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Membrane Domains and the "Lipid Raft" Concept

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Abstract: The bulk structure of biological membranes consists of a bilayer of amphipathic lipids. According to the fluid mosaic model proposed by Singer and Nicholson, the glycerophospholipid bilayer is a two-dimensional fluid construct that allows the lateral movement of membrane components. Different types of lateral interactions among membrane components can take place, giving rise to multiple levels of lateral order that lead to highly organized structures. Early observations suggested that some of the lipid components of biological membranes may play active roles in the creation of these levels of order. In the late 1980s, a diverse series of experimental findings collectively gave rise to the lipid raft hypothesis. Lipid rafts were originally defined as membrane domains, *i.e.*, ordered structures created as a consequence of the lateral segregation of sphingolipids and differing from the surrounding membrane in their molecular composition and properties. This definition was subsequently modified to introduce the notion that lipid rafts correspond to membrane areas stabilized by the presence of cholesterol within a liquid-ordered phase. During the past two decades, the concept of lipid rafts has become extremely popular among cell biologists, and these structures have been suggested to be involved in a great variety of cellular functions and biological events. During the same period, however, some groups presented experimental evidence that appeared to contradict the basic tenets that underlie the lipid raft concept. The concept is currently being re-defined, with greater consistency regarding the true nature and role of lipid rafts. In this article we will review the concepts, criticisms, and the novel confirmatory findings relating to the lipid raft hypothesis.

Keywords: Detergent-resistant membrane, ganglioside, lipid raft, liquid-ordered phase, membrane domain, microdomain, sphingolipid.

THE ORGANIZATION OF BIOLOGICAL MEMBRANES: ORDER IS NOT A SIMPLE MATTER

Cell membrane lipids in vertebrates and many other major phyla consist mainly of glycerophospholipids (GPLs), sphingolipids (SLs) and cholesterol Fig. (**1**). Amphipathic lipids such as GPLs and SLs are the major structural lipids that form the basic matrix of all cellular membranes in eukaryotes because of their aggregative properties; *i.e.*, the tendency of their hydrophobic portions to associate together and exclude water molecules and the tendency of their hydrophilic portions to interact with the extra- and intracellular aqueous environments. GPLs are by far the major structural lipids in cellular membranes, and phosphatidylcholine (PC), which typically accounts for >50% of all cell membrane phospholipids, is the main bilayer-forming lipid. SLs are minor components of cell membranes. Complex glycosphingolipids (GSL), including gangliosides (sialic acid-containing GSLs), are not bilayer-forming lipids; in aqueous solution they tend to form micellar aggregates because of the large size of their polar head groups. However, GSLs can be inserted in the glycerolipid bilayer through their hydrophobic ceramide moiety. Although SLs are minor components of cell membranes overall, their local concentration can be high. They are associated with the external leaflet of the plasma membrane and are particularly abundant in certain cells and tissues such as the myelin sheath and neurons. In cultured cerebellar neurons, SLs comprise ~5% of total amphipathic lipids, *i.e.*, about 10% of total lipids of the outer membrane layer.

The organization of eukaryotic cells is determined largely by their biological membranes, and the structures and functions of biological membranes are based on the intrinsic properties of membrane lipid.

Biological membranes separate the intracellular environment from the extracellular environment and create discrete compartments within living cells. This simple fact has enormous consequences. It permits gradients of water-soluble molecules (ions, enzymes, reaction substrates and products) to be formed, thereby restricting the occurrence of specific biochemical reactions to specific environments. The primary function of biological membranes is to serve as a physical boundary of a hydrophobic nature between different aqueous compartments. Biological membranes also provide a hydrophobic milieu capable of acting as a solvent for water-insoluble (lipophilic or amphipathic) biological molecules, thereby increasing the complexity of chemical reactions occurring in biological environments. A fairly simple molecular organization is apparently sufficient to achieve these functions tasks. Around 1970 (45 years after the observation that biological membranes are composed of a bimolecular sheet [1]), the nature of this molecular organization as a bilayer of amphipathic phospholipids was elucidated [2-4]. The first level of ordered organization of biological membranes, the creation of the lipid bilayer, is lipid-driven; *i.e.*, it is a consequence of the aggregative properties of complex amphipathic membrane lipids. The lipid bilayer is characterized by several basic properties that are relevant to its biological functions; in particular, the bilayer as a whole is quite stable, consistent with its primary function as a physical barrier. On the other hand, the fatty acyl chains of the phospholipids that comprise the bulk of lipid bilayers at 37 °C are in a fluid phase. Thus, biological lipid bilayers under physiological conditions are two-dimensional fluids. The fluid phospholipid bilayer acts as a solvent for membrane proteins such that, similarly to a three-dimensional viscous solution, protein molecules dissolved in the two-dimensional fluid possess a certain degree of lateral motility that permits their free diffusion in the phospholipid bilayer and their distribution along the membrane surface in a random ("aperiodic") arrangement (the "fluid mosaic" model proposed by Singer and Nicolson in 1972 [5]).

As a consequence of the fluidity of the phospholipid bilayer, the components of biological membranes can be arranged in a non-homogeneous lateral distribution, leading to the creation of "ordered structures that differ in lipid and/or protein composition from the surrounding membrane" [6], or "membrane domains". The existence of membrane areas having highly differentiated molecular composition and supermolecular architecture is directly linked to the multiple roles played by biological membranes in addition to that of a boundary. A biological membrane has to serve as a matrix or scaffold for the organization of multimolecular interactions that are dynamic in time and space and to physically and functionally link the different environments that are compartmentalized by the membrane. For example, biological membranes must be able to transduce signals and to translocate molecules and to couple events that take place in the outer and the inner plasma membrane leaflets. They should also have the potential to assume a great variety of

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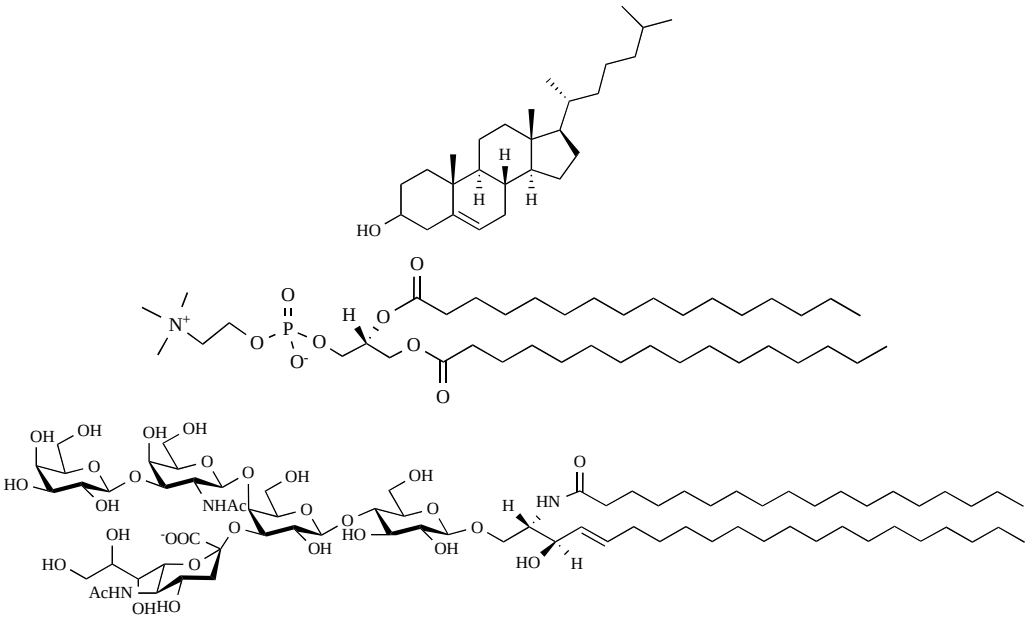

Fig. (1). Chemical structures of cholesterol, phosphatidylcholine (a glycerophospholipid), and GM1 ganglioside (a glycosphingolipid).

different geometries that underlie the dynamic control of the shapes of specialized plasma membrane areas and intracellular organelles and of the inward and outward budding and fusion of membrane vesicles.

These events imply the existence of a second level of order in the organization of biological membranes, made possible by the existence of lateral interactions that stabilize different membrane domains. The non-homogeneous lateral distribution of membrane components at the micron scale is particularly evident in polarized epithelial cells (in which basolateral and apical membrane macrodomains can be distinguished) and polarized neurons (characterized by the presence of somatodendritic, axonal, and synaptic membrane macrodomains). Morphologically distinct domains specialized for particular functions are present at the membrane surface of virtually any cell type; *e.g.*, any mobile cell becomes polarized during movement, with a clearly recognizable leading edge and trailing edge. Lateral heterogeneity in membrane structure is also present at the sub-micron and nanometer scale, as revealed by the finding that even in membrane regions without a morphologically distinguishable architecture, certain proteins do not undergo free and continuous lateral diffusion but rather are transiently confined to small domains at the sub-micron scale ("microdomains") [7].

Membrane macro- and microdomains are characterized in general by the presence of specific subsets of proteins. Differential sorting and trafficking of proteins is considered to be an important mechanism responsible for the creation of distinct domains in intracellular and plasma membranes, and protein-protein interactions have long been regarded as the main factor responsible for the stabilization of membrane macro- and microdomains. Reciprocally interacting membrane proteins or proteins belonging to a common membrane-tethered multiprotein complex formed by direct protein-protein interactions (*e.g.*, the respiratory chain complex in prokaryotes and the complexes organized by certain membrane receptors or ion channels) are obviously limited in their reciprocal diffusion. Certain proteins appear to be specifically designed to serve as scaffolds for multimolecular complexes that trap other proteins. Protein scaffolds [6] can be organized by extracellular proteins (*e.g.*, galectins), intracellular proteins (clathrin in clathrin-coated pits), or membrane proteins (tetraspanins [8], caveolins [9, 10], flotillins [11]). To explain the lateral compartmentalization of the fluid plasma membrane, models such as the "membrane skeleton fence" have been proposed. According to this model, the limitations in lateral diffusion observed for some membrane-bound proteins are due to the formation of compartmental boundaries by actin-based membrane skeleton "fences" that are anchored to the membrane by "picklets" consisting of transmembrane proteins [12]. Based on this and similar models, the formation and stabilization of membrane domains have been regarded by many investigators as protein-driven events.

However, the aggregative properties of complex membrane lipids are responsible for not only the creation of the lipid bilayer but also its lateral and transverse organization, at least to some extent. GSLs in particular possess a high potential for the creation of order in biological membranes [13], as we will discuss later. Membrane lipids, through their cooperative behavior, achieve the complexity of a "non-covalent macromolecule" that is much larger and more adaptive than a protein molecule. Interestingly, this notion was already present in Singer and Nicholson's description of the fluid mosaic model; *i.e.*, some lipid membrane components were assumed not to be in the bulk fluid bilayer phase, but rather to be strongly (specifically?) interacting with membrane proteins, thereby allowing lateral heterogeneity over a short distance (<100 nm). Soon after formulation of the fluid mosaic model (1974-1978), studies of thermal effects on the behavior of membrane lipids suggested that phase behavior of lipid mixtures was responsible for lateral organization in biological membranes, suggesting that the aggregative properties of membrane lipids constituted one of the major driving forces leading to the creation of lateral order [14-16].

The idea that the existence of multiple phases in the membrane lipid environment drives the "organization of the lipid components of membranes into domains" was clearly stated in 1982 [17] and probably represents the first formulation of the "lipid membrane domain" concept. The idea that lipids can organize domains in cellular membranes as a consequence of the limited solubility of lipids in mixtures, leading to fluid-fluid phase separation, was elaborated subsequently by Simons and van Meer to explain the differing lipid composition of the apical and basolateral plasma membrane domains of polarized epithelial cells, and provided the basis for their "lipid raft" hypothesis [18]. The components of these apical and basolateral domains are largely immiscible because of the diffusion barrier formed by the tight junction that separates the domains. The lipid components that are enriched in the apical domain (GSLs and

cholesterol) need to be sorted from the lipids of the basolateral domain (mainly GPLs) at some intracellular site during their trafficking to the surface. The self-associative properties of the apical lipids were proposed as the driving force for such sorting. Experimental proof that SLs and sterols are segregated from GPLs during formation of secretory vesicles in the *trans*-Golgi network was not obtained until 21 years later [19] -- a good example of the difficulties in applying lipidomic analyses to basic cell biological problems. Simons and Ikonen hypothesized in 1997 that the association of a particular protein with lipid clusters ("rafts") could provide a sorting and targeting signal during intracellular trafficking and signal transduction [20]. Although the role of lipid rafts as sorting mechanisms leading to the polarized distribution of lipids and proteins at the surface membrane of epithelial cells remains an elusive and controversial topic even today [21-24], the lipid raft concept has become extremely popular and has been implicated in a wide variety of cellular functions and biological events [25-33], some of which will be discussed later.

The forces that control the formation and dynamics of lipid rafts are not yet fully understood. This is not surprising given the incredible complexity that underlies the apparently simple notion of lipid rafts. It has long been known that a typical biological membrane contains hundreds of different lipid species, but it has been possible to quantify this complexity only recently through the emerging contribution of a sophisticated "lipidomic" approach [34-36]. The level of complexity is particularly evident for SLs, including GSLs, which display great heterogeneity in both their lipid moieties and hydrophilic headgroups. A contribution in modulating the cell signaling has been documented for a category of lipids other than "classical" membrane lipids, *i.e.* cannabinoids [37]. Detailed information on phase behavior is available only for simple mixtures of the most common membrane lipids [38-40]. Translating this information to the phase behavior of natural membranes is extremely difficult. Reductionist approaches such as the use of artificial membrane models are conceptually inadequate to investigate a phenomenon that is based on the maintenance of collective properties [41].

The complexity of membrane chemistry applies to protein components as well as to lipid components. Biological membranes contain a huge number of different proteins that interacts with membranes in very different ways; *e.g.*, transmembrane proteins, proteins with short hydrophobic amino acid sequences, palmitoylated or myristoylated proteins, GPI-anchored proteins [42-44]. Membrane proteins can interact with lipids, or can be partitioned between different lipid phases. Besides fluid-fluid phase separation, interactions between membrane lipids and membrane proteins are clearly relevant to membrane organization.

Biological membranes are systems that are far from equilibrium [45]. However, most of our information regarding lipid phase separation has been obtained from studies of artificial systems in equilibrium. Many studies of lipid rafts in biological systems are based on the putative resistance of raft components to solubilization by non-ionic detergents, as discussed below. Detergent-resistant membrane (DRM) preparations may reflect to some extent the properties of lipid rafts in living cells [46]; however, such systems are obviously driven to equilibrium by the specific experimental conditions used for detergent extraction.

Despite these caveats, phase separation as a consequence of the limited solubility of lipids in lipid mixtures (a phenomenon that can be described using phase diagrams [40]), has been observed in all membrane models that reproduce the other basic properties of cellular membranes [6, 13, 38, 39, 41, 47, 48]. Lipid rafts should therefore be defined as areas of phase separation in biological membrane [6]. Lipid bilayers under physiological conditions generally exist in a liquid-disordered (l*d*) phase characterized by high fluidity, in which the lipid acyl chains are disordered and highly mobile. When the temperature is lowered below the melting point, the lipid acyl chains are frozen in an ordered gel phase (solid-ordered) with very limited freedom of movement. In mixtures comprised of bilayer-forming lipids such as dipalmitoyl-PC and cholesterol (or ergosterol in yeast), a third physical phase, the liquid-ordered (l*o*) phase [49], can be observed. In the l*o* phase, the acyl chains of lipids are extended and ordered, as in the gel phase, but have higher lateral mobility in the bilayer. Sterols can stabilize the l*o* phase by filling in the hydrophobic gaps between the phospholipid or glycolipid acyl chains [39, 50].

Complex membrane lipids are highly heterogeneous in their hydrophobic portions. The prevalence of GPLs that contain unsaturated acyl chains ensures the fluidity of biological bilayers. Glycerolipids with saturated chains are sometimes abundant membrane components [51]. In some classes of complex membrane lipids, *e.g.*, sphingomyelin (SM) and gangliosides in the nervous system, palmitic and stearic acid are the main fatty acids. Lipids with a high content of saturated acyl chains (which have a high melting point and can be tightly packed with a high degree of order in the hydrophobic core of a bilayer) are typically characterized by a high transition temperature within or above the physiological range [39, 51]. Differences in transition temperatures due to the differences in acyl chain composition are most likely one of the major forces leading to phase separation in lipid mixtures and aggregates, including bilayers. Phase separation can be observed in binary mixtures of diacyl lecithins that differ in chain length and/or saturation [52-59]. Complex lipids containing palmitic acid are highly enriched in putative lipid raft fractions prepared from cultured neurons [51, 60].

Phase separation of SM in dimyristoyl-PC bilayers depends on the degree of SM chain mismatch [61]. The distribution of ganglioside GM1 in the fluid phase of a phospholipid bilayer [56] is inversely correlated with the acyl chain length and directly correlated with the degree of unsaturation. Brain gangliosides, usually highly enriched in stearic acid, are typical l*o* phase lipids. Very long ($\geq$ C24) fatty acids are abundant in SLs outside the nervous system. Lipid bilayers of the stratum corneum in the skin are characterized by an extremely high content of long-chain ceramides and are very rigid [62]. Based on neutron diffraction experiments using artificial membranes, it has been proposed that the organization of lipid bilayers in stratum corneum is stabilized by partial interdigitation between the two leaflets [63]. Interdigitated hydrocarbon chains have been suggested to play a role in the stabilization of lipid domains in human neutrophils; these domains are enriched in lactosylceramide having a high content of C24 fatty acid chains [64-66]. Interdigitation of long-chain fatty acid residues of complex membrane lipids may be another feature that favors the separation of phases with a high level of order. Long-chain fatty acid-containing SL have been suggested to form quasi-crystalline structures in GPL bilayers even in the absence of interdigitation [38]. Cholesterol, which has a melting point of 148.5 °C, associates preferentially with ordered acyl chains of complex lipids because of the tight packing of the smooth planar α-face of the sterol ring against the extended acyl chains of l*o* phase GPLs and SLs [38, 41]. Cholesterol (within a wide range of molarity, including physiological concentrations) forms a l*o* phase in dimyristoyl-PC or distearoyl-PC bilayers [67] alone, in phospholipid bilayers in the presence of SM (which mixes better with cholesterol than with PC having the same acyl chain) [68, 69], and in SM vesicles [70]. In the l*o* phase, the sterol molecules are tightly intercalated between the ordered acyl chains of the bilayer-forming lipid [39, 50]. Cholesterol is generally regarded as a key lipid component of lipid rafts [71]. However, understanding the role of cholesterol in stabilizing membrane domains is hampered by the lack of precise information regarding its trans-bilayer distribution, which is largely asymmetric [72]. A large percentage of plasma membrane cholesterol has been reported to be associated with the inner leaflet: 75% in human erythrocytes [73]; 85% in mouse synaptic membranes [72, 74]. Ergosterol, the major

sterol in fungi, is also able to stabilize l*o* phases [75]. The coexistence of l*o* and l*d* phases has been demonstrated in ternary mixtures of cholesterol with a high and a low melting point lipid (*e.g.*, with dipalmitoyl-PC and dioleyl PC [76]), sometimes using a SL as the high melting point lipid [77]. Some studies using mixed monolayers suggested a strong preferential interaction between cholesterol and SM, leading to the formation of liquid-condensed cholesterol- and SM-rich domains [77-79]. However, this idea was refuted by the clear demonstration that there is no specific interaction between cholesterol and SM in phospholipid bilayers [80].

The phase separation of SLs and their association with the l*o* phase in GPL bilayers are favored by two features unique to this class of lipids:

(1) SL are ceramide-based amphipathic lipids that can create a complex network of hydrogen bonds because of the presence in the ceramide moiety of amide nitrogen, carbonyl oxygen, and a hydroxyl group positioned near the water/lipid interface of the bilayer [81]. The hydrogen bond network makes a high energetic contribution to lipid-lipid interactions that stabilize a rigid segregated phase in the bilayer (3-10 kcal per hydrogen bond vs. 2-3 kcal per interaction in the case of van der Waals forces between hydrocarbon chains). The relevance of this factor has been confirmed by recent studies showing that (a) mixtures of natural SM and PC molecular species with comparable fatty acyl chains are largely immiscible at temperatures above the transition temperature of the SM [82]; (b) C18-SM molecules in a dioleyl-PC bilayer display increased order because of the formation of SM nanoclusters stabilized by hydrogen bonds [83, 84].

(2) GSLs, which are present as minor components in all mammalian cell membranes but are abundant in certain tissues (*e.g.*, brain) and cell types (*e.g.*, neurons) and are asymmetrically enriched in the outer leaflet of the plasma membrane, are defined on the basis of their hydrophilic sugar head group. The volume occupied by this headgroup increases with the complexity of the oligosaccharide chain Fig. (**2**). Theoretical calculations of minimum energy conformation show that the hydrophilic oligosaccharide head group of GM1, one of best studied gangliosides, occupies a volume much larger than that of phosphocholine, the bulkiest head group among the phospholipids. According to predictions based on the geometrical properties of GSL molecules, the separation of a GSL-rich phase in a phospholipid bilayer with the concomitant acquisition of a positive membrane curvature implies a minimization of the interfacial free energy required to accommodate the amphipathic GSL molecule in the bilayer. *I.e.*, the geometrical properties resulting from the bulky hydrophilic head group of GSLs strongly favor phase separation and spontaneous membrane curvature [85-87]. These predictions are supported by findings that the degree of ganglioside phase separation in GPL bilayers depends on the surface area occupied by the GSL oligosaccharide chains, which is directly correlated with the number of sugar residues present in the oligosaccharide [52-54]. GM1-enriched domains can be formed in SM bilayers [70], and phase separation was observed in mixed micelles comprised of two different gangliosides (GM2 and GT1b [88], GD1b and GD1b-lactone [89], and GM1 and GD1a [90]) with identical composition of the hydrophobic moiety Fig. (**3**).

Membrane curvature was recently proposed to contribute greatly to reduction of line tension (the energy required to maintain a border between a membrane domain and the surrounding membrane environment) [91]. This general principle could explain the segregation of lipids and proteins in cellular membranes [92].

It has been suggested that clustering of GSL could be further stabilized by the formation of lateral carbohydrate-carbohydrate interactions. Although head-to-head carbohydrate-carbohydrate interactions have been clearly demonstrated for glycolipids [93], direct side-by-side oligosaccharide-oligosaccharide interactions remain hypothetical at present, and NMR studies on ganglioside micelles [94] appear to rule out significant side-by-side intermolecular interactions. The NMR studies also revealed strong interactions between different portions of the GM1 oligosaccharide and solvent water molecules [94], suggesting that a network of water-mediated hydrogen bridges might contribute to the stabilization of glycolipid clusters. In analogy, water bridges between saccharides were shown to help stabilize the three-dimensional structure of hyaluronan [95].

These studies of membrane model systems, taken together, strongly suggest that the separation of a l*o* phase resulting from the fluid-fluid immiscibility of the lipid components of biological membranes is sufficient to create the lateral order and heterogeneous organization of membranes. These models, of course, do not reflect the complexity of the lipid environment in a membranes or the interactions between lipids and proteins. Fluid phase separation has been reported recently in reconstituted versions of biological membranes, *e.g.*, giant unilamellar vesicles formed by lipids from brush border membranes [96] or by lung surfactants [97], vesicles obtained from mast cells, fibroblasts [98-100], and A431 cells [101], and budded HIV virus membranes [102] (an example of natural cell-originated membrane vesicles). Although no current analytical approach is able to identify l*o* phase in living cells, there

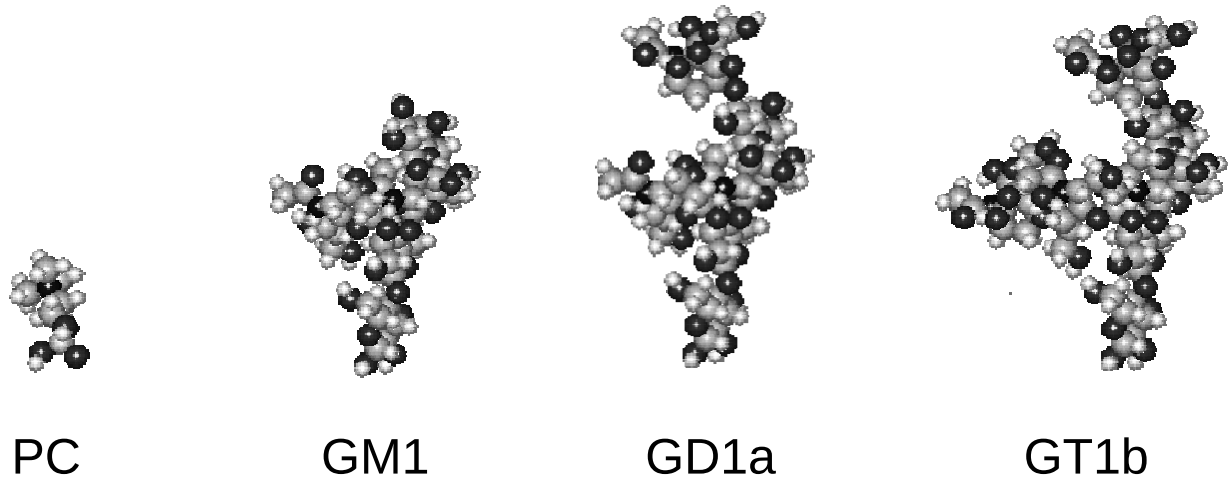

Fig. (2). Schematic representation of the volume occupied by phosphocholine, the headgroup of PC, and by the oligosaccharide chains of three gangliosides of the gangliotetraose series: monosialoganglioside GM1, disialoganglioside GD1a, and trisialoganglioside GT1b. Reproduced with modification from Sonnino S and Prinetti A (2010) Lipids and membrane lateral organization. *Front. Physio.* **1**:153. doi: 10.3389/fphys.2010.00153 [329].

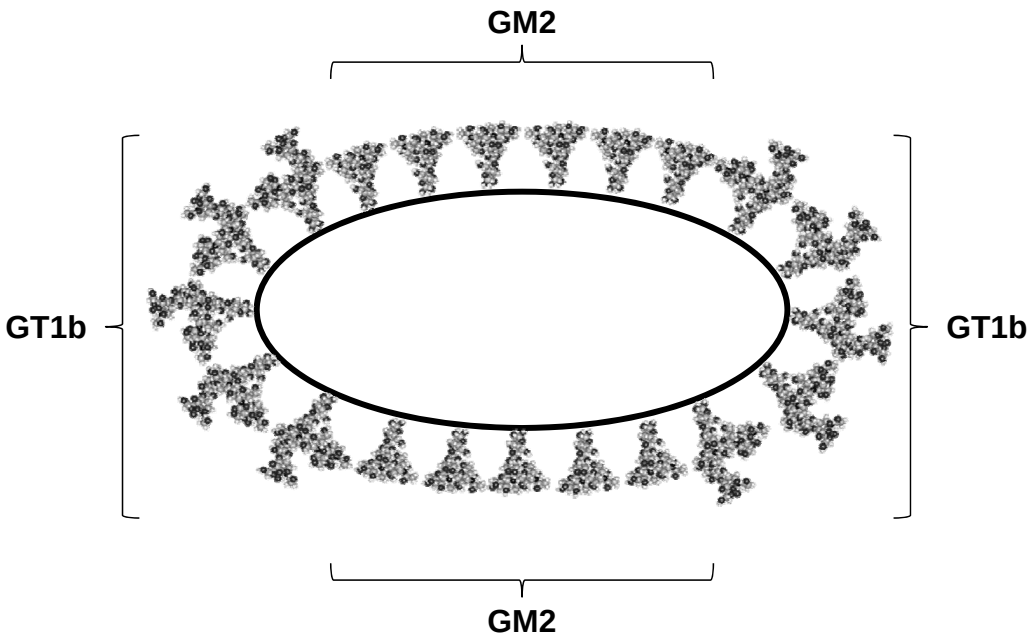

Fig. (3). Schematic representation of phase separation driven by the differences in the oligosaccharide chains in a GM2/GT1b micelle. Reproduced with modification from Sonnino S and Prinetti A (2010) Lipids and membrane lateral organization. *Front. Physio.* **1**:153. doi: 10.3389/fphys.2010.00153 [329].

is persuasive evidence for the ordered nature of lipid-driven membrane domains. In a study of plasma membrane spheres obtained from A431 cells by a cell-swelling procedure, Lingwood *et al.* [101] showed that cross-linking of GM1 using pentavalent cholera toxin at 37 °C resulted in the cholesterol-dependent aggregation of GM1-rich domains and the formation of micrometer-scale domains. The resulting GM1- and cholesterol-enriched phase was characterized by reduced translational diffusion. Fluorescence microscopic and spectroscopic analyses using order-sensitive probes [103] revealed that the degree of lateral order in the GM1 domains was higher than in the surrounding membrane but was considerably lower than in the ordered phase of giant unilamellar vesicles (representative of a l*o* phase). The GM1 domains consequently recruited lipid-anchored proteins regarded as lipid raft markers (but not transferrin receptor, which is not found in lipid rafts) and also transmembrane proteins that are not normally associated with the l*o* phase in model systems or with DRM fractions. Coskun *et al.* [104] reconstituted the human EGF receptor (EGFR) into proteoliposomes having a defined lipid composition to test the influence of phase separation on the well-known ability of GM3 ganglioside to inhibit EGFR autophosphorylation [105, 106]. GM3 was able to inhibit EGFR autophosphorylation without affecting ligand binding in proteoliposomes composed of unsaturated PC, SM, and cholesterol in a molar ratio that allowed the separation of immiscible l*o* and l*d* phases, but not in a mixture of the same lipids in proportions that led to formation of a single l*d* membrane phase.

These studies strongly support the concept that phase separation occurs under physiological conditions and accounts in part for the behavior of ordered membrane domains. The studies also indicate that ordered phases in living cells share some of the properties of -- but are not completely equivalent to -- the l*o* phases observed in model systems, implying that additional forces play an important role in the creation of lateral order in natural membranes. Other lipid-dependent lateral interactions besides liquid-liquid immiscibility indeed contribute to heterogeneity in cell membranes. Protein- and lipid-driven lateral organization have been regarded as somehow mutually exclusive, but it has become clear that they cooperate in the creation of structural and functional heterogeneity in membranes. Certain lipids are components of the quaternary structure of membrane-associated proteins and protein complexes (*e.g.*, beta 2 adrenergic receptor [107, 108], cytochrome bc1 [109]). Specific binding of gangliosides to membrane tyrosine kinase receptors has been known for a long time, although some molecular details of the process were clarified only recently (see for review [110]). Some proteins that are associated with lipid rafts are surrounded by a "shell" of typical raft lipids (SLs, cholesterol) [111]. Such a shell may confer to a membrane protein a higher affinity for lipid rafts, resulting in its partitioning to a phase-separated membrane domain in cooperation with or even in the absence of a specific raft targeting motif. Cholesterol-binding domains and SL-binding domains (that bind to the polar head groups of SLs) have been identified and characterized in several proteins [111, 112]. The binding of lipids to receptors induces conformational changes that affect both ligand binding and signaling pathways downstream of receptor activation. Lipid-to-receptor binding also influences the lateral organization of membrane components in the domain of a membrane-organizing protein. *E.g.*, caveolin-1, a typical scaffold-forming protein, binds strongly and specifically to cholesterol [113, 114], which in turn is essential for the formation and maintenance of caveolae, a flask-shaped type of lipid raft subdomain [115, 116]. Palmitoylation (usually regarded as a lipid raft-targeting motif) of caveolin does not affect its association with lipid rafts, but does affect the interaction of caveolin with cholesterol [117]. The interaction of tetraspanins with cholesterol or SLs does not affect their direct interactions with other membrane proteins (integrins), but does affect their homo-oligomerization and signal transduction through tetraspanin-containing complexes (glycosynapses) [118, 119]. In clathrin-dependent endocytosis, which is regarded as a lipid raft-independent event, phosphatidylinositol(4,5)-bisphosphate serves as the membrane anchor for several proteins that are involved in the formation of clathrin-coated pits [12, 120, 121]. Transient confinement zones bounded by a diffusion barrier created by the anchoring of actin filaments to transmembrane proteins have been proposed as an alternative model to lipid rafts to explain the organization of membrane domains [12]. However, recent studies indicate a close interplay between actin-mediated and lipid raft-mediated events (*e.g.*, endocytosis of GPI-anchored proteins [122, 123]) and suggest that lipid-based domains can be stabilized by the cortical actin network [124, 125].

In summary, our current concepts of membrane organization are heavily influenced by the lipid raft hypothesis, which emphasizes the importance of the collective properties of the cellular lipid environment in determining membrane organization, and the notion that membrane order is highly dynamic and constantly changing. There is increasing evidence that the interplay of different lipid-sensitive mechanisms plays a key role in membrane lateral organization. Models based solely on fluid-fluid phase separation are no longer adequate in representing the overall complexity of lipid-dependent membrane heterogeneity.

FUNCTIONS OF PROTEINS ARE MODULATED BY THEIR ASSOCIATION WITH LIPID RAFTS

Certain membrane-associated proteins are highly concentrated in lipid rafts even though overall protein content in the rafts is very low [126], suggesting that the segregation of signaling proteins within the rafts may modulate signal transduction cascades [127]. Several classes of membrane-associated proteins display a strong association with lipid rafts (see for review [128, 129]). A common raft-targeting motif is the presence of a GPI anchor [130], which is sometimes modified by acylation of the inositol head group or by replacement of the glycerolipid residue with ceramide [131]. A classic example of this phenomenon is the prion protein [32, 132]. Another raft-targeting motif is a lipid modification such as cysteine S-palmitoylation; tandem NH_2 terminal myristoylation/ palmitoylation and double palmitoylation are particularly efficient signals for targeting to lipid domains [133-135]. Modeling studies of transmembrane proteins indicate that, in general, they are not targeted to l*o* phases and are excluded from lipid rafts. However, the presence of a lipid modification (palmitoylation) or of a specific targeting sequence (*e.g.*, a cholesterol-binding or a SL-binding domain such as those present in α-synuclein [136, 137]) within the hydrophobic or the extracellular/ cytoplasmic domains of a transmembrane protein can increase its concentration in lipid rafts [138]. Proteins that lack a specific raft-targeting motif can also be associated or recruited to lipid rafts indirectly via interactions with raft-resident proteins. Certain raft-associated proteins appear to play important roles in organizing multiprotein complexes within lipid rafts; one example is caveolin-1 [9, 10]). The function of a membrane protein can be affected by its association with lipid rafts through three distinct mechanisms [139]:

(1) The association of a protein with an l*o* phase that displays reduced fluidity with respect to the surrounding bilayer may restrict the lateral motility of the protein, thereby favoring more stable interactions with other proteins that are segregated in the same domain. *I.e.*, association with lipid rafts may provide a mechanism that facilitates the co-clustering of different membrane proteins. In some cases, all of the proteins that belong to a signaling complex are resident in lipid rafts in the resting state. In other cases, the activation of membrane receptors is followed by the recruitment to lipid membrane domains of the receptors themselves or of effector proteins that are not located in lipid rafts under basal conditions. Different populations of lipid rafts sometimes aggregate and fuse together upon stimulation, making possible interactions between sets of proteins that were previously physically separated from each other [140]. One argument against this simplistic view is that the surface density of proteins in lipid rafts is very low and that limitations in lateral motility could hamper protein-protein interactions. On the other hand, the trapping of a protein within lipid rafts could prevent it from interacting with other proteins that are localized preferentially in fluid membrane regions; in such cases the association of a protein with rafts could inhibit biological events based on protein-protein interactions.

(2) The association of a protein with a rigid membrane area could induce conformational changes in the polypeptide chain that affect its functional activity, independently from the formation of specific high-affinity lateral interactions with other raft components.

(3) Proteins that are concentrated in lipid rafts are favored in terms of interactions with lipid raft components. GSL, because of their complex oligosaccharide groups, are good candidates for specific lipid-protein lateral interactions. In addition to their association with lipid rafts, GSLs (gangliosides in particular) are well known for their ability to modulate the activity of membrane-associated proteins such as receptor tyrosine kinases (for review see [110, 141, 142]). In most cases, the molecular aspects of GSL-protein interactions that underlie the modulatory effects of GSL remain to be elucidated. The oligosaccharide chain of a GSL inserted in the plasma membrane may interact with a membrane protein in several ways: (a) through the amino acid residues in the extracellular loops of the protein, if the conformation of the polypeptide chain allows these residues to be sufficiently close to the membrane surface; (b) through the sugar residues in the glycans of a glycosylated protein, if the dynamics of the protein oligosaccharide chain allow the appropriate orientation toward the cell surface; (c) through the hydrophilic portion of the anchor in the case of GPI-anchored proteins; this portion is by definition located near the extracellular surface of the membrane.

Early studies showed that many biological effects of gangliosides are due at least in part to the modulation of various protein kinase systems [143-151]. Receptor and non-receptor protein kinases were later found to be highly enriched in lipid rafts, suggesting new models of ganglioside-mediated signal transduction. Coskun *et al.* showed recently [104] that the inhibitory effect of GM3 on EGFR phosphorylation (which is mediated by side-by-side carbohydrate-carbohydrate interaction between the GM3 oligosaccharide and an N-linked glycan bearing multiple GlcNAc terminal residues on the receptor [152, 153]) is possible only in a phase-separated lipid environment.

LIPID RAFTS AND DETERGENT-RESISTANT MEMBRANES (DRMs)

Brown and Rose demonstrated in 1992 that GPI-anchored proteins can be recovered from lysates of epithelial cells in a low-density, detergent-insoluble form. DRM structures enriched in GPI-anchored proteins were also enriched in GSLs, but not in basolateral marker proteins [154]. This was the first evidence supporting Simons and van Meer's 1988 hypothesis regarding the sorting of proteins to the apical domain of polarized cells as a consequence of their association with a GSL-enriched environment (*i.e.*, lipid raft), and strongly influenced subsequent research in this field. There were several biochemical studies on the composition, organization, and biological roles of lipid rafts based on Brown and Rose's method, and many investigators equated *de facto* lipid rafts with a membrane fraction characterized by a particular lipid composition leading to a l*o* or highly-organized phase operationally defined in terms of insolubility in non-ionic aqueous detergents [154]. Most of the components of cell membranes (including GPLs, bulk lipid components, and intrinsic membrane glycoproteins) are solubilized by detergents and chaotropic agents [155]. In contrast, certain components were known to be insoluble in non-ionic (Triton X-100) or zwitterionic detergents (Empigin BB) under certain experimental conditions, and detergent insolubility was used as an analytical criterion or a preparative tool long before the appearance of the lipid raft hypothesis. In early studies, "detergent-insoluble material" (DIM) was shown to be enriched in pericellular matrix proteins (*e.g.*, fibronectin, tenascin, Gp140), in components of cell attachment sites (including cytoskeletal elements) (termed "detergent-insoluble substrate attachment matrix", DISAM) [156], and in GSLs, particularly GM1 (termed "detergent-insoluble glycolipid-enriched material", DIG) [157-159]. Later studies showed that these detergent-insoluble fractions had highly complex compositions,

including other SLs, gangliosides, SM [160-162], cholesterol [161], lipid-anchored proteins (containing GPI anchors or fatty acid modifications) [154, 162-167], and other hydrophobic plasma membrane proteins (*e.g.*, caveolin) [168]. The concept gradually evolved that DIM consisted at least in part of DRMs such as those in the apical compartment of polarized epithelial cells (*e.g.*, MDCK cells) or in the caveolar membrane system and that membrane fractions corresponding to or containing lipid rafts could be separated based on their insolubility in non-ionic aqueous detergents. Treatment with a non-ionic detergent (typically Triton X-100) at low temperature resulting in the solubilization of lipid components present in the membrane (*e.g.*, most GPLs) in a liquid-disordered phase. These lipids are thereby removed from the membrane through the formation of mixed micelles with the detergent ("solubilization") while l*o* phase components remain laterally organized and form microsome-like or planar structures Fig. (**4**). The DIM/ DRM fraction can be separated following the detergent treatment based on its low relative density (buoyancy) [154], using continuous or discontinuous density gradients. The low density of the DIM/ DRM fraction is most likely due to its high lipid content, *i.e.*, high lipid-to-protein ratio.

Low-density, detergent-insoluble fractions were isolated from a wide variety of cultured cells, including almost all the mammalian cell types [51, 126, 168-179] and tissues [180-187] investigated so far, fish [188], yeast [189, 190], protozoan [191-193], and plant cells [194, 195]. The availability of an efficient method to purify lipid raft-containing fractions dramatically advanced the biochemical and compositional characterization of these structures and provided essential pieces of information that helped guide and direct studies on the structure and function of lipid rafts by many research groups.

Consistent with findings from studies in model membranes regarding the segregation of membrane lipids, DRMs were found to be highly enriched in SLs, cholesterol, and palmitic acid-containing GPLs. The composition of a typical DRM fraction prepared from cultured rat cerebellar neurons is summarized in (Table **1**).

Insolubility in Triton X-100 soon became the standard criterion for assessing the association of a given protein with lipid rafts. However, several criticisms were raised regarding the significance of the biochemical data obtained by analyzing DRM fractions (for excellent reviews on this topic, with very different points of view, see [196-199]). One argument was that the apparent detergent insolubility of a given cellular component might result from an artifactual rearrangement induced by the detergent itself. The method was also found to be highly sensitive to the specific experimental parameters (particularly temperature, detergent concentration, and detergent-to-cell ratio). Standardization of the experimental procedures was difficult, and the overall composition of DRM fractions and the association of specific molecules with DRMs appeared to be significantly affected by even tiny modifications of various conditions, including the agents used for membrane disruption (different detergents or detergent concentrations [168, 200-202]), the mechanical procedures used to promote membrane solubilization (sonication, homogenization) [203], temperature [154, 202, 204, 205], pH, and the ratio of the detergent to the biological material [187, 201].

Because of the concern regarding the possible artifactual nature of detergent-prepared fractions, comparative studies were performed using a wide variety of detergents [200, 202, 206, 207]. These studies showed that DRMs enriched in cholesterol and SL and in particular proteins that are regarded as lipid raft markers (*e.g.*, GPI-anchored proteins and acylated proteins) can be prepared

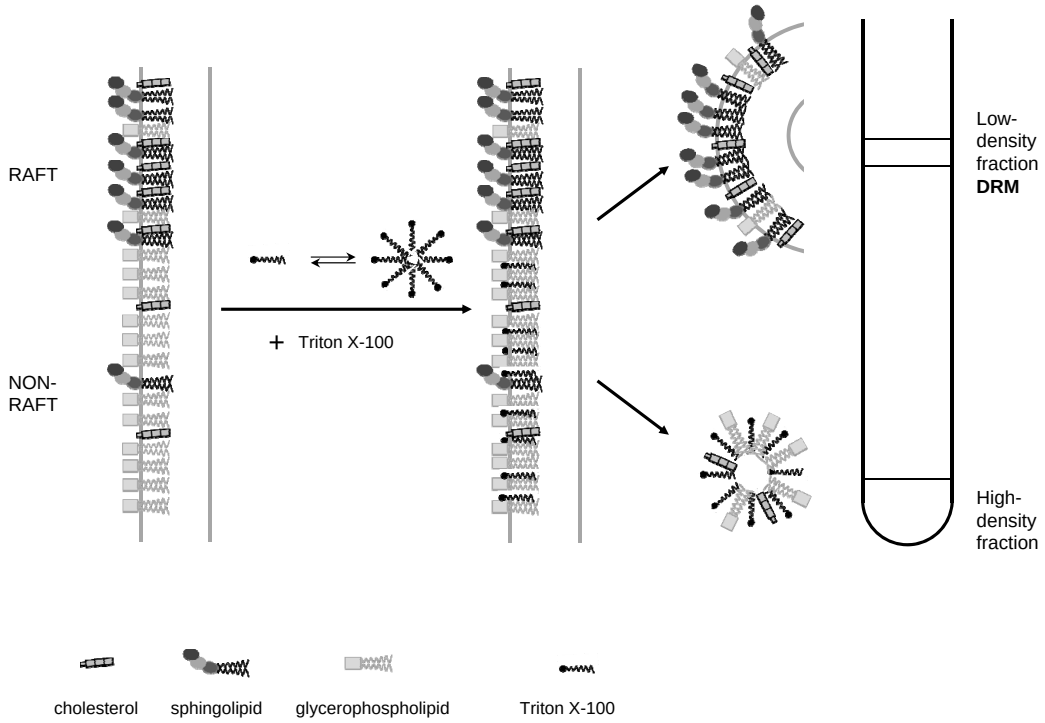

Fig. (4). Insolubility of lipid raft components in the presence of Triton X-100 and preparation of detergent-resistant membranes (DRMs). Detergents in water solutions aggregate as small micelles over a critical micellar concentration (c.m.c.). The c.m.c. for Triton X-100 is 0.31 mM; at a 1% concentration many detergent monomers are therefore present in solution and enter into the fluid ("non-raft") portions of the membrane. Fluid membranes containing Triton X-100 are dissolved and form small mixed micelles that are enriched in detergent, GPLs, and proteins. The detergent does not enter into the less fluid ("raft") portions of membrane, which contain few proteins but are enriched in SLs and cholesterol. The membrane in these areas therefore maintains the bilayer structure. Detergent-resistant and detergent-soluble components can be separated by gradient centrifugation. The figure schematically depicts only the external membrane layer. Proteins are not illustrated, and the relative proportions of the membrane components as shown are not accurate.

Table 1. Composition of DRMs from Rat Cerebellar Neurons Differentiated in Culture. The Composition of the Cell Homogenate is Shown for Comparison [51, 126]

	Homogenate		DRM	
	nmoles/10^6 cells	% of total	nmoles/10^6 cells	% of total
Proteins	1.25	3.05	0.02	0.28
GPLs	32.84	80.29	3.95	55.39
SLs	2.01	5.13	1.25	17.53
Ceramide	0.22	0.54	0.11	1.54
SM	1.00	2.44	0.67	9.39
Gangliosides	0.79	1.93	0.47	6.59
Cholesterol	4.80	11.73	1.91	26.78

using detergents with various stringencies. However, the association of other proteins (particularly integral membrane proteins) with DRM fractions was strongly affected by the type of detergent and by the detergent/protein ratio. The DIMs obtained in the presence of Triton X-100, CHAPS, Brij 96, and Triton X-102 were found to float at different densities, suggesting that the domains insoluble in different detergents were characterized by different lipid content or different lipid/protein ratio. Some studies indicated that the differences observed in the composition of DRM fractions isolated using different detergents might reflect the existence of different levels of order within lipid membrane domains and/or of biochemically distinct lipid membrane domains within the plasma membrane of a given cell [202, 208-211], *i.e.*, the existence of different subpopulations of lipid rafts. *E.g.*, (a) differential solubilization by Triton X-100 and Brij 96 was used to demonstrate that two GPI-anchored proteins in neurons, Thy-1 and PrP, belong to structurally different lipid membrane domains characterized by differing degrees of order [200] and lipid composition [212]; (b) biochemically distinct detergent-specific domains from myelin membrane were separated using different non-ionic (Triton X-100, Brij 96, Triton X-102) or zwitterionic (CHAPS) detergents [202]. Thus, differential detergent solubilization may provide a powerful tool for studying different lipid raft subpopulations or different degrees of lateral order within the same lipid raft. On the other hand, the above findings imply that a DRM may contain membrane fragments derived from the aggregation of distinct lipid membrane domains [200], thus posing a further methodological concern regarding possible artifacts associated with this method. It is clear that detergents, particularly Triton X-100, substantially alter the lateral organization of biological membranes [213]. However, a recent study showed that Triton X-100 did not induce the formation of liquid ordered domains in a model membrane with a composition similar to that of the outer leaflet of plasma membranes, although it increased the average domain size by inducing the aggregation of pre-existing domains [214].

Given that DRMs correspond to phase-separated l*o* domains in membrane bilayers, it is not surprising that temperature critically affects DRM preparations. All of the steps in the preparation procedure are performed between 0 and +4 °C [154]. The low temperature necessary for DRM preparation is an experimental condition that cannot be easily extrapolated to living cells; this fact is one of the major criticisms directed at the physiological relevance of DRM fractions. The working temperature was shown to have a major effect on the composition of DRM prepared from bovine brain myelin. When purified myelin was extracted with Triton X-100 at 20 °C and the extract was then fractionated over a continuous sucrose density gradient, two distinct low-density fractions were isolated [215] which both had higher cholesterol and galactosylceramide levels than the starting preparation but differed from each other in their levels of GM1 and specific protein markers. The l*o*/l*d* phase separation in model membranes occurs at 37 °C [216], and DRM can be prepared from cells or tissues at 20° or 37°C in some cases [201, 202, 204, 215]. Proper adjustment of the ionic composition of the solubilization buffer (*e.g.*, Mg^+ and K^+ concentrations similar to those in the intracellular environment and addition of EGTA to chelate Ca^{2+}) allows the preparation at 37 °C of DRMs that have many of the properties of lipid rafts isolated from brain membranes or cultured cells using Triton X-100 or Brij 96 [217]. These "37 °C DRMs" were larger than lipid rafts prepared at low temperature, indicating that some aggregation may have occurred during the purification. This phenomenon can be avoided by replacing the flotation method for DRM isolation with a magnetic immunopurification procedure, which minimizes the time required for DRM isolation [218].

Although isolation procedures based on different detergents are useful and appropriate for understanding the lateral order of biological membranes [202, 212], the detergent insolubility of a protein is clearly determined in some cases by the protein's intrinsic structural features (*e.g.*, the mode of its association with the plasma membrane) rather than by its association with lipid rafts [206]. Detergent-insolubility *per se* is therefore not a sufficient criterion for establishing the association of a protein with lipid rafts. Biochemical analysis of the complex environment of the protein (particularly of its lipid composition) or complementary approaches in intact cells are necessary to establish the association of a protein with a lipid-rich, highly organized membrane domain.

To avoid problems related to the use of detergents, there have been many attempts to develop "detergent-free" methods for the separation of low-density membrane fractions corresponding to lipid rafts. The rationale for these attempts was that resistance to detergent solubilization might be just one aspect of a more general phenomenon, *i.e.*, resistance to a variety of treatments capable of disrupting the structure of less ordered membrane areas but not that of highly organized, "rigid", and thermodynamically favored lipid membrane domains. Several distinct techniques have been developed for the separation of lipid raft fractions without the use of detergents. The disruption of cells in the presence of high pH or hypertonic sodium carbonate or by mechanical treatments (sonication under carefully controlled conditions) produces membrane fragments that can be separated by density gradient centrifugation [219-226].

Comparative analyses have shown that the compositions of DRM fractions obtained using different detergents vs. detergent-free low-density membrane fractions obtained after cell lysis under very different experimental conditions as described above are very similar but not identical [126, 168, 176, 203, 204, 227-236] This finding suggests that the highly resistant and ordered supramolecular structures that correspond to the native cores of lipid rafts can be isolated using these procedures, although the procedures alter the lateral order of biological membranes to some extent.

DIRECT DETECTION OF LIPID RAFTS AT THE CELL SURFACE

Several techniques are available for the detection of lipid rafts or organized domains in intact cell membranes. These techniques overcome the limitations of analytical methods based on detergent solubilization and add several relevant layers of information to the basic compositional knowledge obtained from the analysis of DRMs. The highly diverse experimental methods used for the identification of lipid rafts at the cell surface are all based on detection of the membrane topology of a putative lipid raft marker (which is usually defined on the basis of the marker's enrichment in DRM fractions) and require the use of a physical, chemical, or biological probe whose nature depends on the experimental approach.

Electron microscopy (EM) allows the ultra-high spatial resolution necessary for the investigation of membrane lateral heterogeneity at the meso scale and is therefore perfectly suited in principle for the detection of lipid rafts. The observation that GSLs form clusters (visualized by immuno-EM) at the cell surface was a key piece of evidence leading to development of the lipid raft hypothesis [237]. GSL clustering in cell membranes has been documented for globoside in human erythrocytes [238], polysialogangliosides in fish brain neurons [239], GM3 ganglioside in peripheral human lymphocytes, Molt-4 lymphoid cells [240], and murine 3T3-L1 adipocytes [241], lactosylceramide (LacCer) in human neutrophils and neutrophilic differentiated HL60 cells [64], and GM1 in dorsal root ganglion neurons [242]. Lipid raft markers can be detected by immuno-EM using colloidal gold particles of different sizes linked to anti-GSL antibodies or glycolipid-binding proteins. This method allows us to define the topology of a putative lipid raft marker in relation to cell architectural features or to other target molecules, and quantitative image analysis allows us to estimate average lipid raft size and the total membrane area occupied by lipid rafts. The fact that the method requires extensive sample manipulation, the use of multivalent probes (*e.g.*, IgM anti-glycolipid antibodies or cholera toxin B-subunit for GM1), and the use of organic solvents and/or of chemical fixatives raises concerns regarding possible experimental artifacts, particularly when the target molecule is a membrane lipid. Immunolabeling should be performed following fixation to prevent the redistribution of SLs after cross-linking with antibodies [243]. Most lipids do not react with the aldehyde fixatives commonly used in EM and can therefore be redistributed within or even removed from the membrane during sample handling [12, 244]. On the other hand, chemical fixatives do not preserve the *in situ* localization of membrane lipids [245]. Standard EM protocols can be modified in order to minimize these concerns. Immuno-EM using anti-LacCer IgM on ultrathin cryosections prepared without organic solvents showed that LacCer is present in clusters (~40 nm diameter) on the plasma membrane of human neutrophils, and double labeling with anti-LacCer and anti-Lyn antibodies showed that 24% of the LacCer clusters are associated with the Src family kinase Lyn [64]. A freeze-fracture replica labeling method was recently established that overcomes some of the disadvantages of conventional immuno-EM and allows quantitative analysis of the distribution of membrane lipids at a nanometer scale [246, 247]. In this method, living cells without pretreatment are flash-frozen using a liquid helium-cooled copper block which prevents any molecular motion in the membrane during the fixation procedure [248]. Samples are kept deep-frozen throughout the freeze-fracture procedure, and the fractured membrane is coated by a thin layer of carbon and platinum by vacuum evaporation, thereby immobilizing and physically fixing the membrane components. The labeling procedure is then performed on the replicas with an appropriate probe at a relatively high temperature (4-37 °C). This method has been successfully applied for nanoscale analysis of the distribution of membrane lipids in the outer (GM3 and GM1) or the inner leaflet (phosphatidylinositol 4,5-bisphosphate) of the plasma membrane. GM3 and GM1 formed independent clusters (<100 nm diameter) at the cell surface [249, 250]. Ganglioside clustering was sensitive to cholesterol depletion, depolymerization of the actin cytoskeleton, and (in the case of GM3) inhibition of Src family kinases [250]. Clustering of GM1 in GM1-null fibroblasts loaded exogenously with GM1 was similar to that of endogenous GM1 [249]. The same method revealed that phosphatidylinositol 4,5-bisphosphate is unevenly distributed in the plasma membrane and highly clustered in the cytoplasmic leaflet at the rim of caveolae; this was the first direct evidence that lipid lateral segregation also occurs in the inner leaflet of the membrane [251].

EM analysis has high spatial resolution power but is not suitable for studies of lipid raft dynamics in time and space. Fluorescence microscopy, which has high sensitivity and is suitable for dynamic studies of living cells, has the disadvantage of poor spatial resolution (the average diameter of a detection spot in confocal microscopy is >200 nm). Several high-resolution techniques that utilize fluorescent probes are now available for studies of membrane heterogeneity in intact cells, allowing the study of lipid rafts *in vivo* [139, 252-255] (Table **2**). Some of these techniques are particularly appealing because of their capability to reveal the dynamics of membrane domains. Such capability is completely lacking in equilibrium-based methods such as those used to study phase separation in model systems and those used to analyze membrane fractions separated on the basis of detergent solubility.

The widely used techniques of fluorescence recovery after photobleaching (FRAP) and fluorescence correlation spectroscopy (FCS) allow us to determine the translational mobility of a fluorophore [256, 257]. Fluorescence resonance energy transfer [258] (FRET) measures the energy transfer between an excited donor fluorophore and an acceptor molecule, allowing us to determine donor-acceptor proximity with high spatial resolution. Single fluorophore tracking microscopy (SFTM) [7, 259, 260], single particle fluorescence tracking (SPFT), and single particle tracking (SPT) follow the translational trajectories of membrane molecules by measuring the motility of a fluorescent label or of a colloidal gold particle (in the latter case, by Rayleigh light scattering) specifically bound to the target molecule [139, 261, 262]. These techniques are promising because of their applicability to dynamic studies, in striking contrast to the biochemical methods based on detergent solubility.

The results obtained from application of these techniques to studies of cell membrane heterogeneity were consistent to some extent with the concept of lipid rafts developed based on DRM analysis. In particular, studies using these techniques revealed a non-random distribution of cell surface molecules leading to a highly hierarchical membrane organization that includes microdomains differing in terms of composition, size, and spatial and temporal dynamics. The studies also confirmed the importance of cholesterol and SLs in membrane domain formation [263]. However, some of the findings obtained using these techniques appeared to contradict the basic assumptions that underlie the lipid raft hypothesis. FRAP studies, for example, indicated that the diffusion coefficients for various membrane proteins were correlated with the mode of association of the protein with the membrane rather than with the protein's detergent solubilization behavior [264]. FRET studies showed no evidence for non-random distribution of GPI-anchored proteins (detergent-insoluble membrane components that are usually regarded as lipid raft markers) [256, 265], and SPT of detergent-insoluble components suggested a behavior consistent with monomer diffusion [266-268]. However, several points must be taken into account when comparing results obtained in intact cells with those obtained from detergent solubility studies: (1) The techniques applied in living cells vary widely in their windows of spatial and temporal resolution [139], and results obtained using different techniques are therefore not directly comparable. (2) The techniques applied in cells rely on the use of a physical or chemical probe or tag to follow the behavior of a membrane molecule. The

Table 2. Techniques Used for Studies of Membrane Heterogeneity in Intact Cells

Technique	Experimentally Observable Events	Selected References
Fluorescence recovery after photobleaching (**FRAP**), Fluorescence correlation spectroscopy (**FCS**)	Translational mobility of a fluorophore	[256, 257, 320, 324]
Fluorescence resonance energy transfer (**FRET**)	Energy transfer between an excited donor fluorophore and an acceptor molecule, allowing the determination of donor-acceptor proximity	[257, 258, 325, 326]
Single fluorophore tracking microscopy (**SFTM**), Single-particle fluorescence tracking (**SPFT**), Single-particle tracking (**SPT**)	Translational trajectories of membrane molecules, allowing measurement of the motility of a fluorescent label or of a colloidal gold particle bound to the target molecule	[7, 139, 259, 260, 262, 327, 328]
Stimulated emission depletion (**STED**) microscopy	Time traces of single molecule diffusion of a fluorescence-labeled probe at the nano-scale	[263, 274]

use of probes introduces several possibilities for generating artifacts because of, *e.g.*, the multivalency of the probe (as in the case of anti-glycolipid antibodies, which are usually IgM, and of the cholera toxin B-subunit), chemical modifications introduced in the marker molecule, or the use of an extrinsic label. (3) The techniques typically follow the behavior of a selected marker molecule but do not provide information on the composition of lipid rafts or on their behavior as an entire entity. (4) A biochemical approach based on detergent solubility defines an averaged, non-dynamic situation that most likely results from the summed activity of many heterogeneous and time-dependent microdomains. Thus, each of the various techniques may be better suited for the detection of a specific subdomain population.

The limitations described above may be overcome in the near future by use of Stimulated Emission Depletion (STED) microscopy, the first and most widely applied in a family of fluorescence-based microscopic techniques that are able to pass the limit imposed by the diffraction barrier, thus scaling the resolution of fluorescence microscopy down to the nano level (some authors have referred to this technique as "nanoscopy") [269, 270]. In STED microscopy, the fluorescence emission of the probe is confined to a region smaller than that corresponding to the excitation spot by the application of a second doughnut-shaped light beam that is able to eliminate the fluorescence in the outer region of the excitation spot. This technique can be applied to the ultrastructural organization of living cells (*e.g.*, imaging of dendritic spines [271]) and even to living animals [272]. Application of STED microscopy to testing of the lipid raft hypothesis [263, 273] demonstrated that putative lipid raft markers, including GPI-an chored proteins, SM, and GM1, were confined to molecular complexes that cover membrane areas with diameters <20 nm. These complexes were transient and had an average lifespan of 10-20 msec. The complexes appeared to be cholesterol-dependent, as the trapping was reduced upon cholesterol depletion. STED microscopy was also used to demonstrate that CD11b integrin and LacCer are associated with the same "nanodomain" in the membrane of living neutrophils and participate in LacCer-mediated phagocytosis of microorganisms [274]. The major limitation of this technique is the necessity of using fluorescent probes or analogues or fluorescence-tagged antibodies against the target molecule, with the usual caveats related to the use of probes [275].

In summary, lipid rafts in intact cells appear to be non-equilibrial structures that can be generated, dissipated, or extensively reorganized in response to a wide variety of biochemical stimuli. The size of these short-term ordered structures in intact cells varies considerably from the nanometer [212, 263, 267, 276, 277] to the micrometer range [278-280], and their lifespan ranges from microseconds [281-283] to milliseconds or seconds [267, 277-279]. Such remarkable variability in the reported features of these membrane domains most likely reflects the great differences in spatial and temporal resolution that are characteristic of the different techniques used. Clearly, however, these observations indicate the coexistence of multiple kinds of non-equilibrial membrane domains the features of which cannot be explained solely on the basis of the lipid raft hypothesis, *i.e.*, fluid-fluid phase separation.

LIPID RAFTS AT WORK: EXAMPLES OF RAFT DYNAMICS AND RAFT-MEDIATED FUNCTIONS

We discuss in this section some examples of lipid-raft mediated cellular events. Our intent is not to provide a comprehensive overview or to list all the biological functions described for lipid rafts, but rather to illustrate the incredible complexity and dynamic nature of lipid raft biology. Other articles in this special issue describe in detail the effects of lipids and lipid rafts on the organization and function of various classes of G-protein coupled receptors.

The nervous system (whose cells, particularly neurons, are among the most highly enriched in SLs and cholesterol) provides many examples of lipid raft-associated signaling proteins and lipid raft-dependent signal transduction [126, 169, 176, 205, 207, 228, 230, 242, 284-305]. Lipid rafts in nervous system cells have been implicated in neurotrophic factor signaling [287-290], cell adhesion and migration [288, 296, 300], axon guidance and neurite outgrowth [242], synaptic transmission [288, 295], neuron-glia interactions [301, 302], and myelin genesis [303].

Several classes of proteins that are involved in signal transduction mechanisms have been reported to be associated with lipid rafts in cultured neural cells (neurons, oligodendrocytes, astrocytes, and neurotumor cell lines) and in various brain regions, myelin, and synaptic plasma membranes. These proteins include: (1) receptor tyrosine kinases (neurotrophin receptors Trk A, Trk B, Trk C, c-Ret, and ErbB, and the ephrin receptor Eph), GPI-anchored receptors (*e.g.*, GDNF family receptor GFRα), G-protein-coupled receptors (*e.g.*, cannabinoid receptors and neurotransmitter receptors such as α1-, β1-, β2-adrenergic, adenosine A1, γ-aminobutyric acid GABAb, muscarinic M2, glutamate metabotropic mGLUR, serotonin 5HT2); (2) non-receptor tyrosine kinases of the Src family; (3) adapter and regulatory molecules of tyrosine kinase signaling; (4) heterotrimeric and small GTP-binding proteins; (5) protein kinase C isoenzymes, (6) cell adhesion molecules, including integrins, Notch1, NCAMs, TAG-1, Thy-1, F3/contactin; (7) ion channel proteins, proteins involved in neurotransmitter release, and postsynaptic density complex proteins.

Two modes of involvement of lipid rafts in neuronal and glial signal transduction have been reported: (1) receptors and effector proteins that are intrinsically present in lipid rafts are activated, giving rise to signal propagation that involves other intrinsic components. Examples of receptors are neurotrophin receptors of the trk family, EGFR, PDGFR, p75NTR, GFRα [287-290], and the neural cell adhesion molecule TAG-1 [228, 304, 306]. Src family tyrosine

kinases are among the effector signaling proteins that are most commonly involved. (2) The activation of membrane receptors is followed by the recruitment to lipid rafts of the receptors themselves or of effector signaling proteins that are not located in the rafts under basal conditions. Alternatively, the activation of receptors that are associated with lipid rafts under resting conditions determines their translocation outside the rafts. Examples of the former case are the receptor tyrosine kinase c-Ret which is recruited into lipid membrane domains by its GPI-anchored co-receptor GFRα [287, 288, 290] and the neuronal adhesion receptor NCAM which is recruited into lipid membrane domains by cis- or trans-interaction with its membrane-bound, GPI-anchored ligand, prion protein [300].

GM3/EGFR Interaction

Long before the lipid raft hypothesis was proposed, observations that the growth of cultured cells was inhibited by exogenous addition of GSLs led to the idea that growth factor receptor function could be modulated by gangliosides [143]. EGFR was identified as the target of the inhibitory effect of GM3 [106]. GM3 was shown to inhibit EGFR autophosphorylation without competing with EGF for receptor binding [143, 307, 308] and without affecting receptor dimerization [105]. Other gangliosides were found to exert much smaller effects on EGFR, indicating that the GM3/EGFR interaction is highly specific [106, 309]. Studies of the purified human recombinant extracellular domain of EGFR indicated that the oligosaccharide sialyllactose was essential for ganglioside-receptor interaction and that its substitution by any other sugar reduced the binding [309]. The molecular basis of the GM3/EGFR interaction was not fully elucidated until 2006, in studies that demonstrated the importance of side-by-side carbohydrate-carbohydrate interaction between the GM3 oligosaccharide and an N-linked glycan bearing multiple GlcNAc terminal residues on the receptor [152, 153]. A 2011 study showed that the membrane-proximal lysine 642 residue of the receptor is essential for receptor interaction with GM3 oligosaccharide [104].

GM3/EGFR interaction is promoted by the enrichment of EGFR in ganglioside-enriched, cholesterol-sensitive, Triton X-100 insoluble membrane domains [310, 311]. Other GSL- and lipid raft-dependent factors can also affect EGFR function. Caveolae [9, 10] and caveolin-1 are involved in the modulation of EGFR signaling [312, 313], EGFR is localized within a caveolin-rich fraction in A431 cells, and EGFR-containing membrane fragments can be separated from caveolae [203, 314]. In a keratinocyte-derived cell line, GM3 overexpression induced a shift of caveolin-1 to EGFR-rich membrane regions, allowing its functional interaction with EGFR and the consequent inhibition of EGFR tyrosine phosphorylation and dimerization [315]. These findings indicate that GM3 influences EGFR signaling through a second distinct molecular mechanism that involves modulation of the EGFR/caveolin-1 association. GM3 also has an inhibitory effect on the ligand-independent cross-talk of EGFR with integrin receptor signaling; the accumulation of GM3 in cultured cells disrupts the interaction of the integrin β1 subunit with EGFR [316].

The 2011 study by Simons' group using recombinant EGFR reconstituted in proteoliposomes with phase separation-prone or -resistant lipid composition demonstrated convincingly that association with an *lo* phase is essential for the inhibitory effect of GM3 on EGFR autophosphorylation. In an *lo* phase, the effect of GM3 is linked to its ability to stabilize the inactive EGFR monomer in the absence of EGFR [104].

GM3 and the Regulation of Insulin Receptor Function

Insulin receptors (IRs) are present in DRMs from normal adipocytes [317] and are partially localized in caveolae in intact cells [318], in which the β-subunit of IR interacts with caveolin-1 through a binding motif that recognizes its scaffold domain [319]. Co-immunoprecipitation, cross-linking, fluorescence microscopy, and FRAP experiments showed that IRs are able to form distinct complexes with caveolin-1 and GM3 within lipid membrane domains [320]. The GM3/IR interaction was shown to be direct (as IR could be cross-linked by a photoactivable GM3 derivative) and specific (as the interaction was abolished by addition of exogenous GM3 to cells prior to the co-immunoprecipitation experiment). The GM3/IR interaction was abolished in IR mutants in which the lysine 944 residue was replaced by arginine, valine, serine, or glutamine. Taken together, these findings suggest that an electrostatic interaction between the negatively charged sialyllactose chain of GM3 and the positively charged amino group of lysine 944 of IR, which is located in close proximity to the transmembrane domain sequence, is essential for the formation of the GM3/IR complex. In 3T3-L1 adipocytes, the induction of insulin resistance by treatment with TNFα was accompanied by the upregulation of GM3 synthase, leading to an increase of cellular GM3 [318, 321] which was accumulated in DRMs. Conversely, the pharmacological inhibition of ganglioside synthesis by the glucosylceramide synthase inhibitor D-PDMP restored insulin signaling in TNFα-treated adipocytes [321]. In insulin resistance, the association of IR with GM3 was increased whereas its association with caveolin-1 and its localization in caveolae were decreased, indicating that the presence of excessive GM3 in lipid rafts leads to the displacement of IR from the complex with caveolin-1 and from caveolae. The association of IRs with caveolae was restored by D-PDMP treatment, suggesting that the regulation of IR/caveolin-1 association by GM3 may be responsible for the changes in insulin response in adipocytes [241, 320]. IR function is thus regulated by its partitioning between two distinct lipid raft populations (GM3-rich, flat membrane domains vs. caveolin-1-rich, invaginated caveolar domains), and this partitioning is in turn regulated by cellular GM3 levels. Based on these findings, Inokuchi proposed that insulin resistance in adipocytes be defined as a "microdomain disease" [322] and that the inhibition of ganglioside biosynthesis is a useful, novel therapeutic approach for this disease [323].

CONCLUSION

We hope that the studies described in this review have convinced the reader that multiple levels of order exist in biological membranes, and that membrane lipids play important roles in determining these levels of order. Clearly, the forces and interactions that underlie lipid-driven order in biological membranes are much more complex than was assumed at the time the lipid raft hypothesis was formulated. The biological consequences of this multi-scale and multi-level order are increasingly important and intriguing, and many crucial and exciting studies in this field remain to be performed.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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ABBREVIATIONS

DRM	=	Detergent-Resistant Membrane
EGFR	=	Epidermal Growth Factor Receptor

EM	=	Electron Microscopy
FRAP	=	Fluorescence Recovery After Photobleaching
FRET	=	Fluorescence Resonance Energy Transfer
GPI	=	Glycosylphosphatidylinositol
GPL	=	Glycerophospholipid
GSL	=	Glycosphingolipid
IR	=	Insulin Receptor
LacCer	=	Lactosylceramide
l*o*	=	Liquid-Ordered
PC	=	Phosphatidylcholine
SL	=	Sphingolipid
SM	=	Sphingomyelin
STED Microscopy	=	Stimulated Emission Depletion Microscopy

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