ω_{AB}. The more positive and large ω_{AB} is, the more complete the separation into A and B domains. If $\omega_{AB} = 0$, random mixing occurs. If $\omega_{AB} < 0$, A and B will mix even more than randomly; if the value is very negative, there will be a tendency to form a checkerboard AB pattern.

If the geometry is more complicated, such as would result from the introduction of a large integral membrane protein in the bilayer, this simple formalism needs to be adjusted; or, if more lipid components are introduced, more parameters of the ω_{AB} type need to be included. However, the basic principle remains the same and this is all we need to understand the thermodynamics of domain formation. The physical origin of ω_{AB} is a different matter, which is addressed in the next section.

3. Physical mechanisms of domain formation: origins of ω_{AB}

One possible mechanism leading to domain formation is the mismatch between the hydrophobic thicknesses of membrane components, lipids and integral membrane proteins, leading to the 'mattress model' of bilayer membranes [57]. This idea is related to the consideration of side-by-side interactions of proteins with lipids and the elastic response of the lipid bilayer to the inclusion of proteins [58–62]. Hydrophobic mismatch, which influences both lipid–lipid and protein–lipid interactions, arises when the length of the membrane-spanning moiety of an integral protein does not match the hydrophobic chain length of the host lipid (Fig. 3C and D) or when two lipids with different chain lengths are placed next to each other (Fig. 3A) [57,63–65].

Early experimental and theoretical work appeared to lend support to the mattress model. For example, in a study of rotational diffusion of rhodopsin reconstituted in fluid bilayers

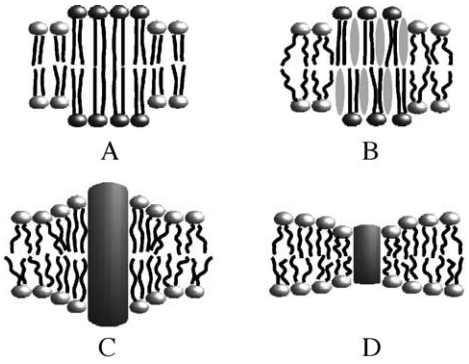

Fig. 3. Hydrophobic mismatch. (A) Mismatch between two lipids with different acyl chain lengths. (B) Mismatch between l_d domains (phospholipids only, on the sides) and l_o domains (central domains, phospholipids and cholesterol (ellipses)). (C) Positive mismatch between an integral protein and lipids, leading to a stretching distortion of the natural thickness of the bilayer. (D) Negative mismatch between an integral protein and lipids, leading to a contraction of the natural bilayer thickness.

of saturated phosphatidylcholines with chain lengths ranging from 12 to 18 carbons, it was found that the protein is monomeric and photochemically active in di14:0PC, but segregates into protein-rich domains in di12:0PC or di18:0PC bilayers [66]. This means that 18:0 chains are not able to shrink and 12:0 chains are not able to stretch to match the hydrophobic core of the protein. This restriction of acyl chain rotational freedom would result in an entropic penalty, which is unfavorable. In di14:0PC the lipid chains match the membrane-embedded protein moiety and there is no thickness mismatch to drive protein aggregation or domain formation. Also, incorporation of photosynthetic reaction centers in a lipid bilayer shifts the T_m of the phospholipid to higher values (+8 °C) in di12:0PC and to lower values (−3 °C) in di16:0PC, which means that the protein partitions preferentially into the gel phase in di12:0PC, but it prefers the fluid phase in di16:0PC, in both cases choosing the phase with a bilayer thickness that matches its own hydrophobic, transmembrane moiety the closest. Furthermore, when two peptides of different lengths where incorporated in bilayers of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) and POPC/Chol 70:30, the longer peptide, which matched the thicker bilayer (POPC/Chol), caused an increase in the observed thickness of the thinner bilayer (pure POPC) [67]. The converse was observed for the shorter peptide, which caused thinning of the thicker bilayer (POPC/Chol), but did not change the POPC bilayer thickness.

The hydrophobic mismatch has also been invoked to explain the retention of integral proteins in the Golgi system according to the length of their membrane-spanning portion [68–70]. Proteins and phospholipids are inserted in the membrane in the endoplasmic reticulum (ER), transported to the cis- and eventually to the trans-Golgi, until they finally reach the plasma membrane. However, some proteins are retained in the Golgi. This can arise because the hydrophobic mismatch selectively leads to retention of proteins, the transmembrane segments of which match the hydrophobic thickness of the Golgi lipid bilayer [69]. A bilayer thickness gradient exists from the ER (thinnest), through the Golgi, to the plasma membrane (thickest), probably determined by the Chol content of the membrane. The hydrophobic mismatch between the length of the protein hydrophobic core and the thickness of the lipid bilayer seems to control the migration of these proteins and their location. This mechanism for selective retention was tested in lipid bilayers of increasing thickness, from di14:1PC to di24:1PC, for two hydrophobic peptides of 16 and 22 residues containing a Leu-based stretch flanked by two Lys residues at both ends [71]. It was found that the greatest peptide incorporation occurred in bilayers with hydrophobic cores that best matched the peptide length. It was also found that the orientation of hydrophobic peptides in lipid bilayers (perpendicular to the membrane plane or surface-adsorbed) was controlled by the matching of the peptide length to the bilayer thickness [72,73]. Furthermore, the selectivity of lipid binding to the bacterial outer membrane protein OmpF was found to be in agreement with the hydrophobic mismatch hypothesis [74]. The best match between the thickness of OmpF and a series of PCs with monounsaturated acyl chains, from di14:1PC to di24:1PC, occurs for di14:1-PC, which is the lipid with the strongest association with OmpF. Using DSC, it was observed that incorporation of synthetic hydrophobic peptides of variable length in di16:0PC bilayers resulted in a decrease in T_m and broadening of the heat capacity function of di16:0PC [75]. The effect was more pronounced for shorter peptides, which are expected to partition preferentially into the liquid-crystalline phase, than for longer peptides, which match the gel phase thickness more closely. Monte Carlo simulation of this experiment, using an interaction parameter proportional to the peptide/bilayer hydrophobic mismatch, produced heat capacity curves that qualitatively resemble the experimental ones [75].

The hydrophobic mismatch between the chains of two different lipid species has also been used to understand the mixing behavior of phospholipids [76,77]. A single interaction parameter related to the extent of chain mismatch could account for differences in experimental phase diagrams of a series of homologous binary mixtures of phosphatidylcholines with varying chain length mismatch (di14:0PC/di16:0PC, di14:0PC/di18:PC, and di12:0PC/di18:0PC) [76]. This is equivalent to making ω_{AB} essentially proportional to the acyl chain length difference for each pair of lipids. Furthermore, using the excimer/monomer (E/M) ratio of an acyl chain-labeled pyrene-PC as an indicator, it was shown that probe clustering was maximal when the host lipid was di14:1PC, and decreased as the chain of the monounsaturated PC was increased from 14:1 to 24:1 [77]. This was interpreted on the basis of chain mismatch forcing the formation of clusters of pyrene-PC.

In more recent work, however, the role of hydrophobic mismatch alone as causing domain formation does not appear to be so clearcut, and there is some conflicting data. Aromatic residues are typically found flanking the helices of integral membrane proteins. Lys-flanked hydrophobic peptides similar to those tested before [71] but additionally flanked at both ends by aromatic residues (Tyr or Phe) were incorporated into the same PC lipid series, increasing the acyl chain length between 14:1 to 24:1. In contrast with the peptides flanked only by Lys,

it was found that the strongest incorporation in the membrane did not occur when the hydrophobic lengths of the peptide and the bilayer matched [78]. Rather, it appeared that these Tyr- or Phe-flanked peptides, even the shorter ones, preferred longer acyl chain lipids, which produce bilayers thicker than the peptide hydrophobic core. Those authors concluded that the presence of aromatic residues at the peptide termini 'buffers' them against sensitivity to membrane thickness [78]. A similar observation was made when Trp-flanked peptides of varying length were incorporated in di12:0PC, di13:0PC, and di14:0PC bilayers [79]. Essentially, there was no effect of peptide on membrane thickness in any of the lipids, when the peptide length was varied between 13 and 19 residues. That hydrophobic mismatch alone was not the only important factor had already become apparent in an earlier study, where the combined effects of the nature of the flanking residues and hydrophobic mismatch on the interactions between synthetic peptides and PC bilayers of varying hydrophobic thickness were examined [80]. For peptides flanked by aromatic residues, peptides longer than the bilayer hydrophobic thickness caused ordering of the lipid acyl chains, but this effect was weaker for peptides flanked by basic residues. For peptides shorter that the bilayer, the nature of the flanking residues does not appear to be as important [81]. When the length of hydrophobic, synthetic peptides and the distance between the Trp residues flanking the helix were varied, it was found that the main factor was the distance between the Trp residues [82]. It appears that the propensity of Trp to reside at the bilayer–water interface overrides the hydrophobic mismatch. When Lys were used as the helix-flanking residues, a peptide with a 37.5 Å hydrophobic length behaved identically to one with 25.5 Å in its interaction with DMPC at 34 °C, which has a hydrophobic bilayer thickness of 22.5 Å [82], indicating that peptides much longer than the bilayer thickness can very well be accommodated. Lys-flanked peptides sufficiently short to place the Lys charges in the bilayer, however, could not be accommodated [82]. While some of these observations appear conflicting, in particular on whether the interaromatic distance works are a hard or as a 'fuzzy' ruler for a preferred bilayer thickness, they point to the fact that peptide partitioning preferences are not determined by hydrophobic mismatch alone, but a significant role is played by flanking aromatic residues.

In phospholipid/Chol mixtures, increasing the content of cholesterol leads to an increase in bilayer thickness [22]. In equimolar mixtures of dioleoylphosphatidylcholine (DOPC)/SM/Chol, which are expected to contain rafts co-existing with a DOPC liquid-disordered phase, it was found that synthetic, Trp-flanked, hydrophobic peptides always partition preferentially to the DOPC phase, regardless of peptide length (from 25.5 Å (23 residues) to 37.5 Å (31 residues) of hydrophobic length), even for peptides the length of which matched the thickness of the rafts much more closely than that of DOPC [83]. In a similar study, two peptides of 23 and 29 residues, containing a central hydrophobic stretch flanked by Lys residues at the helix termini, which were designed to match the hydrophobic thicknesses of the disordered and ordered phases, respectively, were found to partition preferentially to the disordered, DOPC-rich phase rather than the ordered, SM/Chol-rich phase at low temperature (4 °C) [38]. At 37 °C, the 29-residue peptide partitioned significantly more than the 22-residue peptide into the ordered phase, but still not in preference to the disordered phase. These observations were recently confirmed using fluorescence microscopy of GUVs [39].

Additional assessment of the role of mismatch comes from experiments using the E/M ratio of a pyrene-phosphatidylglycerol (pyrene-PG) lipid incorporated into mixtures of PC with 1-palmitoyl-2-oleoyl-phosphatidylserine (POPS). Keeping the phosphatidylserine (PS) structure constant, the effect of varying the PC was examined for: (a) POPC; (b) the monounsaturated, homologous series, di14:1PC-di24:1PC; or (c) the polyunsaturated series, di18:2PC, di18:3PC, di20:4PC, and di22:6PC [33]. Those data closely matched experiments where pyrene-PG was incorporated in PC only, in the same series [77]. In all cases, E/M decreased as the acyl chain of the PC was increased (Fig. 4) [33]. However, while the chain length of the pyrene-PG matched that of POPC, a minimum in E/M was not found for POPC [33]. According to the hydrophobic mismatch concept, ω_{AB} should vary monotonically with the difference in acyl chain between two phospholipids in a binary lipid mixture. Thus, in the monounsaturated series examined, ω_{AB} should be large for the shorter lipids (di14:1PC), reach a minimum at POPC, and increase again as the PC acyl chains became longer. Instead, the E/M decreased monotonically. Furthermore, all the polyunsaturated-chain PCs examined have essentially the same hydrophobic thickness, but the E/M varies significantly. Therefore, the hydrophobic mismatch does not provide the decisive contribution to ω_{AB}. Rather, it appears that other

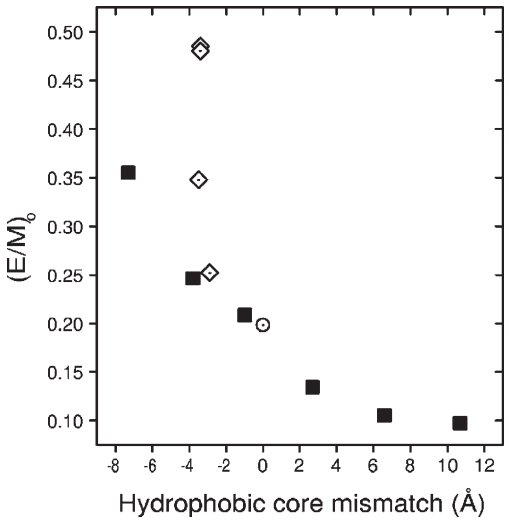

Fig. 4. E/M of pyrene–PG in mixtures of POPS:PC, where the PC is POPC (circle), belongs to the series di14:1PC to di24:1PC (solid squares, in that order from left to right), or belongs to the polyunsaturated series di18:2PC, di18:3PC, di20:4PC, and di22:6PC (diamonds, in that order from bottom to top) [33]. Reprinted with permission from Biochemistry 43, 7102–10. Copyright (2004) American Chemical Society.

factors, including the strength of the van der Waals interactions established by the PC determine the value of ω_{AB} [33].

In addition to the hydrophobic mismatch and van der Waals attractive forces, two additional factors need to be incorporated into ω_{AB}: the lipid chain entropy and the packing of lipid chains against the protein hydrophobic surfaces [61]. Some of these factors, namely, chain entropy, van der Waals interactions (cohesive forces), and bilayer thickness, make important contributions to the bilayer elastic properties [61,60]. Partition coefficients of peptides into membranes are expected to increase as the bending elasticity and the lateral compressibility increase [38]. Therefore, a greater membrane elasticity should favor preferential partitioning into disordered, rather than ordered phases. The effects of these factors have been investigated much less than the hydrophobic mismatch and clearly much research is needed in these areas.

The measurement of ω_{AB} is a difficult experimental problem. One approach is to obtain an experimental measure of domain formation, for example by fluorescence resonance energy transfer (FRET) or E/M, and compare those experimental data with Monte Carlo simulations of bilayers. Varying ω_{AB} in the simulations until a match is obtained between the simulations and the experiment provides an estimate of ω_{AB} [32,33]. A similar approach works well if the heat capacity functions of pure and mixed bilayers are compared with those calculated from Monte Carlo simulations [16,17,46] using the fluctuation–dissipation theorem [84]. A compilation of ω_{AB} obtained from these data is shown in Table 1. An alternative approach has been to use lipids that can be covalently bonded using disulfide bridges [53,54]. The system is initially allowed to equilibrate and then reaction between nearest-neighbors is triggered: this provides a 'quenched' mixture, at that point. The disulfide-bridged lipids are separated and the numbers of different pairs of lipids measured [47–52]. From these data, the differential interactions (ω_{AB}) between different pairs of lipids can be estimated (Table 2). The general conclusion is that the differential interactions between lipids are typically of ω_{AB}= ±100–300 cal/mol. These values are small, at most half the thermal energy at room temperature (kT), in agreement with early estimates [13]. Most interactions are found to be repulsive (ω_{AB}>0), meaning that lipids prefer to interact with like neighbors. However, some are attractive (ω_{AB}<0), notably in the case of long-chain PC with Chol (Table 2) [51].

Table 1
Unlike nearest-neighbor interaction free energies (ω_{AB}) for several lipid pairs calculated from combinations of experiments and Monte Carlo simulations

Lipid A	Lipid B	T (°C)	Phase	ω_{AB} (cal/mol)	Reference
di14:0PC	di18:0PC	–	l_d	+80	[16]
di14:0PC	di18:0PC	–	s	+140	[16]
di14:0PC	di14:0PC	–	l_d/s	+320	[16]
di16:0PC	di16:0PC	–	l_d/s	+280	[17,46]
di18:0PC	di18:0PC	–	l_d/s	+350	[16]
di14:0PC	di18:0PC	–	s/l_d	+370	[16]
di14:0PC	di18:0PC	–	l_d/s	+410	[16]
16:0,18:1PC	16:0,18:1PS	25	l_d	+240	[32,33]
di18:1PC	16:0,18:1PS	25	l_d	+260	[33]
di16:1PC	16:0,18:1PS	25	l_d	+280	[33]
di14:1PC	16:0,18:1PS	25	l_d	+340	[33]

Table 2
Unlike nearest-neighbor interaction free energies (ω_{AB}) for several lipid pairs calculated from data obtained using dimerizable phospholipid analogs (nearest-neighbor recognition method)

Lipid A	Lipid B	T (°C)	mol% Chol	Phase	ω_{AB} (cal/mol)	Reference
di14:0	di18:0	60	–	l_d	0	[47,49,52,53]
di14:0	16:0,18:1	40	–	l_d	+30	[48]
di18:0	16:0,18:1	60	–	l_d	+70	[48]
di14:0	di18:1	40	–	l_d	0	[48]
di14:0	di18:1	60	–	l_d	0	[48]
di16:0	di18:1	55	–	l_d	+70	[48]
di18:0	di18:1	60	–	l_d	+110	[48]
di18:0	di12:0	60	40	l_o	+130	[52]
di14:0	Chol	60	40	l_o	−80	[50,52]
di18:0	Chol	60	40	l_o	−180	[50,52]
di14:0	Chol	60	16	l_d	−50	[50]
di18:0	Chol	60	16	l_d	−20	[50]
di14:0	Chol	30	15	l_d	0	[51]
di14:0	Chol	30	40	l_o	−110	[51]
di18:0	Chol	30	15	s	+370	[51]
di18:0	Chol	30	40	l_o	−240	[51]

4. Domains and phases

The discussion of domains up to this point has been presented from a molecular point of view, with some concepts borrowed from statistical mechanics. The study of inhomogeneous membranes began with the publication of phase diagrams for binary mixtures of phospholipids [8] or of a phospholipid and cholesterol [9]. Those diagrams indicated regions of 'phase' coexistence between gel and fluid [8], or two fluids [9]. In the case of PC/Chol binary mixtures, these two fluid phases were later named liquid-ordered (l_o) and liquid-disordered (l_d) [20]. An example of this type of phase diagram is shown in Fig. 5 for the DMPC/Chol binary system [25]. While in the single-phase regions of the phase diagram the term 'phase' is appropriate, in the coexistence region it may or may not be. If, in a large vesicle, the two regions really separate completely into two large domains, it is appropriate to call them phases. However, the phase diagrams per se do not tell us much about the structure of the coexisting phases: they may be two phases or a 'phase' may be dispersed into a large number of small domains or clusters. In the latter case, from a rigorous viewpoint, the word phase is clearly not appropriate. A phase, defined in the thermodynamic sense, is a macroscopic body of material. The matter has recently been discussed in the literature. The more stringent view advocates that a dispersed phase cannot be called a phase because it is really a system dominated by fluctuations (see, for example, ref [85]), whereas a more relaxed view accepts that one can speak of the domains of a dispersed phase (see for example ref [4]). This was the sense in which it was used when one of us discussed percolation and diffusion in two-phase systems [25,26,86]. To talk about percolation in a two-phase membrane with macroscopic phase separation makes no physical sense in any case. Probably, the best course of action

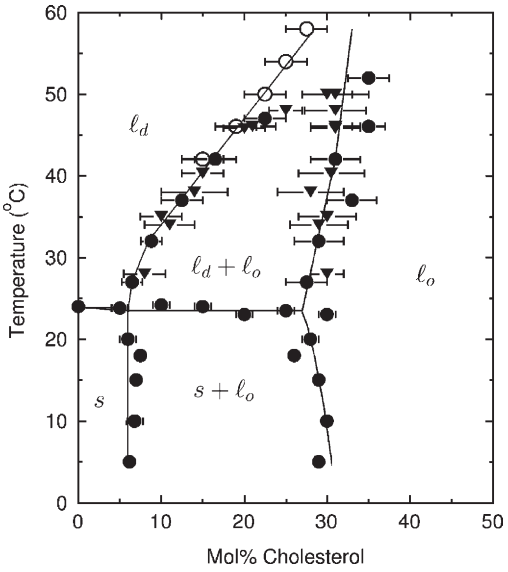

Fig. 5. DMPC/Cholesterol phase diagram [25]. Reprinted (with modifications) with permission from Biochemistry 31, 6739–47. Copyright (1992) American Chemical Society.

would be to talk about solid- and liquid-state domains, reserving the term phase for the pure systems or for when macroscopic phase separation has indeed been demonstrated in two-phase regions of phase diagrams. However, a large body of literature exists regarding phase diagrams of lipid mixtures and this question should not, in our opinion, be given more importance than it deserves: probably, no one will advocate the use of expressions such as 'domain diagrams' just because these mixed lipid systems may not be real thermodynamic phases.

To entirely avoid semantic traps, it is useful, and more productive, to adopt a different point of view, closely following the rigorous approach of Hill on the thermodynamics of small systems [87]. Let us consider a lipid mixture of two components A and B. Suppose that the temperature and pressure are such that a large vesicle with a certain composition exhibits a complete separation between an A domain (major component) and a B-domain (minor component). The two domains make contact at a line (surface, in three dimensions) but the number of A and B molecules at this interface is so small that their contribution to the free energy of the system is negligible compared to the bulk of A and B domains. Those domains are phases: they are large enough to have a constant free energy per molecule or per unit area (or volume, in three dimensions). Now, let us reduce the size of the B-domain progressively. At which point can it no longer be called a phase? The answer is: when it ceases to behave like a macroscopic system and starts behaving like a small system. And that happens when the size of the interface has become large enough relative to the size of the domain, so that its contribution to the free energy of the domain can no longer be neglected. Below a certain size, the free energy per molecule of this, now small, system depends on the extent of the interface and is no longer a constant characteristic of the B phase. But the main point is that, whether it is a phase or not, the domain can still be treated in a rigorous thermodynamic way [87]. In lipid systems, unless the domain sizes become extremely small (of the order of tens of molecules) the properties of the lipids in these domains are not very different from the properties of these lipids in a large phase. For example, the gel phase is characterized by acyl chain order (all-trans conformation) and slow lateral diffusion; the l_d phase is characterized by acyl chain disorder and rapid lateral diffusion; and the l_o phase is characterized by acyl chain order but rapid lateral diffusion [20,21]. These dynamic properties remain valid for domains that are not too small and the acyl chain order definition is valid down to a single molecule.

5. Liquid-ordered phase or condensed complexes

A different question, which has recently emerged in the literature, concerns the distinction between the formation of phases (or domains) and chemical complexes with a defined stoichiometry (compounds) in phospholipid/Chol mixtures. Ipsen et al. [20] proposed that the mixtures of Chol and phospholipids (PC or SM) constitute a monotectic system, with a significant region of coexistence of l_d and l_o phases in the temperature-composition phase diagram. They developed a statistical–mechanical model based on microscopic interactions between Chol and PC in the different states (solid, l_d, or l_o). Essentially, this means that several ω_{AB}-type parameters for the interactions between different PC states and Chol were used together with the differences in enthalpy between states to calculate the most probable states corresponding to the distributions of PC and Chol in the plane of the membrane. The original proposal was later refined to include a set of substates [21]. The model was remarkably successful at explaining the excess heat capacity as a function of temperature for PC/Chol binary mixtures obtained by DSC [88]. Namely, the high-temperature, broad transition typically observed in these mixtures was a natural outcome of the model without any further added assumptions [21]. Support for the l_d–l_o model accumulated during the following decade [22–26,89,90]. In particular, the model naturally explained the physical reason for phase separation in a simple way, as a thickness mismatch between the thinner, l_d, and the thicker, l_o, phase (Fig. 3B) [22]. Recently, strong experimental evidence supporting the coexistence of l_d and l_o phases in SM/Chol bilayers has been provided by an electron spin resonance spectroscopy study, in which the spectrum observed in the mixed-phase regions was demonstrated to be the result of a combination of spectra arising from each separate phase [91]. This observation requires, furthermore, that lipid exchange between the two phases be considerably slower than lateral diffusion.

In the past few years, however, this model has been challenged. On the basis of unusual phase diagrams observed in monolayers of PC/Chol mixtures, which reveal pairs of upper miscibility critical points [92], response to electric fields [93], and an abrupt increase in the chemical potential of Chol when its concentration just exceeds a certain stoichiometric ratio [94], McConnell and co-workers have proposed that Chol forms complexes with phospholipids in defined, stoichiometric proportions [6,95,96]. Several of the previous observations have been re-interpreted in light of the complex formation

model, including measurements using fluorescence probes [6], and especially the heat capacity functions obtained by DSC, in particular those published more recently [90]. The broad high-temperature shoulder was interpreted as thermal dissociation of complexes [95]. However, the complex model does not perform as well as the l_d–l_o microscopic interaction model in the simulation of the experimental DSC curves (compare the heat capacity functions in [21] with those in [95]). In addition, a 'spurious transition' [95] between complex and gel phase appears at low temperatures and high Chol concentrations in the complex model, which is not supported by experiment. A major strength of the model is the ability to make predictions that have been corroborated by experiment, such as the average areas per molecule in monolayers and especially the sudden increase in the rate of β-cylcodextrin-induced Chol desorption at the stoichiometric concentration, which reflects the increase in Chol chemical potential when free PC is no longer available to form complexes [96]. The question arises whether the latter observation could also be explained by the l_d–l_o model, as the two-phase region phase boundary is crossed, into the single l_o region of the phase diagram. Direct demonstration of specific interaction between Chol and SM, which might be expected to support complex formation, has been sought in the form of a possible hydrogen bond between the hydroxyl group of Chol and the amide of SM. This is an old question in the field. According to the most recent evidence, however, no such a hydrogen bond exists [97,98]; the nonpolar moieties of SM and Chol would have to be responsible for the postulated strong interaction. In fact, this is more in agreement with the model of McConnell and collaborators, where formation of the complex is associated with partially compensating, large, negative enthalpy and entropy changes, consistent with the loss of rotational freedom of the phospholipid acyl chains [96]. An alternative explanation for a large entropic contribution to the formation of Chol-rich domains is the need to shield hydrophobic Chol from water, in the context of the 'umbrella model'[99]. More work is necessary in this area before a decision can be made regarding the more appropriate model for Chol/phospholipid mixtures. The question is especially difficult because in many respects the differences are subtle. For example, a system essentially composed of complexes may appear to behave like a liquid-ordered phase [6].

6. Lipid rafts and biological membrane organization

Isolation of cell plasma membranes involves solubilization of the membrane at 4 °C using the nonionic detergent Triton X-100 [100]. Often, a fraction of the membrane preparation is insoluble in the detergent and remains detectable as a low-density band in a linear sucrose gradient centrifugation. This high buoyancy or 'raft' fraction is enriched in Chol, long-chain sphingolipids, glycolipids, and a series of proteins that in many cases are modified with a glycolipid anchor. These observations seem to indicate that eukaryotic plasma membranes are phase-separated systems, just as had been observed in studies with model membranes composed of Chol, long-chain sphingolipids, and unsaturated PC. It was later postulated that the plasma membrane consisted of two major lipid phases: an l_o phase, rich in Chol and sphingolipids, and an l_d phase, rich in POPC. It was suggested that the bulk of the plasma membrane was formed by the l_d phase with the minor, l_o phase as dispersed rafts [1,4,5,7].

More recently, it has been argued that Triton X-100 extraction promotes the formation of rafts, raising the possibility that the isolation of the Chol-rich membrane fraction may have been a detergent-induced artifact [101]. In the meantime, other isolation procedures have been developed, which involve the use of different detergents, higher temperatures, or no detergents at all [102,103]. Although the precise lipid and protein composition of the raft membrane fraction varies somewhat depending on the procedure, the picture remains essentially the same: the plasma membrane fraction is heterogeneous and, under the experimental conditions chosen, is isolated in two fractions [104].

Attempts to directly visualize this heterogeneity in live cells, however, have thus far remained unsuccessful. Lipid probes that preferentially partition into either the l_o or l_d phase have been used to demonstrate phase separation in model systems [105–109], but the same probes have failed to detect phase separation in the plasma membrane of live cells. This could indicate that the model systems used do not adequately represent the behavior of the cell plasma membrane or that the domains formed are too small to be detected by light-based techniques. To date, this principal discrepancy between the behavior of model systems and biological membranes has not been resolved. In the current raft model, l_o–l_d domain separation represents a way to compartmentalize the plasma membrane, with the l_o phase providing a scaffold for the selective interaction of membrane associated proteins [1]. However, a biological membrane is a complex, multicomponent system and herein could lie one reason for the observed structural discrepancy between three component model systems and intact plasma membranes: if proteins or peptides preferentially associate with domain interfaces they will function as detergents, reducing the line tension of these interfaces and leading to domain dispersion [6,110]. The size of these dispersed domains could be well below the detection limit of optical microscopy.

Another concept that has emerged from membrane fractionation experiments is that signaling proteins associate preferentially with rafts (l_o domains) because they co-fractionate with the Chol/SM-rich, detergent-resistant membrane (DRM) fraction. Some of the proteins typically associated with this raft fraction, or peptide fragments derived thereof, have been studied in model membranes [35,36,39,111,112]. The proteins studied were membrane-spanning, lipid-anchored, or were to some degree embedded in the lipid bilayer. Of the proteins examined in model systems, none has been found to have a preference for the l_o phase; on the contrary, they were generally found to partition preferentially into the l_d phase. This observation has also been made repeatedly for surface-associated peptides [37,40–42]. Thus, if proteins do partition to rafts (l_o domains) in vivo, what forces drive that association?

The alternative is that proteins and peptides do not associate with l_o phases, in vivo or in vitro, but always partition preferentially into the l_d phase, in which case the detergent extraction experiments have been misleading. Proteins can appear in DRMs after Triton X-100 extraction for many reasons other than prior localization in putative rafts [113]. The concept of lipid rafts has been extremely important as a driving force for understanding biological membrane organization and rafts have been, at least operationally, assumed to coincide with the DRMs obtained in detergent extraction. However, so far, no raft has been convincingly demonstrated in a living cell.

Other alternatives should perhaps be considered. In view of the lipid composition of eukaryotic plasma membranes, partitioning of signaling proteins into l_d domains makes sense. Plasma membranes typically contain 20–50% Chol, depending on cell type [114]. Mixed, SM/Chol/PC bilayers containing Chol in the same concentration range form either a single l_o phase, or a l_o–l_d mixture in which l_o is the major fraction [115,116]. A membrane model (Fig. 6) in which the bulk of the membrane is formed by a 'sea' of l_o phase dotted with islands of l_d domains [117] is more in accordance with experiments in model systems. Recent studies strongly suggest that the plasma membrane is indeed mainly composed of l_o phase [117]. If this is the case, preferential partitioning of signaling proteins into small, dispersed l_d domains will achieve their selective enrichment and enhance their interaction, just as originally proposed for the raft domains. Partitioning into the major phase does not promote interaction of associated proteins, nor does it allow easy control of domain size.

We conclude this section by examining three examples that illustrate the difficulties and subtleties involved in understanding protein partitioning and interaction in mixed membranes with l_d–l_o domain coexistence. Most often, co-clustering of proteins in certain domains has been interpreted in terms of their preference for that type of domain. Another factor, however, is protein exclusion from domains. Thus, if two proteins cluster together in one of the phases of a two-phase system, this may simply mean that they are both excluded from the other phase but have no particularly high affinity for each other or for the lipid components of the phase they are found in. This type of exclusion has recently been suggested for the mechanism of interaction of a bacterial peptide with red blood cell membranes [42]. As our first example, we consider caveolin, the standard 'marker' for caveolae, which are domains related to rafts that form specialized membrane structures involved in endocytosis and a variety of other cellular transport events [118,119]. Just as many of the proteins thought to be raft-associated are acylated, caveolin is a triply-palmitoylated protein, which co-extracts with the Chol-rich lipid fraction during cell membrane fractionation. The isolated protein from animal cells was found to always contain tightly bound Chol [120]. The levels of caveolin in cells are closely related to those of Chol, and depletion of Chol leads to the disappearance of caveolae, where caveolin resides [118]. It appears, therefore, that the concentration of Chol in caveolae is very high and that this is essential for caveolin association with those structural domains [118]. However, these are specialized structures and caveolin may not interact well with the l_o phase in general; in fact, if the cell membrane is mainly in the l_o state, caveolin exclusion from bulk l_o phase appears necessary for it to concentrate in caveolae, where it forms extensive aggregates.

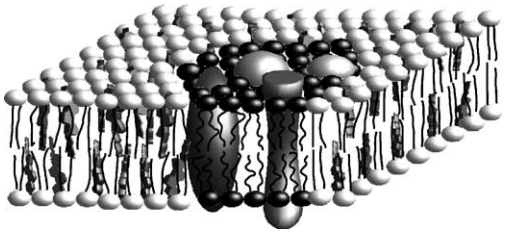

Fig. 6. Schematic representation of a biological membrane that is mainly in the l_o phase with dispersed l_d domains where integral and peripheral proteins partition preferentially.

As the second case, we consider experiments where green fluorescent protein (GFP) was found to co-cluster with caveolin if modified with a lipid anchor consisting of two acyl chains (palmitoyl, myristoyl, or one of each) [121]. Dual prenylation of GFP, on the other hand, led to its clustering but not to co-clustering with caveolin. Thus, double acylation was thought to target GFP to rafts, as opposed to prenylation which seems to prevent raft association [121,122]. However, acylation alone is not sufficient to target peptides to rafts and even acylated peptides supposed to be raft-associated do not partition preferentially into the l_o phase in model, mixed-phase bilayers [111,112]. Alternatively, co-clustering of acylated GFP with caveolin could occur if palmitoylated and myristoylated GFP were excluded from the l_o phase along with caveolin. The prenylated versions may mix even worse and form domains of their own, with a minimal amount of phospholipid.

Third, the IgE receptor, FcεRI, was shown to recruit a GFP-modified Lyn tyrosine kinase following receptor activation at 37 °C [123]. FcεRI co-fractionates with raft lipids upon activation and dimerization and is thought to partition into the l_o (raft) phase [123]. GFP coupled to a palmitoyl/myristoyl double anchor was recruited to the same spot, which agrees with the observations on co-clustering of lipid-anchored GFP and caveolin [121]. However, other lipid-raft markers, such as glycolipid-coupled proteins and fluorescent probes derivatized with long, saturated acyl chains, appeared to be uniformly distributed throughout the membrane, rather than co-clustered with FcεRI. It seems that the lipid probes are either not localized in rafts at 37 °C, or that their co-fractionation with the IgE receptor during membrane fractionation is fortuitous. Clearly, activation of FcεRI causes a reshuffling and clustering of membrane components that is not based on acylation alone, but whether FcεRI moves into and recruits other proteins to rafts in vivo remains unanswered.

7. Conclusion

Several lines of evidence summarized in this review point to an alternative to the raft hypothesis regarding the organization of eukaryotic plasma membranes: that the membrane exists predominantly as a mixed-phase system in which the l_o phase

represents the major fraction [117]. The minor fraction would consist of l_d domains (of which there are probably more than one type) dispersed throughout the l_o phase. As a rule, integral membrane proteins and peptides are excluded from the l_o and partition preferentially into the l_d phase [35–39]. The same is true of surface-binding peptides [37,40–42]. The l_d domains may be further stabilized by peripheral proteins that selectively associate with their lipid components [32,33] or by proteins that associate with domain interfaces [110]. Any protein that partitions preferentially to the interface will stabilize it. A more stable interface corresponds to weaker repulsive interactions between the two domains, which means that the extent of interface can increase. The summed interface of many small domains is larger than that of a few large ones. Therefore, protein partitioning to the interface would explain why they are not observed in cells by fluorescence microscopy: if the domain size is less than half the wavelength of the light used, the domains will not be detected. For example, the IgE receptor could be localized at domain interfaces in its inactive, monomeric state. Upon dimerization and aggregation it could be excluded from the interfacial region which would cause several small l_d domains to coalesce in order to minimize the interfacial free energy. Cross-linking of peptides in lipid vesicles has recently been shown to trigger formation of large domains where none could be observed before by fluorescence microscopy [124]. In this concept, proteins would play a very dynamic role in modulating the size of l_d domains.

If the protein–lipid interactions are altered following receptor dimerization, aggregation or phosphorylation, the chemical potential of the lipid species involved will change. Re-equilibration of the lipids in the plane of the membrane and across the bilayer will occur until the chemical potentials are again equal. In this manner, changes in the domain composition of the outer leaflet of the bilayer can be chemically coupled to changes in lipid distribution in the inner leaflet. For example, clustering of negatively charged lipid species in the inner leaflet, which could arise in response to membrane association of PS-binding proteins, such as annexins [125] or C2 domains [32,33], could then be communicated to the outer leaflet of the bilayer through changes in the chemical potential of one lipid common to both sides of the bilayer. Subsequently, other membrane proteins could be recruited to these domains, because they bind to the newly formed lipid domain or to other proteins already associated with that domain. Given the fluid character of the biological membrane (l_d and l_o areas), it will be able to respond rapidly to an incoming signal through the formation and dissipation of structurally and compositionally distinct lipid domains. Lipids interact with other lipids through nearest-neighbor contacts. Most important, each lipid interacts with multiple neighbors, typically about six. This allows for a change to be communicated and a cooperative response to follow. The lipid–lipid interactions are typically small in magnitude (about 200 cal/mol), but because of cooperativity they can lead to large changes in protein and lipid distributions on the membrane surface [32]. Moreover, because these interactions are weak, domain formation is a reversible process and can constitute a response to a signaling event. This response can be triggered or shut down by a small variation in the concentration of Ca^{2+}, a hormone, or a cytokine [33].

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