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MODEL SYSTEMS, LIPID RAFTS, AND CELL MEMBRANES[1]

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■ **Abstract** Views of how cell membranes are organized are presently changing. The lipid bilayer that constitutes these membranes is no longer understood to be a homogeneous fluid. Instead, lipid assemblies, termed rafts, have been introduced to provide fluid platforms that segregate membrane components and dynamically compartmentalize membranes. These assemblies are thought to be composed mainly of sphingolipids and cholesterol in the outer leaflet, somehow connected to domains of unknown composition in the inner leaflet. Specific classes of proteins are associated with the rafts. This review critically analyzes what is known of phase behavior and liquid-liquid immiscibility in model systems and compares these data with what is known of domain formation in cell membranes.

CONTENTS

[1]Abbreviations: AFM, atomic force microscopy; DOPC, 1,2-dioleoylphosphatidylcholine; DPPC, 1,2-dipalmitoylphosphatidylcholine; DPPE, 1,2-dipalmitoyl phosphatidylethanolamine; ESR, electron spin resonance; F-DOPE, N-fluoresceincarboxamido-1,2-dioleoylphosphatidylcholine; F-DPPE, N-fluoresceincarboxamido-1,2-dipalmitoylphosphatidylcholine; FRET, fluorescence resonance energy transfer; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; SpM, sphingomyelin; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine.

INTRODUCTION

The lipid bilayer that forms cell membranes is a two-dimensional liquid, the organization of which has been the subject of intensive investigations for decades by biochemists and biophysicists. Although the bulk of the bilayer has been considered a homogeneous fluid, there have been repeated attempts to introduce lateral heterogeneities, lipid microdomains, into our model for the structure and dynamics of the bilayer liquid (22, 37, 38, 89). The identification of boundary lipids around proteins created excitement in the 1970s but disappeared into the ESR realm of research (48) when it was shown that on the timescale of NMR experiments the boundary lipids could not be observed.

The realization that epithelial cells polarize their cell surfaces into apical and basolateral domains with different protein and LIPID compositions in each of these domains initiated a new development that led to the "lipid raft" concept, which has stirred up commotion and a lively controversy in the field (78, 80). The concept of assemblies of sphingolipids and cholesterol functioning as platforms for membrane proteins was promoted by the observation that these assemblies seemed to survive detergent extraction (8). This operational breakthrough caused a flood of papers in which raft association was equated with resistance to Triton-X100 extraction at 4°C. The addition of another criterion, depletion of cholesterol using methyl-β-cyclodextrin (33, 72), leading to loss of detergent resistance, added even more papers to the field. But at the same time these new developments added further controversies. Do lipid rafts exist in membranes or are they just artifacts of detergent extraction? Despite, or maybe because of all this controversy, the field has matured and many new methods have added substance to the definition of lipid domains in cell membranes and have significantly improved our understanding of heterogeneity in membrane systems. There is now increasing support for a role of lipid assemblies in regulating numerous cellular processes including cell polarity, protein trafficking, and signal transduction. As Edidin (16) aptly put it, "Despite great reservations about the interpretation of classical operational definition of lipid raft components and function, we are left with. . .the stubborn insistence by cells that raft lipids can be organized and segregated into membrane domains." In this review we try to integrate what we have learned about phase immiscibility in model systems with what we know of lipid domain organization in cell membranes today.

WHAT DO WE LEARN FROM MODEL SYSTEMS?

Cell membranes are two-dimensional liquids. Thus, lateral heterogeneity implies liquid-liquid immiscibility in the membrane plane. Hydrated lipid bilayers undergo phase transitions as a function of temperature. These transitions, which occur at defined temperatures for each lipid species, always involve some change in the order of the system. The most important of these transitions is the so-called main or chain-melting transition, in which the bilayer is transformed from a highly ordered quasi-two-dimensional crystalline solid to a quasi-two-dimensional liquid. It involves a drastic change in the order of the systems, in particular the translational (positional) order in the bilayer plane and the conformational order of the lipid chains in a direction perpendicular to this plane. Translational order is related to the lateral diffusion coefficient in the plane of the membrane, and conformational order is related to the *trans/gauche* ratio in the acyl chains. The main transition has been described as an ordered-to-disordered phase transition, so that the two phases may be labeled solid ordered (s_o) below the transition temperature and liquid disordered (l_d) above that temperature. An important advance was the realization that cholesterol and phospholipids could form a liquid-ordered (l_o) phase that could coexist with a cholesterol-poor liquid-disordered (l_d) phase, thereby permitting phase coexistence in wholly liquid phase membranes (34, 35). Sterols do so as a result of their flat and rigid molecular structure, which imposes a conformational ordering upon a neighboring aliphatic chain (68), when the sterol is the nearest neighbor of the chain, without imposing a corresponding drastic reduction of the translational mobility of the lipid (56). Owing to the fact that the sterol does not fit exactly in the crystalline lattice of an s_o (gel) lipid bilayer phase, it will (if it dissolves within this phase) disrupt the crystalline translational order without significantly perturbing the conformational order. Thus, cholesterol at adequate molar fractions can convert l_d or s_o lipid bilayer phases to liquid-ordered (l_o) phases. This conceptual and experimental progress in model membrane research set the stage for detailed analysis of the molecular basis for liquid-liquid immiscibility.

The degree of translational freedom (lateral mobility) of the lipid molecules in an l_o phase is similar to that in an l_d phase, the lateral diffusion coefficient being only reduced by a factor of about 2–3 in the former compared with the latter (2). The conformational order of the lipid hydrocarbon chains in an l_o phase is, however, more similar to that of the s_o phase (21). Exactly by how much the conformational order of an l_o phase differs from that of an s_o or an l_d phase has yet to be defined for most lipid bilayers that can serve as models for biological membranes. This is particularly true for the interaction of cholesterol with lipids containing unsaturated acyl chains where it is not clear whether cholesterol configurationally orders the *cis*-unsaturated chain. POPC, for example, forms an l_o phase with cholesterol only at a molar fraction of $\geq$40 mol % cholesterol (49), and the translational diffusion coefficient is about twofold lower in this phase than in the l_d phase formed from pure POPC (95). The same effect is achieved in bilayers formed from DMPC, DPPC,

or SpM (above the transition temperature) at ~25–30 mol % cholesterol (1, 67, 91). It also remains to be defined in exactly what respect l_o phases formed from saturated and unsaturated lipids are different from or similar to each other. In spite of these uncertainties, there is broad agreement that liquid-ordered and liquid-disordered domains can coexist in hydrated bilayers containing cholesterol and saturated phospholipids and that cholesterol plays a key role in l_o phase formation (1, 67–69, 91).

There has been considerable discussion in the literature as to how large these domains can be. Feigenson & Buboltz (18) have shown that the domains can be large enough to be visible under the light microscope (i.e., larger than 500 nm across) or have dimensions that are below the limit of resolution of the optical microscope. There is no fundamental requirement that any phase in a heterogeneous system not be divided into several parts or domains, although their exact thermodynamic description becomes unreliable when the domains become too small. When phases coexist, there is a mismatch of properties at the interface where two phases meet. This mismatch results in a tension (a line tension in two-dimensional systems such as lipid bilayers, and a surface tension in three dimensions) that works to reduce the interface of mismatch and, as a result, causes a macroscopic phase separation. In lipid bilayers, the dipole moments of the lipid molecules in each monolayer are, because of geometric constraints of the system, oriented in the same direction so that a repulsive interaction between the lipid molecules results. This repulsion works against the line tension at the phase boundary so that the domain size and shape is a balance between these two contradictory forces (84). Additionally, effects such as "surfactancy" (the ability of surface-active agents to reduce the interfacial surface tension at the interface between two phases as is commonly seen in emulsions) can reduce the interfacial tension to the point that it no longer drives the system to a macroscopic phase separation. The extreme complexity of the chemical composition of biological membranes makes surfactancy at the domain boundaries a likely possibility. Many of the protein and lipid components of biological membranes might actually preferentially partition into domain boundaries.

HOW DOES CHOLESTEROL INTERACT WITH NEIGHBORING PHOSPHOLIPIDS?

McConnell and coworkers (51, 52), in a series of papers published over the past four years, have suggested that cholesterol can form reversible oligomeric chemical complexes with phospholipids with a fundamental stoichiometry of between 3 phospholipids per 2 cholesterols and 2 phospholipids per cholesterol in a cooperative manner with a cooperativity factor of between 4 and 12. Thus a single complex, depending upon the exact fundamental stoichiometry and the cooperativity factor, could have between a maximum of 60 molecules, 24 of which would be cholesterol, and a minimum of 12 molecules, 4 of which would be cholesterol.

The formation of these complexes has been proposed to lead to the "condensing" effect of cholesterol, by which the surface area occupied by the phospholipid is decreased by interaction with cholesterol. The condensed complex is proposed to be stable at temperatures below the phase transition temperature of the phospholipids and to dissociate endothermically above this temperature, resulting in the broad endothermic process seen in calorimetric studies and generally attributed to a transformation of an l_o phase to an l_d phase. Effectively, in this view, the micelle-like cholesterol-lipid aggregates would exist in an l_o phase below the phase transition temperature of the lipid. These conclusions are derived from studies on mixed monolayers, which show phase diagrams with two upper miscibility critical points, and a proportional increase in the chemical potential of cholesterol above the complex stoichiometry. It is assumed that the behavior of monolayers can be extrapolated to bilayer membranes.

Huang & Feigenson (32) have presented another model of cholesterol-phospholipid mixing on the basis of multibody interactions instead of pair-wise additivity of nearest neighbor interactions. They propose a model in which nonpolar cholesterol relies on shielding by the lipid polar head groups to avoid the energetically unfavorable contact with water. The phospholipid head groups effectively act as "umbrellas" for the cholesterol molecules below them. As the concentration of cholesterol increases, acyl chains and cholesterol become more tightly packed because they share the limited space under the phospholipid head groups. When the head groups cannot cover additional cholesterol molecules, the solubility limit is reached and cholesterol precipitates to form a separate cholesterol monohydrate crystalline phase (31). The interactions of glycerophospholipids with cholesterol decrease in the following order: phosphatidylcholine > phosphatidylserine > phosphatidylethanolamine. Cholesterol has a preference for interaction with lipids that have fully saturated aliphatic chains when compared with lipids that have one or more unsaturated chains (75). The former can be more conformationally ordered by the molecular flatness of the rigid sterol ring structure than the latter. Also, it is to be expected that the position of a double bond in an aliphatic chain and its configuration will be of significance with regard to the interaction of this chain with cholesterol. Unsaturation beyond the fourteenth to fifteenth carbon atom in the chain is likely to have little if any effect upon interaction with cholesterol, since the chain conformation beyond this position is not likely to conflict with the rigid and flat ring structure of the sterol.

Multiple unsaturation of the fatty acyl chain significantly decreases the interaction with cholesterol in both bilayers and monolayers. This can be observed by comparing the areas of phospholipid molecules at the air-water interface in the absence and presence (at a molar ratio of about 1:1) of cholesterol in monolayers at pressures equivalent to those in cell membranes. POPC condenses from an area of 0.61 nm^2 to an area of 0.42 nm^2; saturated DPPC condenses from an area of 0.46 nm^2 to an area of 0.39 nm^2; 18:1-SpM condenses from an area of 0.57 nm^2 to an area of 0.40 nm^2; and egg SpM condenses from an area of 0.49 nm^2 to an area of 0.40 nm^2 (81).

A considerable amount of data demonstrate that cholesterol interacts more favorably with SpM than with other phospholipids such as PC in both bilayers and monolayers (9, 57, 63). The rate of cholesterol desorption from SpM-containing bilayers is slower than desorption from membranes with saturated and acyl chain-matched PCs. Cholesterol preferentially abolishes the phase transition of SpM in binary mixtures with other phospholipids, indicating a preferential interaction with the SpM molecules. The water permeability is lower in SpM/cholesterol bilayers than in PC/cholesterol membranes, indicative of denser lateral packing in the former system. In monolayers, the oxidation of cholesterol by cholesterol-oxidase was reduced in SpM-containing monolayers compared with PC-containing monolayers. Another sensitive indicator of SpM-cholesterol interactions is the measurement of the interfacial elasticity in monolayers (9). Recent studies (82) demonstrate that the measured elasticity-reducing effect of cholesterol on SpM is significantly stronger than on PCs with fully saturated and matched acyl chains.

The reason for the preference of cholesterol for SpM rather than PC lies in the structural differences of these lipid molecules. SpM is a derivative of sphingosine (D-*erythro*-2-amino-*trans*-4-octadecene-1,3-diol). A fatty acid is attached to the 2-amino group of sphingosine by formation of an amide. In mammalian sphingolipids, this fatty acid is variable but is generally a 16- to 24-carbon saturated chain with a small fraction of $24{:}1^{\Delta 15 cis}$ chains depending upon the source tissue. Thus, in terms of the apolar part of the molecules, these lipids present rather long fully saturated aliphatic chains (with a *trans* unsaturation in the sphingosine base). When a *cis*-unsaturation appears, it is located deep down toward the bilayer midplane. Monounsaturated PCs, the major species in mammalian cell membranes, usually have a *cis* double bond in the Δ^9 position of one of the fatty acids and may have more double bonds lower down the acyl chain. Therefore, in terms of what was discussed above in regard to cholesterol interactions with lipids, sphingolipids present ideal partners for interaction with cholesterol. The polar portions of the molecules are also of interest from this perspective. At the membrane-water interface the sphingosine-based lipids have a hydroxyl group and an amido nitrogen, both of which can act as hydrogen-bond donors as well as acceptors. These groups can, together with the fatty acid carbonyl functions, hydrogen bond to water and to the other lipids. In comparison the PCs, having only hydrogen-bond acceptors in the form of the ester carbonyl functions and oxygen atoms, have a lower versatility in hydrogen bonding with the water molecules in the aqueous interface and are not able to directly hydrogen bond with their lipid neighbors in the bilayer. If hydrogen bonding is assumed to be a structure-stabilizing interaction, it must be concluded that sphingolipids have a greater propensity for ordered structures in the membrane. The most abundant sphingolipid in membranes is SpM, which has a phosphorylcholine head group attached to the 1-hydroxyl group of the acyl-amidosphingosine (ceramide). This phosphorylcholine head group, analogous to the phosphorylcholine head group in PCs, can eventually serve as an effective umbrella in terms of favoring the solubilization of cholesterol in a SpM bilayer.

Systematic alteration of the functional groups in the SpM molecule have identified the amide linkage to be important for the interaction with cholesterol, possibly

owing to hydrogen bonding to the cholesterol 3-OH group. Recent infrared spectroscopic studies on egg SpM showed that the amide-I band was shifted to lower wave numbers in the presence of cholesterol. This was interpreted to be indicative of a change in the hydrogen-bonding pattern of the SpM amide group, possibly connecting to the hydroxyl group of cholesterol (90). However, results from NMR (24) and fluorescence (29a) spectroscopies indicate that there may be no chemical complex formed between SpM and cholesterol.

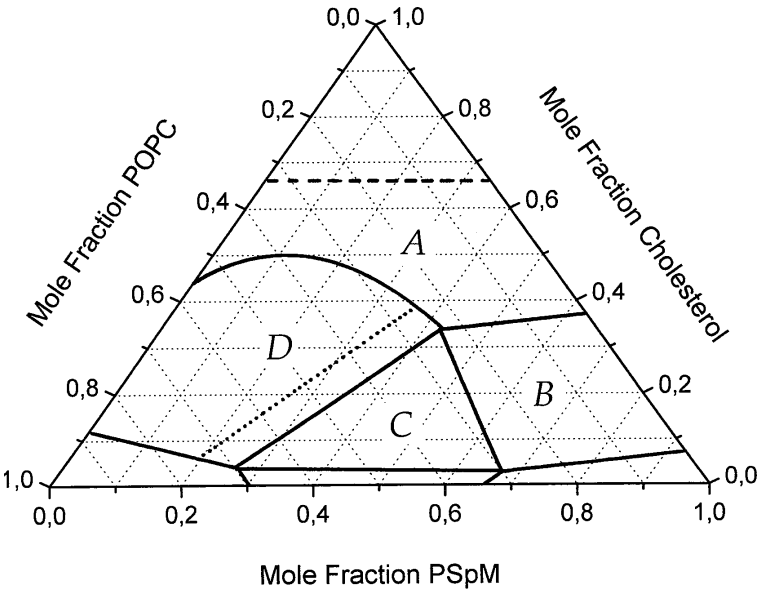
The differential interaction of cholesterol with SpM and unsaturated PC is convincingly reflected in the recent ternary phase diagram of palmitoyl-SpM, POPC, and cholesterol (11). This is the first phase diagram of lipid bilayers formed from the ternary mixture of the three major lipid components of the exoplasmic leaflet of mammalian plasma membranes and, although rather simple in compositional terms compared to a biological membrane, it is a good starting point for our understanding of the physical complexity of the exoplasmic leaflet of the plasma membrane. The phase diagram (Figure 1) shows that only l_d and l_o phases coexist at concentrations mimicking the composition of the outer leaflet of cell plasma membranes. Both coexisting phases contain all three chemical constituents of the system, the l_o phase richer in SpM and the l_d phase richer in POPC.

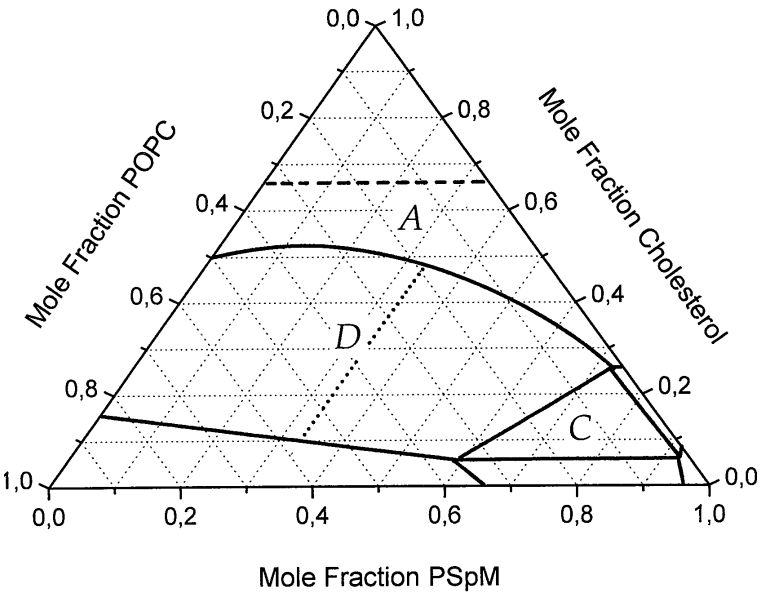
Other studies, notably those of Silvius et al. (76) and Feigenson & Buboltz (18), have also shown liquid-liquid (l_o/l_d) immiscibilities in ternary mixtures containing cholesterol and two PCs, one of which had long saturated acyl chains. In the latter study, the liquid-liquid coexistence was shown to form domains that were visible in the light microscope in certain parts of the phase diagram and domains of dimensions below the limit of resolution of optical microscopy in other regions of the diagram. When fluorescent probes that partition preferentially into one of the coexisting liquid phases and optical microscopy were used, l_o/l_d phase coexistence has been visualized in monolayers at the air-water interface, in supported monolayers and bilayers, in planar lipid bilayers, and in giant unilamellar vesicles containing SpM, unsaturated PCs, and cholesterol (13, 14, 66). Even lipid extracts from epithelial brush border membranes form monolayers or bilayers, which display liquid-liquid immiscibilities (13). Liquid-liquid immiscibility and the formation of domains clearly depend on cholesterol concentration. Treatment of such membrane systems with methyl-β-cyclodextrin abolishes the domains. Dietrich et al. (14) demonstrated that two raft components, the ganglioside GM1 and the GPI-anchored protein Thy-1, significantly partitioned into the raft-like liquid-ordered domains in supported monolayers. In similar monolayers the partitioning of monomers and antibody cross-linked dimers of the fluorescein-labeled lipids, F-DPPE (saturated acyl chains) and F-DOPE (unsaturated acyl chains), between domains of an l_o and an l_d phase were compared. Both probes partitioned preferentially into the l_d phase. The estimates for the equilibrium partition coefficients between the l_o and l_d phases were 0.07 for F-DOPE and 0.14 for F-DPPE. A report from the same laboratory (13) indicated that F-DPPE partitioned about equally between l_o and l_d domains in supported bilayers. Dimerizing the probes with antifluorescein antibody resulted in a preferential partitioning of the F-DPPE into the l_o domains, whereas the F-DOPE remained in l_d domains (14). This is

an interesting finding because it could be interpreted to mean that cross-linking (dimerization or multimerization) of some membrane constituents can drive them into rafts. Silvius and coworkers (92) used a fluorescence-quenching assay to study the partitioning of lipidated peptide species into liquid-ordered domains in lipid bilayers composed of DPPC, a spin-labeled unsaturated PC, and cholesterol. They demonstrated that peptides incorporating isoprenyl groups or multiple unsaturated acyl chains showed a low affinity for l_o domains. However, peptides containing multiple S- and/or N-acyl chains or a cholesterol residue plus an N-terminal palmitoyl chain displayed significant partitioning into l_o domains under the same conditions.

Another interesting tool for studying lipid domains is AFM because it displays the surface landscape of a supported monolayer or bilayer with or without proteins. When this technique is used, it becomes possible to visualize l_o/l_d phase coexistence as well as to study the partitioning of gangliosides and GPI-anchored proteins in bilayers that show this phase coexistence. An l_o domain in a lipid bilayer composed of $C_{18:0}$-SpM and cholesterol should have a thickness of 4.6 nm, and a bilayer containing di-$C_{18:1}$-PC is 3.5 nm thick. Inclusion of cholesterol increases the thickness of the di-$C_{18:1}$-PC bilayer to 4.0 nm. In contrast, cholesterol does not affect the thickness of a SpM bilayer. Studies using AFM on supported bilayers have been able to visualize domain formation by the height difference of l_d and l_o domains. Using 1:1 mixtures of DOPC and SpM, Rinia et al. (64) saw domains increasing in size and area coverage with increasing concentration of cholesterol. The height difference between the ordered domains and the surrounding fluid bilayers decreased from 1 nm in the absence of cholesterol (s_o phase SpM domains) to 0.8 nm at 50 mol % cholesterol (l_o phase SpM-cholesterol domains). Milhiet et al. (54) used POPC and SpM (1:3) and observed microscopic separation up to 25 mol %

Figure 1 Phase diagrams for fully hydrated lipid bilayer membranes prepared from ternary mixtures of palmitoyl-SpM (PSpM), POPC, and cholesterol (adapted with permission from Reference 11). An experimentally obtained phase diagram is shown for 23°C (*upper panel*) and a hypothetical phase diagram is shown for 37°C (*lower panel*). In each panel, the dashed line at 66 mol % cholesterol is the hypothetical limit of cholesterol solubility in the bilayer phase. Above 66 mol %, excess cholesterol separates out as a cholesterol monohydrate crystalline phase (32). The major regions in the phase diagram have been labeled *A* through *D* to avoid cluttering. Starting from region *A*, which corresponds to a single l_o phase, and proceeding clockwise, the regions along the axes of the diagram correspond to a two-phase region with $s_o + l_o$ phase coexistence (marked *B*), a single s_o phase region (unmarked), a two-phase region with $s_o + l_d$ phase coexistence (unmarked), a single l_d phase region (unmarked), and a two-phase region with $l_d + l_o$ phase coexistence (marked *D*). The central tie-triangle (marked *C*) is a three-phase region with $l_d + l_o + s_o$ phase coexistence. In the region marked *D*, a possible tie-line passing through the 1:1:1 composition is indicated by a dotted line in both panels. For further details the reader is referred to the original work (11).





cholesterol with height differences of the domains of 0.3–0.4 nm. Both studies were performed on compositionally symmetric bilayers. Yuan et al. (96) used bilayers of SpM/DOPC/cholesterol 1:1:1 to which 1% of the ganglioside GM1 was added. In this case, a successive deposition of two monolayers onto the support formed an asymmetric supported bilayer in which the first (lower) monolayer was of DPPE and the second (upper) monolayer was prepared from the SpM/DOPC/cholesterol mixture. They saw little height difference between the ordered SpM/cholesterol-rich and the disordered DOPC-rich phases in the bilayer. The GM1 molecules could be visualized as small islands of 200–300 nm in diameter and about 2 nm above the bilayer matrix. Thus, ganglioside formed small aggregates presumably within the ordered phase not detected by AFM. Saslowsky et al. (70) studied the incorporation of GPI-anchored placental alkaline phosphatase (PLAP) into supported bilayers with AFM. As in the previous studies, they could see the formation of domains in equimolar mixtures of SpM, DOPC, and cholesterol that had a height difference of 0.7 nm. When PLAP was reconstituted into liposomes made from this mixture and these liposomes were transferred to a mica surface to form a supported bilayer, lipid domain formation was seen with AFM and PLAP was seen protruding from the elevated liquid-ordered domains. PLAP mostly occupied a molecular volume corresponding to a dimer. However, larger protruding particles that could correspond to PLAP oligomers were also seen. In all the AFM studies, domain formation was also seen with the SpM/PC mixtures without cholesterol. These domains probably correspond to s_o/l_d phase coexistence in which the s_o phase was rich in SpM and the l_d phase was rich in DOPC.

AFM studies and optical microscopy on model bilayers have also demonstrated that the outer and the inner leaflets in the liquid-ordered domains are coupled in bilayers prepared from mixtures containing SpM, unsaturated PCs, and cholesterol. However, in cell membranes, the inner leaflet is not composed of the same lipids as the outer leaflet. The inner leaflet contains phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphoinositides. Wang & Silvius (93) studied mixtures of inner leaflet lipids together with cholesterol and could not detect formation of segregated liquid-ordered domains employing a fluorescence-quenching assay. Keller et al. (40), on the other hand, could see phase segregation when small amounts of SpM were present. This is interesting because although SpM is asymmetrically distributed in cell membranes, a fraction is in the inner leaflet.

HOW DO THE MODEL MEMBRANE STUDIES RELATE TO CELL MEMBRANES?

The studies with model monolayers and bilayers have demonstrated that mixtures of lipids mimicking the composition of the outer leaflet of plasma membranes exhibit liquid-liquid immiscibility and segregate into l_o and l_d domains. SpM, which carries mostly saturated hydrocarbon chains, preferably partitions

with cholesterol into l_o phase domains, segregating from unsaturated PCs, which are the major constituents of domains of the l_d phase. The size of l_o domains seems to vary greatly depending on temperature, pressure, and composition. The domains can be large enough to be visible in the optical microscope or smaller than the limit of resolution of light microscopy (18). Cell membranes on the other hand have an extremely complex lipid composition and the fact that lipids are continuously being added to or removed from the membrane does not simplify the situation. A typical mammalian cell plasma membrane is constituted from about eight major classes of lipids including cholesterol (94). In each of these classes, with the exception of cholesterol, there is a great variation in the acyl chain composition so that the total number of chemical species that composes the lipid bilayer of these membranes is large. Recent mass spectrometric analysis demonstrated that only for PC were there close to 100 molecular species present in MDCK cells (16a). The binary and ternary lipid systems studied so far can provide only the boundary conditions within the framework of which we may attempt to extrapolate toward understanding the complexity of cellular membranes.

Rafts have been proposed as lipid platforms of a special chemical composition (rich in SpM and cholesterol in the outer leaflet of the cell membrane) that function to segregate membrane components within the cell membrane. As discussed below, they are understood to be small (estimates of size vary considerably) but they can be coalesced under certain conditions. An estimate of the number of rafts in a cell plasma membrane (based upon lipid composition, total membrane size and size of the raft domains) would be on the order of 10^5 to 10^6 (77). Their specificity with regard to lipid composition is reminiscent of phase separation behavior in heterogeneous model membrane systems. In fact, many of their properties with regard to chemical composition and detergent solubility are similar to what is observed in model systems composed of ternary mixtures of unsaturated PC, SpM (or a long-chain saturated PC), and cholesterol (11). Rafts could be considered domains of an l_o phase in a heterogeneous l phase lipid bilayer composing the plasma membrane. It is not clear at this time what the other coexisting phase(s) is. There is consensus that the biological membrane is a liquid, so s_o phase coexistence may be ignored for most cases. Whether the other phase(s) is an l_d or l_o phase depends upon the chemical identity of the phospholipids that constitute this phase(s) and the molar fraction of cholesterol in them. At this point it would appear most correct to equate rafts with a liquid-ordered phase and refer to the rest of the membrane as the nonraft liquid phase.

Although there is increasing consensus that lipid rafts do exist in cell membranes (7, 16, 45, 46, 75, 79), this field of research is still in its infancy. There is an ongoing debate on the size and lifetime of rafts (20, 54b). This is not understood even in model systems, but there is evidence that domain sizes of l_o phases may depend on their composition. When proteins are incorporated into these domains, still another level of complexity is introduced. In fact, there are contradictory views of how rafts organize themselves. One view maintains that raft proteins act as

nucleation sites for raft domains. Anderson & Jacobson (4) suggested that raft proteins organize "shells" composed of maximally 80 molecules of cholesterol and sphingolipid around themselves. Assuming a GPI anchor for the raft protein, the shell would have about 4 to 5 layers of lipid around the anchor. The shells are postulated to be what facilitates the proteins to eventually integrate into large rafts. This hypothesis raises certain fundamental questions: First, the association of the first layer of lipids with the protein membrane anchor would require a rather strong interaction of the shell lipids (which presumably are the same as those typically encountered in rafts) with the anchor. No such specificity has yet been demonstrated, and all that is known about "boundary" lipids hardly indicates that such a strong interaction is probable. Boundary lipid-protein interaction, limited on the average to less than one millionth of a second, has not been demonstrated, with the exception of a few cases, to show any significant specificity (48). Second, formation of the succeeding layers in the shell would require that the lipids of the shell have a rather strong tendency to associate. If this were the case, one wonders why the raft protein would be needed at all. An alternative is to view the proposed "shell" as a solvation shell around the raft protein, in which case its composition should reflect the chemical composition of the entire lipid bilayer phase into which the protein is inserted. It is not clear in this case why this shell would have any particular affinity for larger rafts.

Another view is based on studies by Kusumi and coworkers (86), who have developed high-speed video microscopy to study lipid and protein movement in the plasma membrane of living cells. The diffusive walk of 40-nm beads coupled by antibodies to lipids and to GPI-anchored lipids was monitored at the time resolution of 25 μs. Subczynski & Kusumi (86) propose that raft proteins such as GPI-anchored proteins form a small raft containing only a few raft lipids with a lifetime of less than 1 ms. These dynamic entities can coalesce and cluster to stabilized or signaling rafts.

A third view of raft organization maintains that lipid rafts are manifestations of the thermodynamic properties of the lipid bilayer of the membrane, namely, that this lipid bilayer is a heterogeneous system (78). The phase coexistence is manifested as the existence of domains of the coexisting phases. A subset of these domains, corresponding to one particular phase with defined physical and chemical properties, are the rafts. Not only are these domains proposed to be condensed complexes of cholesterol and sphingolipids around proteins, but they are also larger. How large? The use of different techniques has given variable size estimates ($<$700 nm) for lipid rafts in membranes (15, 41, 61, 62, 74). However, if one analyzes the different size determinations, it becomes obvious that they reflect different states of rafts. The large, 700-nm size was obtained from measurements by single molecule tracking on the plasma membrane of human aortic smooth muscle cells (74). Rafts of this size could not be seen in other cell types and might have been due to raft clustering into caveolar or other large raft domains (G. Schütz, personal communication). An estimate of 200-nm raft size was obtained by single particle tracking. This work, however, used beads to which multiple antibodies

were bound, and multiple contacts with GPI-anchored proteins on the cell surface could have caused raft aggregation (15).

So what could the intrinsic raft size in the resting state (nonclustered) be? When photonic force microscopy (30) was used, a larger raft size was obtained (61). This method employs a laser trap to confine the motion of a bead bound by antibodies to raft and nonraft proteins into a small area ($<$100 nm) and uses high-resolution single particle tracking to estimate the local viscous drag upon the particle. Multiple attachment of the bead to the plasma membrane was carefully avoided by titration with soluble antigen so that one single antibody was assumed to bind to the surface antigen. The viscous drag measured for the three different raft-associated proteins, two with a GPI-anchor and one transmembrane protein, was independent of the type of membrane anchor and was significantly larger than the local viscous drag of the nonraft proteins. After cholesterol depletion by methyl-β-cyclodextrin, the viscous drag upon the raft-associated proteins decreased to the level of the nonraft proteins. In contrast, these latter proteins did not change their behavior after cholesterol depletion. The mean diameter of the raft protein assemblies obtained from these measurements was 52 ± 26 nm, the platform diffusing as a single entity for minutes. Recently, Prior et al. (62) used a completely different method based on expression of a lipid raft marker partitioning into the inner leaflet of plasma membrane rafts and on immunoelectron microscopy, coupled with spatial point pattern analysis. Their results indicated a diameter of 44 nm for the rafts, occupying 35% of the plasma membrane area. A platform of this size would contain around 3000 lipid molecules and probably not more than 10 to 20 protein molecules. Kusumi's measurements (see above) give an even smaller size. A small raft size that results in a platform that accommodates less than two copies of the same protein species might explain the lack of FRET between GPI-anchored proteins in the experiments reported by Kenworthy et al. (41). On the other hand, Mayor and colleagues (87, 74a) have observed FRET between GPI-anchored folate receptors using a more sensitive detection method, and this may be due to a minority population containing clusters of receptors. Also Saslowsky et al. (70) saw some PLAP oligomers in their AFM experiments with reconstituted proteoliposomes.

Whatever the intrinsic size of rafts in cell membranes may be, the important question is what prevents the raft from coalescing to a microscopically visible phase separated by the smallest possible boundary from the other coexisting phase or phases. A part of the answer may lie in the dimensionality of the system. The line tension/mass ratio in a two-dimensional domain may not be as high as the surface tension/mass ratio in a three-dimensional liquid. It is the line tension in two dimensions and the surface tension in three dimensions that promote spontaneous domain coalescence. As discussed above, another part of the answer could lie in surface-active effects at the interface between coexisting domain boundaries in lipid membranes. These effects, in which the role of proteins and minority lipids that may accumulate at the domain boundaries is important, would effectively reduce domain size somewhat analogously to what happens in a three-dimensional micro-emulsion. The raft phase composition may also constitute another reason.

For example, unsaturated phospholipids are components of the raft phase. These lipids prefer the nonraft phase and this may limit the domain size when the probability of encountering them in the raft domain perimeter exceeds a certain value.

Although there is disagreement on the size and the lifetimes of the lipid assemblies in which raft proteins are embedded while floating in the nonraft liquid bilayer phase, there is consensus that lipid rafts can cluster into larger domains that are visible in the light microscope after oligomerization or cross-linking. There are numerous examples of such a raft clustering process, one of which is the formation of caveolae (3, 43). Caveolae are flask-shaped surface invaginations of 50- to 100-nm diameter, containing raft lipids and the protein caveolin. The caveolins form oligomers and formation of caveolae is probably driven by protein polymerization in the membrane phase.

Another process involving raft clustering is influenza virus envelope formation. The membrane of this virus is acquired by budding through the host cell plasma membrane. Two glycoproteins, hemagglutinin and neuraminidase, are tightly packed in the viral membrane. Both are raft-associated; hemagglutinin was shown by photonic force microscopy to be in 50-nm rafts (61, 71). The viral membrane is enriched in cholesterol and SpM compared with the host cell membrane from which it was formed. The budding process is thought to be driven by association of the cytoplasmic tails of the viral membrane glycoproteins with the influenza virus matrix M1 protein that drives the budding process (23, 97). The working model is that the binding of the M1 protein to the cytoplasmic tails protruding from the inner leaflet of the plasma membrane facilitates docking of the M1 protein into an assembly complex. The complex then interacts with other M1 protein subunits associated with the ribonuclein particles to form a complete virus particle. Clustering of rafts containing the viral glycoproteins is assumed to be accomplished by M1 protein polymerizing with itself. Other rafts and raft proteins are excluded from the bud because they do not interact with the M1 protein lattice and are sterically hindered from entering the growing viral envelope. An alternative mechanism could involve association of the M1 protein with the domain on the inner leaflet of the raft domain similar to the way in which spectrin associates with phosphatidylserine on the inner surface of the erythrocyte membrane. Because this protein has a propensity to polymerize with itself, this polymerization leads to an aggregation of the inner leaflet domain of rafts and a consequent aggregation of rafts in the outer leaflet. The viral glycoproteins, hemagglutinin and neuraminidase, partition favorably into rafts and therefore get concentrated in this aggregated domain. A favorable interaction between the cytoplasmic tails of these proteins and the aggregated M1 matrix attached to the cytoplasmic leaflet further stabilizes the entire structure. Association of the viral ribonuclein particle with the M1 polymer matrix will induce a curvature of the membrane that, together with the mismatch at the boundary of the patched raft phase with the other coexisting phase or phases, will favor the budding process (44).

The specificity of raft clustering is nicely illustrated by experiments in which raft clustering is induced by cross-linking with specific antibodies on the surface

of living cells. If two raft proteins are cross-linked by antibodies, they will form overlapping patches in the plasma membrane. However, simultaneous patching of a raft protein and a nonraft protein leads to the formation of segregated patches. Copatching of the two raft components depends on the simultaneous addition of antibodies to both raft proteins to the cells (28). If antibodies are added sequentially, segregated patches predominate. Notably, the copatching behavior depends on cholesterol. Before cross-linking, both the raft and the nonraft components were completely dispersed over the cell surface. These examples illustrate an important property of raft domains in cell membranes. Whatever the size of the resting state, for rafts to function in cellular processes, they are usually specifically clustered together by different means such as oligomerization or scaffolding by other proteins. For two different raft proteins to meet in a raft, they have to be brought together. Clustering of rafts by antibodies to a GPI-anchored or transmembrane cell surface antigen also brings protein attached to the inner leaflet of lipid rafts, such as doubly acylated src kinases into the raft cluster (28). The "passive" dragging of raft residents during raft coalescence depends on concentration of the resident in the membrane and in rafts. This is not easy to explain by the "lipid shell model" of raft protein behavior. However, it is easily understood by clustering small preexisting rafts and protein partition behavior (including that of proteins associated with the inner leaflet). This assumes coupling of outer and inner leaflets of individual small rafts, presumably stabilized by transmembrane raft proteins or perhaps by the interdigitating long chains of the sphingolipids between the two leaflets of the membrane. The asymmetric distribution of SpM would leave a fraction on the cytoplasmic side ($\sim$15%) that could preferentially be raft-associated. It should be remembered, however, that little is known about the properties of the inner leaflet surface interactions with the cytoplasmic milieu. Interactions with cytoplasmic proteins and electrolytes may promote heterogeneity in the inner monolayer of the cell membrane that might not be observable in model membranes of the same lipid composition.

How rafts cluster to perform functions in membrane trafficking, signal transduction, and cell polarization is still poorly understood. However, there are now many instances of such processes in cellular life, both in health and disease, so that there is bound to be progress just by the sheer volume of research that is being carried out.

PROTEIN INTERACTIONS WITH LIPID RAFTS

One key issue is how proteins associate with rafts. Lipid rafts contain specific sets of proteins (7, 79). These include GPI-anchored proteins, doubly acylated proteins such as tyrosine kinases of the src family, G_α subunits of heteromeric G proteins and endothelial nitric oxide synthase, the cholesterol- and palmitate-linked hedgehog protein and other palmitate-linked proteins, and transmembrane proteins. It is fairly straightforward to understand that proteins with attached saturated acyl

chains and cholesterol can be associated with liquid-ordered raft domains. Studies with model membranes have confirmed that peptides containing such lipid modifications associate with liquid-ordered domains (92). It should be noted that the GPI anchors differ in their fatty acid composition. Some GPI anchors contain unsaturated acyl chains, and how these interact with lipid rafts remains to be studied. Transmembrane proteins, however, do pose a problem. It could be imagined that because they cross the bilayer they should disrupt the packing of the liquid-ordered domain, but it must be recalled that the l_o phase is a liquid phase and therefore does not have long-range order in the membrane plane. Association of proteins with lipid rafts can be viewed as a simple solubility problem described by an equilibrium partition coefficient for partitioning of the protein between two coexisting phases, or it can be understood to require some chemical affinity for raft lipids. Several proteins interact with cholesterol. Caveolin is the prime example (55). There are also examples of receptor proteins interacting with glycosphingolipids including gangliosides (26). A structural protein motif has been identified for binding to sphingolipids (47). Recent results also demonstrate that proteins can exist in different states depending on the membrane environment. Glutamate receptors, which are G protein–coupled heptahelical transmembrane proteins, are in a low-affinity state when reconstituted into membranes lacking cholesterol. The receptor changes its conformation in liquid-ordered cholesterol-containing membranes and now binds its ligand with high affinity (17). The EGF receptor is activated by interaction with the ganglioside GM3 and inactivated by cholesterol depletion (54a). The receptor seems to depend on the lipid environment for high-affinity binding capability.

One way to view this differential behavior (88) would be to consider the protein as a solute in the bilayer solvent of the membrane. If the lipid bilayer has two phases, each phase is a different solvent. The protein has a conformation that depends on its environment and therefore depends on the bilayer solvent phase in which it is dissolved. So one can expect that in a nonraft domain it will have one conformation, and in the raft domain it will have another. The receptor activation would depend on the partition coefficient between the different lipid domains in the bilayers and upon phase coexistence. Another issue is the length of the transmembrane domains of the protein because a liquid-ordered bilayer is thicker than a liquid-disordered one. These parameters play a role in protein sorting to the cell surface (5). But how precisely the transmembrane domains should be matched with the thickness of the bilayer is an open issue. So far, no detailed analysis has been carried out of how different transmembrane proteins having different transmembrane domain lengths partition into liquid-ordered and liquid-disordered domains. The transmembrane domains of single-span transmembrane proteins in the plasma membrane are usually longer than the transmembrane domains of proteins that reside in the Golgi complex or in the endoplasmic reticulum (5). It is also worth considering how oligomerization of proteins affects their affinities for a raft domain. If oligomerization does not affect the partition coefficient of each monomer in the oligomer, the partition coefficient of the oligomer is a product of the partition coefficients for the monomers that constitute it. A partition coefficient that

is weakly in favor of a raft phase (say $K_P \approx 2$), in the case of homo-oligomerization, will lead to a substantially increased affinity of the oligomer for this phase ($K_P \approx$ 4 for a dimer, 8 for a trimer, 16 for a tetramer). This effect is shown in Figure 2. The increased affinity of the raft-partitioning proteins after oligomerization can also be a driving force for aggregation of raft domains and their stabilization. Only careful reconstruction studies will shed light on conformation and/or solubility in lipid phases and what structural characteristics will define the principles for raft partitioning.

Nevertheless, the problem remains of how transmembrane proteins will disturb the packing in liquid-ordered domains. Will the protein be located at the raft boundary where they could work as surfactants and decrease the line tension and consequently raft size? Would raft size in cell membranes depend on their raft protein constituents? As discussed above, there can be a substantial interfacial energy associated with the interface between liquid phases that results in a line tension at the domain interfaces. An interface with a large line tension would be a trap for impurities; thus, McConnell & Vrljic (52) postulate that if there were liquid-liquid interfaces in cell membranes, these would be decorated by specific proteins and/or lipids.

One interesting class of proteins is the group of GAP43-like proteins, which include GAP43, LAP23, MARCKS, and MacMarcks (10, 53). These are highly hydrophilic and contain a basic domain that binds acidic phospholipids including PI(4,5)P2. When binding to the inner leaflet at sites containing PI(4,5)P2, these proteins seem to organize lipid raft-like domains visible in the light microscope. These sites recruit WASP and ERM proteins to promote actin polymerization and filament assembly. The GAP43-like proteins are myristoylated or palmitoylated, and it has been postulated that the association of multiple GAP43-like proteins through their binding to PI(4,5)P2 and the intercalation of multiple saturated fatty acyl chains lead to nucleation of a raft domain.

WHAT DOES DETERGENT RESISTANCE TELL US ABOUT LIPID DOMAINS?

One of the stickiest issues in the lipid domain field is the issue of how detergent resistance can be used to define lipid raft composition. There is no doubt that the finding of Brown, Rose, and colleagues (7, 8, 46) demonstrating that a GPI-anchored protein became resistant for Triton X-100 solubilization at 4°C in the Golgi complex during transport to the cell surface was a breakthrough in the field. The detergent-resistant membrane fraction (DRM) floated to low density and was enriched in sphingolipids and cholesterol. The subsequent work of Brown and London (7, 46) attributed the property of detergent resistance to liquid-ordered domains, while liquid-disordered bilayers were solubilized by Triton X-100. This set in motion a wave of research in which DRMs were used to define raft association. This was coupled with cholesterol depletion using methyl-β-cyclodextrin;

solubilization of raft proteins after this treatment became a standard tool for specifying whether a protein was involved in raft processes. The usefulness of this methodology is attested to by the fact that most proteins that have a substantial fraction in DRMs are shown to associate with rafts also when analyzed by other methods. However, it should be noted that the number of raft proteins that have been carefully investigated by additional methods is still rather small.

There are several caveats that have to be kept in mind when using DRM methods to analyze raft association. First, not all proteins that are in rafts are in DRMs. One such example is the vesicular stomatitis virus G protein. It is Triton X-100 soluble but copatches with raft markers using antibody cross-linking (28). The G protein is also involved in phenotypic mixing, a process based on mingling of raft-associated proteins in virus envelope formation during budding from the host cell plasma membrane (6, 58). Furthermore, recent studies have demonstrated that Triton X-100 may promote liquid-ordered domain formation in model membranes (29). The addition of a detergent to a membrane results in its initial insertion into that membrane. When sufficient detergent has been inserted, the detergent becomes an additional chemical constituent of the system, thereby altering its phase behavior. It is surprising that the detergent-resistance criterion has turned out to be as useful as it has. This may be related to the kinetics and/or thermodynamics of mixing of detergent with l_o phase bilayer and speaks in favor of a pre-existence of domains of this phase in the membrane. Hence, DRMs should not be expected to extract lipid rafts from cell membranes precisely reflecting their detailed composition. Triton X-100 addition may not only enhance liquid-ordered domain formation, it may also lead to fusion of existing rafts to large confluent membrane aggregates. The kinetics and thermodynamics of detergent interaction with membranes that contain an l_o phase, analogous to the raft phase in biomembranes, will have to be studied in detail in order to answer some of these questions.

A number of other mild detergents have been introduced to extract lipid domains from cell membranes. Several of these detergents were compared in a recent study (73) that demonstrated that detergents of the Lubrol and the Brij series extracted DRMs with different lipid and protein composition when compared with that of Triton X-100-DRMs. CHAPS behaved more like Triton X-100. This study emphasizes that the use of different detergents to extract DRMs is only a first step in defining possible membrane domains and should be followed up by other methods to define domain existence and composition. Song et al. (85) introduced a detergent-free method on the basis of sonication, pH 11 treatment, and density gradient centrifugation to isolate what they called the caveolar membrane fraction. This fraction is highly enriched in caveolin protein but it also contains membranes from other cellular compartments. A recent proteomics study concluded that 75% of the proteins detected in this membrane fraction did not correspond to raft components as defined by inclusion in Triton X-100 DRMs and sensitivity to cyclodextrin treatment (19). Seven hundred three proteins were identified in DRMs and 585 in the pH 11–resistant fraction. Of the 703 proteins 392 were quantifiable, revealing that 241 were sensitive to cholesterol depletion. Analyzing the

proteins found that both the DRMs and the pH 11 fractions contain proteins, such as ribosomal proteins, that should not be there, only demonstrating that care must be taken when using these criteria for defining raft association. Smart et al. (83) introduced another method in which a crude plasma membrane fraction was first isolated. This fraction was sonicated to release lipid rafts (and caveolae) that were isolated by flotation in a continuous Optiprep gradient. The detailed protein composition of this fraction has not yet been analyzed. This fraction has been analyzed for lipid content by mass spectrometry (59). In contrast to Triton X-100 DRMs, these membranes are enriched in arachidonic acid-containing ethanolamine plasmalogens. These lipids with polyunsaturated fatty acids would not be expected to partition into liquid-ordered domains. On the other hand, myelin, which is a specialized raft-like phase, contains high amounts of ethanolamine plasmalogens, mostly composed of two 18:1 fatty acyl chains (65). The plasmalogens have not yet been carefully studied in model systems and their propensity for forming liquid-ordered domains should be explored.

The use of cyclodextrins to deplete cholesterol is also not without pitfalls. Treatment of living cells with methyl-β-cyclodextrin not only leads to dissociation of raft components, but has side effects as well. Cholesterol depletion affects the function of the plasma membrane by decreasing its permeability and changing its behavior in unpredictable ways (25, 60). Thus, effects of cholesterol depletion alone cannot be used to define raft function. There are also membranes such as the apical membrane of epithelial cells that remain resistant to cyclodextrin extraction in living cells (73). However, when a membrane fraction prepared from the same epithelial cells was treated with methyl-β-cyclodextrin, release of raft-associated proteins from the apical membrane could be demonstrated after Triton X-100 extraction. The apical membrane is especially resistant to intervention probably because of its high raft lipid concentration, the membrane being practically covered by rafts. An interesting new approach for isolating clustered rafts in cell membranes is the immuno-isolation of activated T-cell receptors and associated signaling molecules in plasma membrane subdomains (27). Receptor-activating antibodies attached to magnetic beads were used to cluster rafts on cell membranes that were then disrupted by nitrogen cavitation. The clustered raft fraction was isolated with a magnet.

PERSPECTIVES

The lesson that we have learned from studies of model membranes prepared from binary and ternary lipid mixtures is that there can be liquid-liquid immiscibilities in lipid monolayers and bilayers. This observation conforms to the general idea that more-ordered liquid domains rich in sphingolipids and cholesterol segregate from less-ordered liquid domains composed of mainly unsaturated phospholipids. The size and behavior of these domains are sensitive to the chemical composition of the membranes as well as to pressure and temperature. Cell membranes,

although they contain hundreds of lipid species, also exhibit the formation of ordered liquid domains, rafts, which are rich in sphingolipids and cholesterol, and segregate from less-ordered liquid domains composed of unsaturated phospholipids (and cholesterol). It is indeed surprising how well results obtained from the model systems studied correlate with postulated and experimentally verified properties of sphingolipid-cholesterol rafts. The study of model systems prepared from lipid mixtures relevant to cell membranes is providing increasing support for the existence of liquid-liquid immiscibility in cell membranes, which manifests itself as a segregation of liquid-ordered raft domains in a fluid bilayer. However, the detailed lipid composition of lipid raft domains is not known. Neither is the coupling between the outer and inner leaflets understood. Also, the dynamics of raft behavior need to be analyzed. The cell membranes are nonequilibrium systems, in which lipids and proteins are constantly being removed and added by intracellular transport. The plasma membrane is also constantly releasing cholesterol to lipoproteins in the extracellular medium. One possibility is that the liquid-liquid immiscibilities are undergoing constant fluctuations because of these perturbations. But, how could such metastable behavior be regulated? Is it compatible with the functions that cell membranes have?

We favor the view that the complex lipid composition of cell membranes has evolved to be buffered against such fluctuations. The little evidence that exists (12, 39, 50) indicates that multiphase bilayers respond slowly to perturbations of phase equilibria. Perhaps the cell membrane needs such a large number of lipids precisely to avoid rapid responses to perturbations of its state. The complexity serves the need to pack lipids such that stable but fluid lipid rafts of defined size can coexist in a fluid matrix. Also, the liquid-disordered matrix in cell membranes might be less permeable and more tightly packed than a simple two-component unsaturated phospholipid-cholesterol model membrane. Israelachvili (36) postulated early on that lipid complexity is required to fill the holes the integral proteins create in membrane bilayers. Perhaps lipid complexity also helps to construct liquid membranes in which the bilayer space is tightly fitted to make it impermeable. However, at the same time the lipid composition is such that liquid-ordered-like domains can be formed. We postulate that these move about as individual rafts of discrete size but they can be clustered by protein-protein cross-linking to form raft clusters. As a consequence, proteins must also have evolved to either function in the fluid matrix or within the rafts. Movement of proteins in and out of rafts demands structures capable of raft inclusion through simple partitioning, conformational changes leading to preferential partitioning or vice versa, or by scaffolding interactions with resident raft proteins. There are significant advantages in terms of enhanced/reduced bimolecular interactions to be had from dynamic compartmentalization of the lipid bilayer (42, 88).

How proteins interact with lipid rafts can only be decided by careful reconstitution studies in model systems that are designed to mimic cell membranes in an increasingly authentic fashion. One outstanding parameter is lipid asymmetry. If model systems are to give more detailed insights into how biological

membranes work, it becomes urgent to develop methods that permit bilaterally asymmetric model membranes to be prepared and studied. The rapid progress in identifying and characterizing lipid flippases may provide new tools to construct asymmetric membranes. The characterization of such reconstituted membranes by new sophisticated tools such as solid-state NMR will provide answers as to how proteins interact with lipids in membranes. Despite the perplexing complexity facing us, there is no escape from accepting that membrane biophysics should take its inspiration from cellular systems. This means that our community has to support attempts to overcome the technical difficulties involved. We are optimistic. The influx of new methodologies and young blood from both the physics and biology community is a sign of hybrid vigor. The broadening horizon of membrane research is opening up exciting vistas for the future.

POSTSCRIPT CONCERNING TERMINOLOGY

A considerable amount of confusion in the field is created by the somewhat arbitrary use of terminology. Much of the terminology used in describing physical phenomena that occur in biological membranes is borrowed from the physical/chemical description of model membrane systems. Many of these terms have a precisely defined meaning in the context of the physics/chemistry of model membranes and may not be directly applicable to biomembranes. Other terms do not have precise physical definitions and care must be taken in their utilization. A lipid bilayer "phase" is a physical state of a lipid bilayer characterized by structural and dynamic properties. The structure of an isolated and fully hydrated single lipid bilayer can be characterized by the positional and orientational order of the lipid molecules in the plane of the layer (defined in a laboratory coordinate system as the *xy* plane) and by the thickness of the layer in a direction perpendicular to this plane (defined as parallel to the *z* axis of the same coordinate system). The dynamics of the bilayer have to do with the temporal behavior of the position of the lipid molecules (translational order), their orientation (rotational order), and the configuration of the chemical bonds in each lipid molecule (configurational or conformational order). The translational order is measured in terms of the hopping frequency (the number of times per second that a lipid molecule exchanges places with its neighbors) and is related to the translational diffusion coefficient. Rotational order is measured in terms of an angular velocity, usually for gyration around the long axis of the molecules that is perpendicular to the bilayer plane, and is related to the rotational diffusion coefficient. "Flip-flop" is a special case of rotational dynamics around the *x* and *y* axes of the coordinate system with a limited translational step in the *z* direction. The configurational order results from *trans-gauche* isomerism and is measured in terms of an order parameter. In structural terms, the lipids in biomembranes are in a lamellar lipid bilayer phase. In dynamic terms, the bilayer membrane can exist as an ordered solid (s_o) or liquid (l)

phase. An s_o phase is characterized by high degrees of translational (translational diffusion coefficient on the order of 10^{-11} cm^2 s^{-1} or lower) and configurational (high *trans/gauche* ratio in the acyl chains) order, whereas an *l* phase is characterized by lower translational (translational diffusion coefficient on the order of about 10^{-8} cm^2 s^{-1}) and configurational (low *trans/gauche* ratio in the acyl chains) order. An *l* phase with a high molar fraction of cholesterol (varies from about 0.25 to about 0.5 depending upon the chemical identity of the other lipids in the bilayer) has been classified as being in a liquid-ordered phase, l_o. This l_o phase has been shown to have a slightly increased translational order compared to a cholesterol-free *l* phase (translational diffusion coefficient about two times lower) and a configurational order that is comparable to that of an s_o phase. To distinguish the l_o phase from the *l* phase bilayer without cholesterol, the latter has been termed a liquid-disordered phase, l_d. Using the criteria of translational and configurational order, *l* phase bilayers with a low molar fraction of cholesterol also qualify as l_d phases. There are, however, no exact definitions in terms of the hopping frequencies or order parameters that rigidly distinguish l_d phase from l_o phase bilayers. Due to the increased *trans/gauche* ratio in an l_o phase, this phase (and its domains) are thicker than an l_d phase.

Within the framework of thermodynamics a phase is always a macroscopic system that consists of large numbers of molecules. However, in lipid bilayers the phases often tend to be fragmented into small domains (often only a few thousand molecules), each of which, per se, may not have a sufficient number of molecules to strictly satisfy the thermodynamic definition of a phase. In the absence of a better description for this sort of mesoscopic state, and assuming that there are a large number of domains in a given system, the domains may be treated as if they were a part of a macroscopic phase so that the same properties are attributed to the domains that would describe the phase. This definition is probably adequate as long as the domains do not get too small. The liquid-ordered raft phase thus comprises all the domains (small or clustered) of the raft phase in the membranes. The rest of the membrane surrounding the rafts, the liquid phase, may be a homogeneous percolating liquid phase or may be further subdivided into liquid domains not yet characterized.

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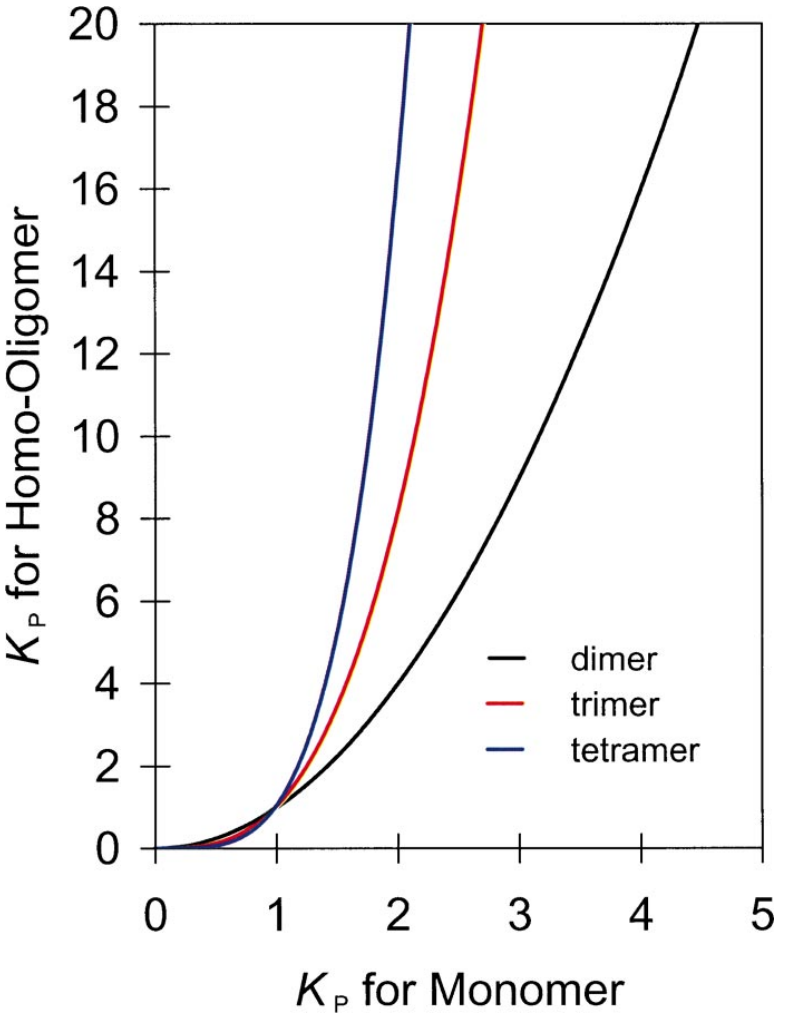

Figure 2 The effect of cross-linking upon the partitioning of membrane components between domains of coexisting phases. Homo-oligomerization is assumed and it is also assumed that cross-linking does not alter the individual partition coefficients of each monomer in the oligomer.

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CONTENTS