Biological membranes are not structurally passive solvents of amphipathic proteins and lipids. Rather, it appears their constituents have evolved intrinsic characteristics that make homogeneous distribution of components unlikely. As a case in point, the concept of lipid rafts has received considerable attention from biologists and biophysicists since the formalization of the hypothesis more than 10 years ago. Today, it is clear that sphingolipid and cholesterol can self-associate into micron-scaled phases in model membranes and that these lipids are involved in the formation of highly dynamic nanoscale heterogeneity in the plasma membrane of living cells. However, it remains unclear whether these entities are manifestations of the same principle. A powerful means by which the molecular organization of rafts can be assessed is through analysis of their functionalized condition. Raft heterogeneity can be activated to coalesce and laterally reorganize/stabilize bioactivity in cell membranes. Evaluation of this property suggests that functional raft heterogeneity arises through principles of lipid-driven phase segregation coupled to additional chemical specificities, probably involving proteins.

Lipid rafts are currently defined as dynamic sterol-sphingolipid-enriched nanoscale assemblies that associate and dissociate on a sub-second timescale [1,2]. Protein localization to these membrane environments is primarily mediated by a GPI (glycosylphosphatidylinositol) anchor, acylation or certain transmembrane domains [3]. Although still structurally elusive, biologically, it appears that rafts provide a means for cell membranes to form dynamic platforms within the bilayer, functioning in membrane trafficking, signal transduction and cell polarization [4,5]. In the present paper, we review the concept as it relates to our present understanding of functional heterogeneity in cell membranes.

Key words: chemical interaction, cholesterol, lateral self-organization, membrane order, sphingolipid.

Abbreviations used: CTB, cholera toxin b-subunit; DRM, detergent-resistant membrane; GPI, glycosylphosphatidylinositol; GPMV, giant plasma membrane vesicle; GSL, glycosphingolipid; GPI, giant unilamellar vesicle; Ld, liquid-disordered; Lo, liquid-ordered; PMS, plasma membrane sphere; STED, stimulated emission depletion; TCR, T-cell receptor; TM, transmembrane.

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Origins
The raft hypothesis was originally developed as an explanation for the enrichment of GSL (glycosphingolipid) in the apical membrane of polarized epithelial cells [6]. Unlike the specific signals used by proteins, there is no known soluble factor that can organize the lateral distribution of membrane lipids [7]. However, asymmetric enrichment of GSL implies that, during their journey to the largely immiscible apical and basolateral surfaces [8], lipids are somehow divided in the plane of the membrane. Indeed, lipodomics of post-Golgi carriers from yeast now reveals that glycerophospholipids are segregated away from GSL and sterol during trafficking to the plasma membrane [9]. Simons and van Meer [6] hypothesized that the self-associative properties of GSL in vitro, i.e. their capacity for hydrogen bonding [10–12], could lead to the formation of membrane domains as a basis for lipid sorting. The discovery of cholesterol-dependent Lo (liquid-ordered)–Ld (liquid-disordered) phase separation in wholly liquid model membranes [13–15] would later change the focus away from lateral heterogeneity by hydrogen-bonding to domain formation by differences in lipid order parameters [16,17]. In this case, Lo phase formation occurs...
via preferential association between cholesterol and longer saturated lipid hydrocarbon chains [18]. Although both forms of lipid assemblage potential now appear to be important (see below), it is worth mentioning an often forgotten fact: unlike previous descriptions of membrane domain formation [19,20], the raft hypothesis was uniquely geared to explain a functional biological phenomenon, i.e. lipids are sorted during membrane trafficking. Indeed, later discoveries of raft-mediated changes in lateral dimensionality and its consequent modulation of membrane bioactivity (e.g. signalling foci [21]) stress the need to relate principles of heterogeneous self-organization to the most likely evolutionarily selected need for functional association within cell membranes.

**Detergent-resistance**

The first working definition of lipid rafts was suggested by Brown and Rose [22] who reported that sphingolipids and GPI-anchored proteins from cell membranes were insoluble in Triton X-100 at 4°C and floated to a characteristic density following equilibrium density gradient centrifugation. Coupled with the observation that this detergent-insolubility was cholesterol-dependent and enriched for Lα phase constituents [23], these preparations (broadly defined as detergent-resistant membranes or DRMs) quickly became the ruling method for assigning lipid and protein raft affinity. However, using the standard Triton X-100 preparation, DRMs from animal cells are obtained at 4°C, but not 37°C, indicating that these Triton-insoluble complexes are not isolations of pre-existing membrane structures [24]. DRMs involve the artificial coalescence of raft constituents into an insoluble residue by process in which the physicochemical parameters are not fully understood [25]. Nevertheless, it is possible to work within the confines of this artefactual system via an analysis of ‘paired’ differences. Detergent-solubility/insolubility are not strict criteria in themselves; however, many of the major breakthroughs in the raft field originate from observations of changes in DRM association upon induction of physiologically relevant stimuli [26,27]. Such analyses of DRM partitioning changes have served as major workhorses for the evaluation of many raft-related phenomena, including assessing roles in disease pathogenesis [28], membrane trafficking [22] and identifying ‘raftophilic’ peptide moieties [24].

**Membrane patching and the uncovering of raft activation and functionality**

A major advance in the field came from the discovery that following antibody (homo-) cross-linking at the cell surface, the GPI-anchored placental alkaline phosphatase, the TM (transmembrane) raft protein haemagglutinin and the raft ganglioside GM1 (monosialotetrahexosylganglioside) exhibited a micron-scale co-patching distribution that excluded the transferrin receptor, a classical non-raft protein [29]. Similar selectivity in coalescence behaviour has now been demonstrated for a number of other laterally associating raft proteins and lipids [30–32] and can be transmitted through to the cytosolic membrane leaflet [33–35]. Selective coalescence is taken as strong evidence that clustered membrane constituents occupied a similar lipid environment before multimerization. More specifically, it suggests a means through which raft lateral organizing potential is functionalized. In the context of membrane trafficking, multimerization is an important lateral factor that promotes recruitment of GPI-anchored proteins into sphingolipid- and cholesterol-enriched carriers, most notably in clathrin-independent endocytosis [36]. Selective raft-based domain clustering can also occur naturally, as in the case of T-cell activation where lymphocytes restructure their cell surface to form membrane domains at TCR (T-cell receptor) signalling foci and immunological synapses [37]. Lipidomics of immunoisolated TCR activation domains now shows that, in comparison with membranes clustered by anti-transferrin antibodies, the activation site accumulates more cholesterol, sphingomyelin and saturated phosphocholine [38], all consistent with a raft-selectivity for lipid sorting in the membrane plane. Rafts in this ‘activated’ or coalesced condition are envisaged as being a more ordered membrane environment wherein lipid acyl chains are longer and more saturated. In combination with tighter cholesterol interdigitation, the result is a more condensed assemblage, a membrane environment that is separate from the surrounding more disordered unsaturated glycerophospholipid milieu [18].

**The resting state**

Rafts appear functionalized as lateral sorting platforms when activated to coalesce; however, of continual contention is the size and distribution of the so-called ‘resting state’ of lipid rafts. With the exception of caveolae, unperturbed lipid microdomains are too small and transient to be observed directly in unperturbed living cells [39]. As such, this debate has largely become the realm of researchers who employ high-resolution non-invasive microscopic techniques including single-particle tracking and microscopy [40–43], nanometric aperture-based FCS (fluorescence correlation spectroscopy) [44–46], high spatial and temporal resolution FRET ( Förster resonance energy transfer) microscopy [47–49], and the recently applied STED (stimulated emission depletion) far-field fluorescence nanoscopy [50]. A synthesis of this literature paints a picture of small non-random GPI-anchored protein assemblies, residing in ~10–20 nm areas of the plasma membrane. These nanoscale assemblies are highly dynamic, fluctuating on a sub-second timescale. Assembly formation is cholesterol-dependent; however, in some cases, an obligate role of the underlying actin cytoskeleton has been assigned [41,51]. Other techniques indicate that this nanoheterogeneity is actin-independent [46]. Moreover, the situation for TM raft proteins is unknown, so a coherent picture of ultrafine membrane organization is still lacking. In the traditional view of lipid rafts, the selective associative properties of cholesterol and sphingolipid would play a major role in domain formation. Indeed, STED microscopy now
shows that that, unlike glycerophospholipid, sphingolipid exhibits cholesterol-dependent confined diffusive behaviour, transiently dwelling in <20 nm diameter areas of the plasma membrane [50]. Although the effect of lipid hydrocarbon chain length was not tested directly, the glycerophospholipid and sphingolipid probes of this study had similar (and sometimes identical) acyl chain length and saturation indices. The differences in diffusivity were therefore suggested as being due to the differential hydrogen-bonding capacities of glycerol- compared with sphingosine-based lipids.

**Model membranes and the relevance of L₀ phases**

The re-emergence of hydrogen-bonding as a principle of raft organization raises an issue as to the degree to which lipid ordering, as it is understood in model membranes, accounts for raft-based membrane heterogeneity in the biological condition. In model membranes, it is clear that L₀ phase separation depends on the interaction of cholesterol with longer saturated lipid [52–58]. This phase separation is often visualized by the macroscopic coexistence of two immiscible liquid phases in ternary lipid mixtures [59,60]. However, it is becoming apparent that nanoscale assemblies of L₀ phase conformation exist well below the ~300 nm spatial resolution limit set by the diffraction of light [61,62]. An interesting example is the emerging concept of critical behaviour in biological membranes [63,64]. Before stable microscopic L₀–L₄ phase separation, membranes can exist in a 'supercritical state', wherein compositional fluctuations of L₀ phase within an optically uniform membrane can be observed by fluorescence microscopy. At higher temperatures above the critical point, sub-micron fluctuations are expected. Whereas the form of optically unresolvable L₀ behaviour remains unclear, micron-scale phase separation is thought to result from coalescence of nanoscale L₀ domains once a certain line tension (greater than thermal energy) has been reached [62]. In this situation, L₀–L₄ phase miscibility decreases until it is no longer possible for L₀ phase membrane to persist below optical resolution and microscopic phase separation results. Line-tension-driven L₀ phase formation has been suggested as an explanation underlying coalescence of raft domains in cell membranes [65,66]. Indeed, GPMV (giant plasma membrane vesicle) isolated by a formaldehyde blebbing procedure can be induced to phase separate into L₀- and L₄-like domains by cooling [67]. Moreover, this system displays similar critical point behaviour to that of model membranes [68]. Now the question arises: does L₀ formation behaviour account for raft assembly and functional clustering in cell membranes?

A contention of the lipid raft hypothesis is that the metastable raft resting state can be stimulated to coalesce into larger more stable raft domains by specific lipid–lipid, protein–lipid and protein–protein interactions [69]. When clustered, bilayer components are thought to be laterally stabilized according to their underlying affinity for pre-existing raft domains, i.e. clustering enhances the inclusion of proteins associating both strongly and weakly to rafts and excludes further those that segregate away [18,69,70]. Indeed, a lipid basis for clustering has been identified in model membranes: uniform GUVs (giant unilamellar vesicles) containing sphingomyelin and cholesterol can be induced to macroscopically phase separate into G₃M₁-enriched L₀ phases via cross-linking by quinquivalent CTB (cholera toxin b-subunit) [71]. However, in this situation (and for all cases of phase separation in model membranes) raft TM proteins are excluded from the L₀ phase [72–74]. This phenomenon is also seen for the cold-induced L₀–like phase of the formaldehyde-isolated GMPVs [75]. It appears that TM proteins are excluded physically by the energetic constraints of packing membrane-spanning α-helices into rigid membrane phases. This property does not appear to correlate with the co-patching behaviour seen for clustered TM raft proteins and raft lipids at the cell surface. The discrepancy between TM protein inclusion in rafts and exclusion from ordered phases in model systems has been unclear for some time.

A possible answer to this question has come from recent work suggesting that L₀ phases are not equivalent to stabilized raft domains at physiological temperature [76]. We have developed a cell-swelling procedure to separate PMSs (plasma membrane spheres) from the cytoskeletal/endocytic/exocytic influence in A431 cells, an epidermoid carcinoma cell line expressing the raft ganglioside G₃M₁. At 37°C, clustering of G₃M₁ by quinquivalent CTB, induces the cholesterol-dependent micron-scale coalescence of G₃M₁ domains on the surface of previously uniform PMS. The G₃M₁ phase exhibits slower translational diffusion, enriches for cholesterol and recruits both TM and exoplasmic/cytoplasmic lipid-anchored raft proteins, but not the transferrin receptor. These data indicate that, at physiological temperature, biological membranes possess the compositional capacity to access underlying raft-based connectivity and amplify it to the level of a distinct membrane phase. Moreover, the selective inclusion of TM proteins suggests that this phase possesses a quality in addition to the lipid basis for L₀–L₄ phase separation seen in model membranes. Indeed, the membrane-order-sensing dye Laurdan has now indicated that although this G₃M₁ phase (TM protein selective membrane) is more ordered than the surrounding PMS membrane, the level of condensation is far below that which is seen in the L₀ phase (TM protein excluding membrane) regions of phase-separated GUVs [77]. Therefore phase separation, as it is understood in model membranes, does not completely account for raft coalescence behaviour at 37°C, at least in activated PMSs.

**Perspectives: rafts as assemblies functionalized by both physical and chemical specificities**

The context for heterogeneity by rafts must be understood within the complex chemical background of biological membranes. In model systems, phase separation arises from interactions between different lipids that leads to a liquid–liquid
immiscibility in the membrane plane, a concept valid for a number of lipid species and simple lipid/protein preparations [18]. However, cell membranes encompass a multitude of specific biochemical structure–function relationships that generate a lateral interaction diversity not described by these systems. Occupying over 20% of cell membrane area [78], proteinaceous sources of membrane heterogeneity are also potentiated: oligomeric protein assemblies dominate over monomers [79]; variations in hydrophobic domain dimensionality create membrane regions of varying thickness and composition [80,81]; and large ectodomains cover lipid and produce steric restrictions [79,82]. A particularly interesting area of this complexity is the specific binding of lipid to protein. Here, the discovery of the peptide boundary layer unveiled the capacity for direct lipid sorting by protein [83]. Moreover, X-ray crystallography now shows that specific lipids are selected as integral components of the quaternary structure of many membrane protein complexes [84–89]. These data underscore the capacity for a coherent membrane structure formed through both chemical and physical parameters. Lipid–protein interactions alone cannot account for lipid rafts as has been suggested [90], because this excludes the possibility for selective lipid–lipid association. Both lipids and proteins have the potential to specifically organize features of the membrane plane. In this respect, coalesced rafts are likely to be the products of lipid phase separation principles coupled to specific lateral associations governed by standard biochemical ligand interactions, i.e. hydrogen bonds, van der Waals attractions, hydrophobic/hydrophilic interactions and electrostatic forces. Under this scheme, functional raft-based membrane heterogeneity depends on both lipid physical parameters and specific interactions that may include or even require TM proteins. The importance of raft functionality was highlighted in the opening of the present review: heterogeneity in cell membranes did not evolve to laterally sort membrane constituents through a purely physical lipid phase separation that is devoid of protein, but through raft phase separation that is selectively and functionally inclusive. Here the principles of lipid phase separation are unified or ‘wetted’ to specific lipid–protein–protein interactions, not just as cross-linkers of lipid domains, but also as structural features of the domain itself. Consequently, the entity produced upon raft clustering at 37 °C is not exclusively a domain in which proteins and lipids partition according to order preferences, but is a chemical complex scaffolded by specific chemistries of association. In this view, protein-based heterogeneity joins with cholesterol–sphingolipid assemblage-potential to functionalize rafts in living organisms. Rafts, as we define the term, refers to the operational assembly present in living cells, assemblages wherein bioactivity is dependent on lipid and protein.

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