Critical Lipidomics: The Consequences of Lipid Miscibility in Biological Membranes

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Abstract There is growing evidence that cell plasma membranes exhibit significant lateral heterogeneity in the composition of lipids and concentration of proteins. These domains have sub-micron dimensions and have been implicated in vital cell functions. Similar liquid domains are also observed, with fluorescence and non-perturbative techniques such as NMR, in model bilayer membrane mixtures that mimic cellular lipid compositions. This chapter overviews the physics, biological evidence, and consequences connected to liquid immiscibility in phospholipid membranes. The presence of phase transitions close to physiological conditions and concentrations directly implies a wide phenomenology of spontaneous lipid organization and dynamics on different length-scales. The interplay of this spontaneous lipid ordering due to the miscibility transition, with protein function and other regulatory, structural, biochemical, and mechanical membrane processes, is still an open area of investigation.

Keywords Lipid rafts · Lipidomics · Membrane proteins · Phase separation

1 Introduction

A basic aspect of cell membranes is to provide a barrier to partition volumes, and regulate the transport of charged molecules; they also act as a two dimensional substrate for membrane bound proteins, and indeed a large fraction of protein biochemistry in a cell takes place on or near membranes [1]. In infectious disease, parasites (viruses, bacteria, apicomplexans) bind and penetrate the cell through the membrane. It is crucial for all these functions that cell membranes are in a liquid

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state, where proteins and lipids are able to diffuse laterally. Over the years, a better understanding of the physics of lipid mixtures has shed light on the fact that the lipid component of cell membranes plays a quite active regulatory and functional role [2–4]. Biological membranes vary significantly in topological complexity from small, highly curved single bilayer structures (e.g., synaptic vesicles) to extended highly convoluted organelles (e.g., cubic membranes in endoplasmic reticulum [5], and the Golgi apparatus), extended single bilayers (e.g., the plasma membrane), and "bulk" liquid-crystalline phases (e.g., the myelin sheath). The energy related to curvature and the intrinsic curvature of bilayers are important in defining the shape of the biological membrane [6]. Many vital cell processes involve dynamic interconversions between these different morphologies, for example, by membrane fusion, fission, or budding, on time-scales spanning milliseconds to days or longer (10^{-3}–10^{6} s). Lipid asymmetry across the bilayer, lateral organization into domains, and curvature are all known to play crucial roles in maintaining these structures and their associated functionalities [7]. Cells maintain lipid homeostasis not just globally, but within each compartment of a very dynamic environment characterized by constant flow of lipid vesicles between different membranes [7].

As well as the properties that determine structure and stability, the physical parameters linked to transport and dissipation within the membrane also play an important regulating role; in particular the membrane viscosity determines how fast objects confined to the membrane can move. Typical "objects" are the membrane proteins, and assemblies of these proteins. Their function is to allow processes both within the membrane and also in the bulk fluid inside and outside the cell [8]. For example, there are pumps that regulate the concentration of calcium, potassium, pH, etc. across the membrane. Other proteins promote the formation and budding off of small membrane vesicles, which are essential for the directed transport of molecules to particular areas of cells [9]. A very important class of membrane proteins are those that act as receptors, binding to specific chemicals, and triggering a particular downstream response; the sequence of such responses within the cell is called a signaling pathway (or transduction cascade). The molecules that relay signals from receptors on the cell surface to target molecules in the cell cytoplasm or nucleus inside the cell are called second messengers (the first messengers being the signal molecules that arrive on the cell) [8]. While many of these processes are very specific, and the biochemical details are different for each set of coupled chemical reactions, signaling pathways are themselves an area where general physical principles are important.

Many phases exist in lipid monolayers and bilayers, each characterized by differing symmetry in the lipid order (these are liquid crystal phases), and authors have used a variety of notation. The main phases we discuss here are: the disordered and highly fluid phase that occurs when pure lipids are above their chain melting temperature; the solid like gel phase below the lipid's chain melting temperature; the intermediate "liquid ordered" phase that occurs when a significant fraction cholesterol is mixed with saturated lipids, which has been postulated to occur both above and below the main transition temperature. For notation, consistent with many papers, we will use L_α to denote the disordered phase in the absence of cholesterol

(e.g., in the phase separation that can take place between saturated and unsaturated binary lipid mixtures), and L_o/L_d (ordered/disordered) to indicate the two liquid phases, of differing order and physical properties, that can occur in the presence of sterols. The subject of this chapter is the discovery, over the last few decades, that the composition of eukaryotic plasma membranes of cells is tightly regulated such that the system is close (and the proximity is seen to be changed in various cell regulatory transitions) to a thermodynamical critical point for demixing into coexisting L_o/L_d liquid phases, containing different lipid and protein components.

Physical systems in the vicinity of critical points have general behavior that depends only on the symmetry of the order parameter and the dimensionality of the system, and not on the detailed molecular interactions. In general, near a second order phase transition (such as the demixing that takes place in the ternary lipid mixtures) the susceptibility diverges, and fluctuations in the order parameter become large and long lived; this is referred to as "critical behavior." There are specific laws (common to wide classes of systems) to describe the critical behavior of thermodynamic parameters as a function of the distance to the critical point, in particular here the temperature difference [10]. Laterally separated liquid domains also spontaneously form in model bilayer membranes with certain lipid compositions, allowing the thermodynamic transitions, and organization on multiple length-scales, to be well characterized.

We argue in this chapter that the thermodynamic proximity of the critical point is biologically relevant, entailing a variety of behavior well understood from the physics of phase transitions, including spontaneous formation of transient domains. Also, the formation of more permanent domains can occur with a modest energetic cost. It is known from various communities, through experiments using a variety of methods, including recent direct observations in living cells, that saturated lipids and cholesterol in cell membranes laterally organize into domains or "rafts," affecting protein function. It is likely that phase separation is the key physical concept underlying raft formation in biological membranes, and that the related mechanisms are exploited by cells as important regulators of membrane biochemistry.

2 Lipid Phases and Structure in Biological Membranes

Three major classes of lipids are present in biological membranes: phospholipids, glycolipids, and sterols. Phospholipids are a major component of cell membranes, and are composed of a head group and phosphate group; these are covalently linked to two hydrophobic chains through either a glycerol (glycerophospholipids) or sphingosine (sphingophospholipids) backbone [1, 11, 12]. The hydrocarbon chains can each vary in length (number of carbons) and in the level of unsaturation (number of double bonds): these are the main aspects that determine the main chain transition temperature, and mixing behavior between different species. Common head groups are choline (phosphatidylcholine, PC), serine (PS), and ethanolamine (PE). Among the sphingophospholipids, sphingomyelin (SM) is commonly found in cell membranes.

Glycolipids have a sugar group (e.g., glucose) in place of the polar head, and then like phospholipids their backbone can be glycerol or sphingosine. In both phospholipids and glycolipids, the head group is exposed to the aqueous environment, and is responsible for specific chemical affinity to proteins. Sterols are present in plant and animal cells, and absent in most prokaryotes. They are also strongly amphiphilic molecules, with a very small hydrophilic region (O–H). A typical sterol is cholesterol, where the hydrophobic region is made up of a rigid, planar, ring structure, and a short hydrocarbon chain which terminates in two methyl groups. The amount of cholesterol in biological membranes of eukaryotes varies significantly, increasing concentration from the endoplasmic reticulum (ER) where lipids are synthesized, through the "secretory pathway," up to the plasma membrane [13]. Sterols generally can partition in the hydrophobic region of phospholipid bilayers, altering the physical properties, and in some cases giving rise to phase separation or formation of physically distinct phases [14].

2.1 Lipid Bilayer Phases

Membranes made of lipids with long, saturated chains tend to tightly pack and form gel (S_o) phases at room temperature (note, this classification is a simplification, considering together various possible distinct solid phases). In the S_o phase, lipid chains are ordered and extended, molecules are arranged in a hexatic lattice with a correlation length of approximately 290 nm [15], and individual lipids diffuse slowly in the plane of the membrane, with a diffusion constant of approximately $10^{-10}\,\mathrm{cm}^2/\mathrm{s}$ [16]. Above the chain melting temperature (T_m), membranes of pure phospholipids are in a liquid-crystalline (L_α) state, or, in other words, T_m is the boundary between S_o and L_α phases. The L_α phase is characterized by fast diffusion ($D \simeq 10^{-8}\,\mathrm{cm}^2/\mathrm{s}$ [16]), short lateral correlation lengths, and highly mobile hydrocarbon chains. Bilayers in the L_α phase are thinner (38 Å in L_α vs 44 Å in S_o, for DPPC [15]), and individual lipids occupy more cross-sectional area ($\simeq 70\,\text{Å}^2$ (L_α) vs. $\simeq 50\,\text{Å}^2$ (S_o) for DPPC [15]), than in S_o phase membranes of the same lipid species. Lipids with unsaturated hydrocarbon chains have low chain melting temperatures and tend to form L_α phases. The double bond restricts the mobility of the chain and prevents tight packing of the lipids into a gel state. Most lipids in cell membranes contain unsaturated bonds, contributing to the high fluidity of the membrane.

SM lipids often have high chain melting temperatures (T_m for 16:0 SM is 41 °C [17]). In cells, SM usually constitutes a large fraction of saturated lipids in the plasma membrane. Cholesterol is a ubiquitous molecule in mammalian cells, where it often makes up 20–40 mol% of the lipids in the plasma membrane. The planar ring structure of cholesterol is known to disrupt lipid packing when mixed with lipids below their chain melting temperature, while ordering the chains of lipids for $T > T_m$ [18].

A third phase of bilayer lipid membranes that will be discussed is called liquid-ordered (L_o). The L_o phase often contains saturated lipids and cholesterol. In this phase, the hydrocarbon chains of saturated lipids are more ordered and extended than in the L_α phase, though membranes remain in a liquid state where lipid diffusion constants are high ($D \simeq 5 \times 10^{-9}\,\mathrm{cm}^2/\mathrm{s}$ [16]), such that there is lateral disorder.

2.2 Lipids in Cell Membranes

In cells, lipids are found in bilayer membranes which provide a substrate for membrane bound proteins. Proteins are bound to the membrane either through hydrophobic peptide segments that span both leaflets (transmembrane protein) or are anchored to one leaflet through a few hydrophobic amino acids (e.g., cytosolic protein). Some proteins are acylated (modified with hydrocarbon chains) or directly bind to specific lipid species (e.g., GPI linked protein [19]). Some proteins are associated with the membrane through direct binding to a membrane bound protein (peripheral protein). Many lipids and proteins in the extracellular (outer) leaflet are decorated with carbohydrate moieties that contribute to an extracellular matrix. Some proteins that are exposed on the cytoplasmic face of the membrane are attached to the actin cytoskeleton (either directly or indirectly) and contribute to the structural stability of the membrane.

An added complexity is that lipids are distributed asymmetrically in the plasma membrane of living cells [7, 20]. Charged PS and PE lipids are actively pumped to the inner leaflet, while PC and SM lipids are found mainly in the outer leaflet. The loss of this asymmetry is a signal of cell death and can lead to degradation of the cell by the immune system.

2.3 Liquid-Ordered Phases and the Origin of the Raft Hypothesis

With the discovery of the liquid-ordered phase in mixtures of saturated lipids and cholesterol in the 1970s–1990s, physical scientists began to speculate that cell membranes might contain coexisting L_o and L_d phases (e.g., [21, 22]). It is more appealing to assume the existence of an L_o phase rather than an ordered S_o phase because S_o phases are rigid and would not be good substrates for membrane bound proteins. It was demonstrated that membrane lipid composition [23] and phase behavior [24] could affect protein function. Work by Simons and Van Meer [25] and Brown and Rose [26] in epithelial cells brought these ideas to the cell biology community and created a link between model and cellular systems [27]. In 1997, a landmark paper coined the words “Lipid raft” and described these biological

entities as small (<100 nm) L_o domains of saturated lipids and cholesterol in a "sea" rich of unsaturated lipid L_d phase [28]. Since then the field of lipid rafts has exploded, and many cell processes have been associated with these cholesterol and saturated lipid rich microdomains, including immune cell response, viral entry, cell polarity, protein sorting, endocytosis, cholesterol regulation, apoptosis, and many cell signaling pathways (reviewed in [29]). There is evidence for lipid rafts in a wide variety of cell types, including yeast [30], plant cells [31], and neurons [32].

The immense popularity of the raft hypothesis is due in part to the simple biochemical assay that is used to determine protein raft association. Lipid rafts are generally too small to resolve by confocal or widefield optical microscopy, but are thought to be biochemically isolated by exposing membranes to non-ionic detergents [26, 27]. The part of the membrane that remains after detergent extraction is thought to contain raft domains. This detergent resistant membrane (DRM) fraction is easy to isolate and biochemically analyze, and it has been shown that DRMs are enriched in saturated SM lipids, cholesterol, and certain membrane bound proteins [28]. While there is some evidence from model studies that DRMs contain lipids in a liquid-ordered state [33, 34], the interpretation of DRMs is strongly questioned by biologists and physicists alike. Detergent extractions are usually conducted at low temperature where more ordered phases may exist even if they are not present under physiological conditions. Also, it has been shown that the addition of detergent can promote phase separation [35]. Biologists have noticed that the composition of DRMs can vary when different detergents are used [36], or the same detergent is used in different concentrations [37]. In addition, proteins resident in internal membranes are often found in the DRM fraction, even though lipid rafts are thought to only exist in the outer plasma membrane of eukaryotic cells [38]. Other experimental methods are often combined with detergent extraction to support observations of lipid rafts, but they also have associated artifacts. For example, a protein is deemed "raft associated" if a fluorescently labeled version co-patches with known "raft markers" such as the ganglioside GM1 [39]. Unfortunately, this assay typically involves massively crosslinking both the raft markers and proteins of interest, begging the question if co-clustering is purely a consequence of this aggregation procedure and not reflective of the native state [38].

A second commonly used experimental method is cholesterol depletion. It is accepted that cholesterol is vital for raft formation, and therefore removing cholesterol should disrupt lipid rafts and their associated biochemical pathways. Cholesterol can be removed from the membrane by various molecules, disrupting protein organization and altering biochemical pathways. While it is possible to quantitatively measure the amount of cholesterol removed from the cell, in most cases it is not possible to determine the cholesterol concentration in the plasma membrane [38] because cholesterol can reside in internal membranes, and can be rapidly synthesized by the cell [40]. In addition, it has been shown that cholesterol depletion can have secondary effects that can alter protein organization. For example, a reduction in plasma membrane cholesterol can lead to disruption of the actin cytoskeleton which, in turn, alters the organization of both raft and non-raft proteins [41]. These experimental problems with raft assays led to skepticism

regarding the validity of the raft hypothesis, until better experimental methods were developed.

In its current form, lipid rafts are postulated to be small and dynamic regions of heterogeneous membrane composition, but can become larger and more stable in response to stimuli [42, 43]. This is consistent with a range of experimental data, including domains that form in immune cells after receptors are cross-linked with multivalent antigen [44]. A major criticism remains that the raft hypothesis lacks a firm mechanistic basis, as well as experimental methods to reliably probe consequences of membrane organization in cells [45].

3 Experiments on In-Vitro and Ex-Vivo Lipid Mixtures

3.1 Early Work on Binary Mixtures with Cholesterol

Much work was carried out from the 1970s on phase diagrams of binary mixtures (particularly, on phospholipid/sterol systems) [46–48], elucidating the quite complex role of cholesterol. Vist and Davis were the first to use the experimental methods of DSC and 2H NMR in concert to yield consistent results, and obtained the partial phase diagram for binary mixtures of DPPC-d62 and cholesterol shown in Fig. 1a [49]. By DSC, two peaks are detected in mixtures of saturated lipids and cholesterol. One of these peaks is sharp and corresponds to the main chain transition of the saturated lipid, while the other is broad and has been interpreted as demixing of liquid-crystalline disordered (L_d) and liquid- ordered (L_o) phases. The sharp transition remains fixed just below T_m and decreases in intensity with increasing cholesterol concentration. At the same time, the broad component increases in intensity, shifts to higher temperature, and becomes increasingly broad. The main chain transition is not observed in membranes with greater than 25% cholesterol, and the broad component can no longer be resolved when cholesterol exceeds 10–40%. Deuterium (2H) NMR directly measures anisotropic motions of the hydrocarbon chains. Distinct 2H NMR spectra can be acquired for membranes in the S_o, L_d, and L_o phases and are shown in Fig. 1b. Clear superposition of S_o and L_o spectra are observed in mixtures of DPPC and between 5 and 25% cholesterol at low temperatures ($T < T_m$). These results are in good agreement with DSC measurements which indicate an S_o–L_o coexistence region between membranes of 5–25% cholesterol. At higher temperatures ($T > T_m$), the interpretation of 2H NMR spectra is more difficult: No clear superposition of spectra are observed, instead 2H NMR spectra lose resolution in the proposed L_d–L_o coexistence region inferred by DSC. The loss of resolution is attributed to lipids exchanging between lipid environments on a time-scale comparable to the measured 2H NMR frequency differences ($\sim 10\,\mu s$). Since lipids exchange between domains via normal diffusion, this puts a limit on domain size of $<100\,nm$. At high temperatures and high cholesterol concentrations, the resolution of 2H NMR spectra is restored and

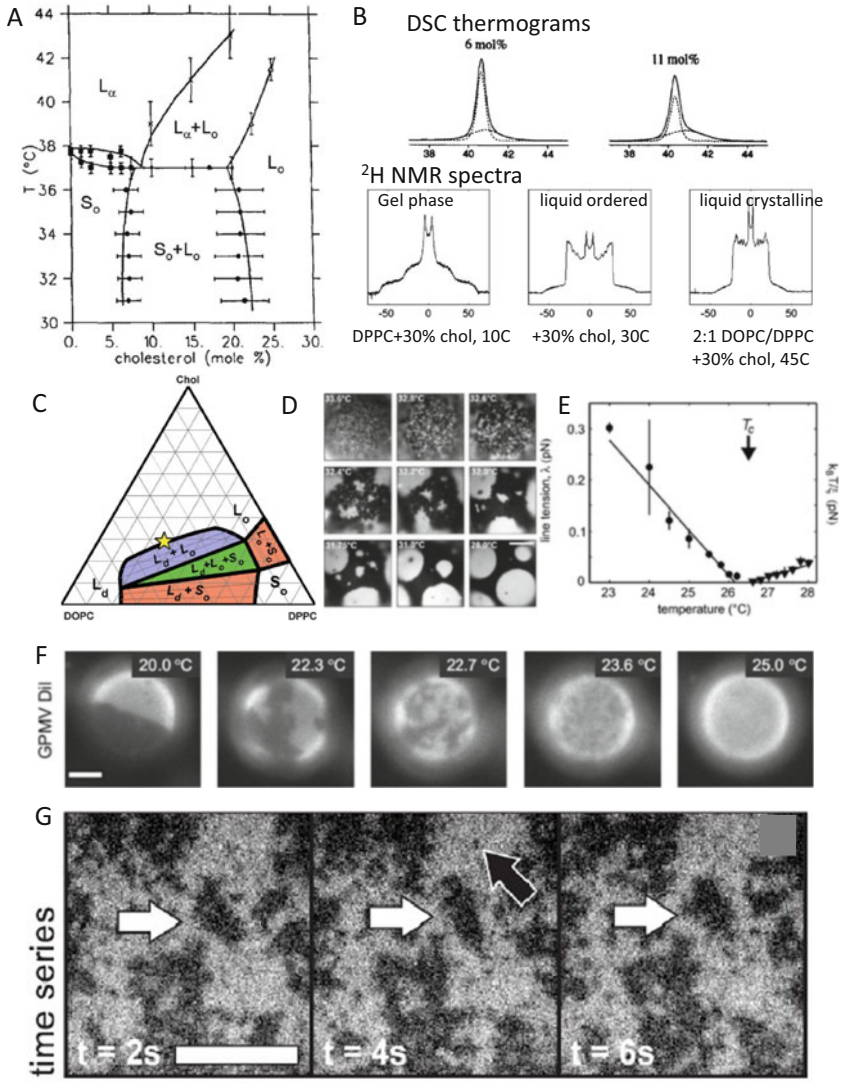

Fig. 1 Phase diagrams obtained in monolayers and membrane bilayers at concentrations mimicking the plasma membrane show phase separation and critical phenomena, and similar results are also obtained with GPMV extracted from cells. (**a**) Early work on binary mixtures of DPPC and cholesterol [49]; (**b**) DSC and NMR both allow determination of phase transition boundaries [50]; (**c**) composition phase diagram typical of a wide variety of ternary mixtures, at close to physiological temperature. Increasing T, the $L_d + L_o$ coexistence region shrinks and eventually vanishes. The yellow star marks the critical point at a particular T. (**d**, **e**) Changing T, and observing vesicles or GPMV in fluorescence microscopy, it is possible to measure the vanishing of line tension, and the growth of critical fluctuations, around the critical T [51]. (**f**) This $L_d + L_o$ coexistence and critical behavior are also observed in GPMV [52]. (**g**) The large critical fluctuations are transient, and their characteristic lifetimes (as well as their structure) depend on the proximity to the critical point in a way that is common to many other physical systems [53]

membranes are thought to be in one uniform phase. Additional evidence for the L_d–L_o phase boundaries is seen by analyzing trends in the moments of 2H NMR spectra as a function of composition and temperature. Phase diagrams like the one shown in Fig. 1a have also been evaluated by examining the lipid environment around spin probes (ESR [46]), by measuring diffusion constants of fluorescently tagged lipids (FRAP [54]), and by detecting short range interactions between fluorescent probes (FRET [55]). FRET experiments have also put a limit on domain size in the L_o–L_α coexistence region. Even though there are indications of thermodynamic transitions using experimental methods of DSC, NMR, ESR, and FRET, all phase separations detected in mixtures of saturated lipids and cholesterol are on a small length-scale [56, 57]. Fluorescence microscopy on membranes of binary mixtures of DPPC and cholesterol shows no $>1\,\mu$m arrangement of lipids, though there is evidence for $<1\,\mu$m lipid organization in membranes with $<25\%$ cholesterol below the chain melting temperature of DPPC [57]. In addition, a large change in area per molecule is detected in GUVs as temperature is scanned through T_m [58]. It is not obvious that small-scale lipid organization constitutes a true thermodynamic phase separation in mixtures of phospholipids and cholesterol. On one hand, the limits on domain size are large on the lipid length-scale (over 15,000 lipids could be contained in an 80 nm domain). On the other hand, these inhomogeneities are dynamic entities with interfacial regions that are not negligible. In addition, it is not known what governs the length-scale of <100 nm lipid domains. The nature and role of lipid domains have been addressed by the biophysical community for over 20 years [56, 59–61].

3.2 Recent Developments and Current Questions

Proximity to the critical point is a general mechanism that will lead to composition heterogeneity, but it should be noted that other mechanisms can also be at play: for example, stable nanodomains can arise in particular points of the phase diagram [4] (but perhaps not physiologically relevant to the plasma membrane concentration of sterols), and the coupling of composition with curvature can stabilize lipid heterogeneity (as well as then rapidly recruiting a variety of curvature sensing proteins) [60]. Many factors and organizing principles have been proposed and highlighted in isolation, and an interplay between these factors (lateral membrane heterogeneity, cytoskeleton pinning, clustering of lipids around proteins, and curvature) can result in very rich functional behavior. Many questions have been posed, and answered to differing degrees, out of this wide field of investigation: What types of lipids are needed to have a miscibility transition? How does composition affect transition temperature? What about phase morphology? Which lipids are found in the coexisting phases? And is one phase really liquid-ordered (L_o)? Also, are these phase separated membranes related to lipid rafts? If so, what do we learn about lipid rafts by studying miscibility? What regulatory roles can rafts play in the cell membrane? The remainder of this chapter describes the state of

work through these questions, focusing mainly on the effects that are expected from proximity to phase separation: from the determination of phase boundaries, characterization of the coexisting phases, exploration of cell membrane extracts, to the most recent experiments finding correlation (and in some cases direct links) of this phenomenology to cell biochemical protein processes.

The last two decades saw great progress in understanding liquid immiscibility in model lipid membranes, in large part made possible by the use of fluorescence microscopy to visualize phase morphologies in giant unilamellar vesicles (GUVs) [62], and the rationalization of results within the context of thermodynamic free energies of mixing. Fluorescence microscopy, coupled to suitable membrane dyes (and fluorescent antibodies and protein fusions for cell work), has become a tool of choice (validated in various ways by NMR, DSC, and AFM): it can directly highlight critical fluctuations and domain structure in GUVs (for example, artificial binary mixture vesicles [63], ternary systems [64], or cell membrane extracts [52, 65]). By this method, it has been shown that if the mixtures are allowed to phase separate then domains can grow to be large ($>1\,\mu$m), and S_o phases take on a variety of shapes that depend on lipid structure, whereas the liquid L_d and L_o phases form circular domains. Some fluorescent probes have spectral characteristics that are sensitive to the ordering of the hydrocarbon chains [63]. When these probes are used, lateral organization and chain order can be measured simultaneously.

The key initial studies were two reports of coexisting liquid domains in membranes with at least three lipid components. The first was by Dietrich and colleagues [34] where they directly observed coexisting liquid domains in giant unilamellar vesicles and supported bilayers by fluorescence microscopy. These domains differed from those observed in binary mixtures with cholesterol because domains were large ($>10\,\mu$m) and could be clearly resolved using fluorescence microscopy (e.g., see Fig. 1d, f). They characterized the phases as liquid, by quantifying diffusion constants, and showed that the more ordered phase was resistant to detergent at low temperature. A second study by Samsonov et al. [66] used black lipid membranes (a bilayer membrane spread over an aperture) and extended this work to additional lipid mixtures, varying cholesterol concentration, and making the connection between the miscibility transition and the chain melting temperature of the saturated component.

3.3 Direct Imaging Experiments Show Criticality in Model Systems

Many results on the properties of lipid mixtures near their critical point of demixing have been obtained by fluorescence microscopy, on giant unilamellar vesicles (GUVs) or on membrane extracts (giant plasma membrane vesicles, GPMVs). The resolution of fluorescence microscopy is sufficient to detect with good accuracy the miscibility transition temperature (T_{mix}) and also to extract length-scales and

fluctuation properties, so that the parameters of the criticality can be measured. Most observations to date are well described within thermodynamic models of the membrane, as reviewed in [67]. With recent advances in super-resolution imaging, it is becoming possible to investigate co-localization in vivo of lipid species and membrane proteins [44].

3.3.1 Giant Unilamellar Vesicles (GUVs)

Giant unilamellar vesicles (GUVs) of diameters 10–100 μm are most often prepared in >18 MΩ/cm water or non-ionic solutions, by the process of electroformation as described by [58, 68], with modifications to increase yield and compositional uniformity [69]. This specific method produces a high yield of compositionally uniform, single-walled bilayer vesicles when phosphatidylcholine (PC) or sphingomyelin (SM) lipids are used and when vesicles are grown at temperatures well above the chain melting temperature. It is possible to make bilayers of many lipid species, and typically a small molar fraction of one lipid species is tagged with a fluorescent marker; this trace species will in many cases partition proportionally to the compositions of the L_o/L_d phases. Other methods of making GUVs have been proposed, and have advantages in controlling size monodispersity, or enabling asymmetric compositions in the bilayers; however, they are challenging to fine tune for multicomponent mixtures, where each species differs in its amphiphilic character and adsorption dynamics.

3.3.2 Giant Plasma Membrane Vesicles (GPMVs)

Giant plasma membrane vesicles (GPMVs) can be derived from various types of mammalian cells. These GPMVs are thought to be free of cytoskeletal constraints and have been shown to display the same geometries and phase behavior of giant unilamellar vesicles (and once harvested, they can be worked on with similar methods). Fluorophores with preferential partitioning can be used to highlight fluid phases. The protocols differ slightly depending on cell type, but typically cells are grown to high density (sometimes to confluency) in tissue culture flasks, then washed and treated with formaldehyde and DTT [65, 70], causing blebs to form. With further incubation and gentle shaking GPMV detached from cells, and can be decanted. GPMVs are usually allowed to settle on ice before collection. By using this method, a single confluent 25 cm^2 flask yields sufficient GPMVs to create several dozen microscopy samples. In an alternative protocol, formaldehyde and DTT can be replaced by 2 mM N-ethylmaleimide, a reagent previously shown to cause GPMV formation [56]; all other steps are identical [71]. This alternative reagent is thought to induce less lipid/protein crosslinking, and generally to cause a milder chemical perturbation of the membrane; the resulting critical temperature in GPMVs obtained with this method is much lower, but the critical lipid phenomenology is the same. As a downside, the yield of GPMV is lower (more cells detach under these

conditions), and the mixing temperature close to freezing makes characterization of GPMVs more challenging.

3.3.3 Critical Behavior Near Liquid–Liquid Demixing

The phase diagram of ternary mixtures of saturated and unsaturated lipids, plus a sterol, has the general phases shown in Fig. 1c. On heating, the liquid–liquid region shrinks, meaning that phase separation (or more generally the proximity to the critical point), and hence the whole array of critical effects can be initiated by either changing temperature or by altering lipid composition. When lowering temperature through T_{mix}, small domains initially form in the membrane. These domains are circular, diffuse freely, and their edges fluctuate indicating that both the domain and the background phase are liquid [51, 72]. The vesicle ripens as domains collide and coalesce to form larger circular domains [73]. In most cases, vesicles completely phase separate at long times after the temperature quench (>1–30 min, depending on membrane composition, temperature, and state of tension). Alternatively, molecules can be added to the vesicle solution to initiate the miscibility transition at fixed temperature by changing lipid composition, for example, methyl-β-cyclodextrin (MBD, a carbohydrate molecule that binds cholesterol and removes it from the membrane) has proven useful.

Transition temperatures can be identified at a glance observing when recognizable domains appear (disappear) as temperature decreases (increases). More precise determinations of T_{mix}, analogously to what is done in other experiments of critical phenomena, can be obtained once the character of the phase transition is known. For example, in ternary mixtures the phase transition to L_o/L_d phases has been shown to exhibit a critical point and to be second order with Ising universality [10]; with this knowledge one can quite precisely find T_{mix} from the zero of reduced temperature by extrapolating some critical property like the domain line tension to zero (or in other cases finding their temperature of divergence, e.g., correlation lengths).

Even in the absence of protein interactions, it is possible to form metastable or even stable equilibrium phases with L_o/L_d domains of finite size. This can happen because of coupling with curvature, at low enough membrane tension [74, 75], or if the situation arises that the line tension is extremely small, akin to microemulsions [59]. Ultra small (radius < 5 nm) domains at physiological concentrations have also been reported recently, inferring their existence from FRET experiments [61].

There is biological significance in including sphingomyelin and POPC ((16:0–18:1)PC) in model vesicle systems. Phase diagrams for membranes of POPC mixed with palmitoyl SM (PSM) and cholesterol are strikingly similar to phase diagrams of mixtures that substitute DOPC for POPC (POPC/PSM/Chol), or dipalmitoyl PC (DPPC) for PSM (DOPC/DPPC/Chol) [76].

The system of diPhytanoylPC/DPPC/Chol has been studied in detail and has become a reference system, with a quantitative characterization of its properties near the critical point of demixing [51]. The choice of roughly equimolar concentrations of the three components positions the system close to its critical composition. Then,

above the critical temperature, the system is in a homogeneous phase; concentration fluctuations occur only below a characteristic size, which diverges (and domains take over the whole system) as the temperature is lowered to the critical temperature, see Fig. 1c. The divergence of this characteristic size is linear with temperature, consistent with critical behavior (Ising class universality) expected of physical systems in two dimensions [10]. As a counterpart to this behavior, it has been observed that below the demixing temperature, the circular domain shape is determined by a line tension; this tension vanishes (again, linearly) as temperature approaches mixing, from below. A subsequent study also established that the dynamics of these concentration fluctuations near their critical point is also analogous to that of simpler condensed matter systems [53]. These experiments allow the extrapolation of length- and time-scales beyond the experimentally accessible window.

Interestingly, GPMVs, which have the complex and rich composition of the plasma membrane, also exhibit liquid–liquid phase separation: the behavior appears critical-like (see Fig. 1f), and with apparently the same universality properties as in the model ternary mixtures [52, 65]. Critical fluctuations, and phase separation, are observed on cooling GPMVs below physiological temperature. Because of the invasive nature of the protocols used to produce GPMVs, one should not put too much weight on the exact temperature for phase separation; however, what is remarkable is that this temperature is robust for cells grown in identical conditions, and furthermore that consistent shifts in the phase separation temperature are observed as a consequence of perturbing the cells via drugs or signaling molecules [77–80]. This shows that the composition of the plasma membrane in cells is maintained close to the critical point, and the correlation of T_{mix} with pharmaceutical and biological stimuli is indirect proof that critical behavior is biologically relevant.

4 Cell Membrane Heterogeneity

Over the past few years, efforts from both membrane biology and biophysics communities have focused on the role lipids play in membrane organization [81–83]. In cell membranes, lipid rafts are currently thought to be localized regions that are on the order of 10–100 nm in diameter, possibly transient and rapidly dynamic over time, in which certain proteins and lipids are concentrated. Both the raft domains and the surrounding lipid matrix are liquid [83]. Lipid rafts have been associated with important biological processes such as endocytosis, adhesion, signaling, protein transport, apoptosis, and cytoskeleton organization [29, 84–86]. Since rafts in cell membranes have not been directly observed by standard microscopy, most current assays employ either indirect methods (e.g., detergent resistance), or crosslinking of rafts into larger aggregates (e.g., colocalization) [39]. Lipid-driven lateral separation of immiscible liquid phases is likely a factor in the formation of rafts in cell membranes. There is mounting evidence that the plasma membrane of many cells is inhomogeneous. Raft domains in cell membranes are thought to preferentially contain cholesterol and saturated lipids, as well

as specific lipids (e.g., sphingomyelin and the ganglioside GM1) and proteins (e.g., certain receptors and proteins with palmitoyl or glycosylphosphatidylinositol anchors) [28, 87]. The list of important physiological processes in which rafts are thought to play a role is long (see [88], and the references therein).

There is also ample evidence for biological tuning of plasma membrane lipid composition: It is well established that cells alter their lipid content in response to their environment. For example, bacteria and higher organisms change their membrane composition and physical properties when grown at different temperatures [89–93], yeasts alter their lipid content to counteract the membrane fluidizing effects of ethanol produced during fermentation [94–96], and mammalian cells adjust their lipids during the cell cycle [97–100] and differentiation [101, 102], and in response to stress or disease [103–105]. Some of these changes reflect the cells' effort to retain a robust and flexible barrier at the cell periphery.

Our current thinking, see Fig. 2, is that cells adjust their membrane composition also to maintain a functionally useful level of membrane heterogeneity, by tuning to be in close proximity to a miscibility critical point, a concept we think of as "critical lipidomics."

4.1 Lipids and Membrane Proteins

About 50% by mass of a biological membrane is composed of proteins (as little as 25% in the insulating myelin sheath, and as much as 75% in the membranes of mitochondria and chloroplasts). Some membrane proteins are transmembrane, extending across the bilayer. Others reside in the cytosol, and are anchored into a leaflet by one or more fatty acid chains, or anchored covalently to a lipid in the leaflet. Many membrane proteins are common between prokaryotes and eukaryotes. The transmembrane section of proteins is often one or more α-helix segments, which are relatively hydrophobic. The β-barrel is also a common motif, as in the porins, which are discussed below [8].

In order for a membrane protein to feel the effects of lipid heterogeneity, it must prefer to be surrounded by certain lipid types along their boundary. If these boundary conditions are specific enough, then the protein's localization and function can couple to local structure in the membrane bilayer. There are several ways that a cell might accomplish coupling to a more ordered local lipid composition. A common mechanism could be through posttranslational modification with saturated acyl groups such as palmitoylations and myristoylations. It is also hypothesized that proteins with greater hydrophobic thickness tend to prefer more ordered lipid local environments, since lipid chains in these regions tend to be more ordered and extended [107]. A third general mechanism to couple proteins to more ordered local lipids could be direct binding between proteins and specific lipids. Numerous "raft" associated proteins have proposed cholesterol binding sites, and some transmembrane proteins such as GPCRs have been shown to have preferential

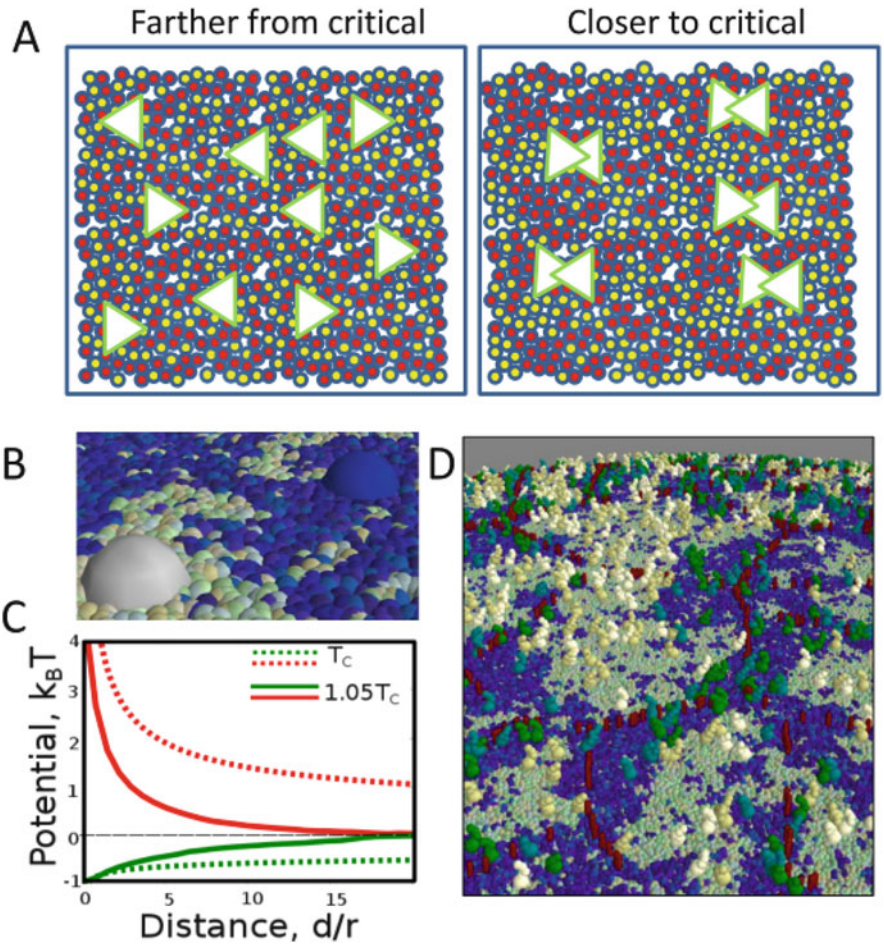

Fig. 2 Cells change membrane lipid composition, regulating the distance from the critical point. Critical lipidomics can profoundly affect cell membrane biochemistry, through a variety of mechanisms discussed in the text. One aspect is illustrated schematically in (**a**): closer to the critical point lipid domains form spontaneously, and can recruit proteins with a specific affinity, thus enhancing the local receptor protein concentration and changing the dimerized fraction. Conversely, association of proteins that require a particular lipid micro-environment is also facilitated closer to a miscibility transition. Lipid-mediated interactions between proteins can be tuned by adjusting $T - T_c$. (**b**) Schematic of two membrane inclusions (grey and blue caps) that prefer different local lipid environments feel a repulsion because they don't want to share the same local lipids. (**c**) The magnitude and range of this interaction is related to the proximity to the critical point. (**d**) Fluctuations within the membrane can couple to cortical cytoskeleton (drawn in red in the diagram), possibly also contributing to corralled diffusion [106]

interactions with polyunsaturated lipid chains [108]. Membrane proximal regions of proteins can have specific interactions with local lipids, such as through poly basic stretches on the cytoplasmic face which specifically interact with anionic lipids or glycolipid binding domains on the extracellular face. Recent studies also suggest

specific amino acid sequences within transmembrane helices can influence protein partitioning into ordered local environments [109].

Some membrane lipids are themselves directly involved in reactions: for example, binding of the protein AKT to the phospholipid PIP_3 is a signal that can trigger a range of events downstream, including the onset of cell growth and protein production [110]. PIP_3 is a minority component, accounting with PIP_2 mentioned above for around 1% of membrane phospholipids. Yet they are involved in a variety of processes [111]. The localization of PIP_3 has been studied in depth, in relation to the question of cell polarization and eukaryotic chemotaxis [112]. In response to a weak chemotactic gradient, a phase separation process is triggered, localizing PIP_3 on the plasma membrane side exposed to the highest chemoattractant concentration, and the PIP_3-degrading enzyme PTEN and its product PIP_2 to a complementary pattern [113].

A number of membrane proteins respond to the global mechanical properties of the membrane in which they are embedded; this "mechanics" (e.g., state of tension, state of curvature, flexibility to bend) can in turn be determined by composition, thus providing another mechanism coupling protein function to composition. An important example of this is represented by the mechano-sensitivity of ion channels [114, 115]. Taken together, cells have a number of different mechanisms at their disposal to regulate protein association through lipid composition.

5 Critical Lipidomics in Biomedical Scenarios

Section 3 overviewed what is known about criticality in model systems, and Sect. 4 gave a flavor of activity at the cell membrane, and how lipids, with their heterogeneity, can impact on proteins, and hence on a variety of functions. Very generic mechanisms should be at play in the membrane: the spontaneous formation of small domains, and hence tendency to recruit a higher concentration of proteins compared to a homogeneous membrane; the complementary side of the same mechanism, i.e., possibility of proteins to surround themselves in patches of specific lipid composition, see Fig. 2. Note the key importance (through mass balance kinetics) of the *local* protein concentration, for any process which (as typical of transmembrane receptors) requires a dimerization step, and even more for other processes requiring self-assembly of large protein structures (e.g., vesicle coating proteins). This is perhaps not fully or quantitatively appreciated in the literature.

Many protein processes are generic to many cell types (e.g., endocytic traffic) and represent what we can consider a basal cell activity. The lipid behavior consequent to the composition being close to a critical point is likely to be an important feature in these constitutive processes. However, in order to elucidate the importance of lipid composition, it is particularly interesting to look at some cell biology situations in which it is known that significant lipid composition changes occur. This has been observed (generally data exists from experiments involving mass spectrometry measurements on cell populations) in a variety of situations where cells differentiate,

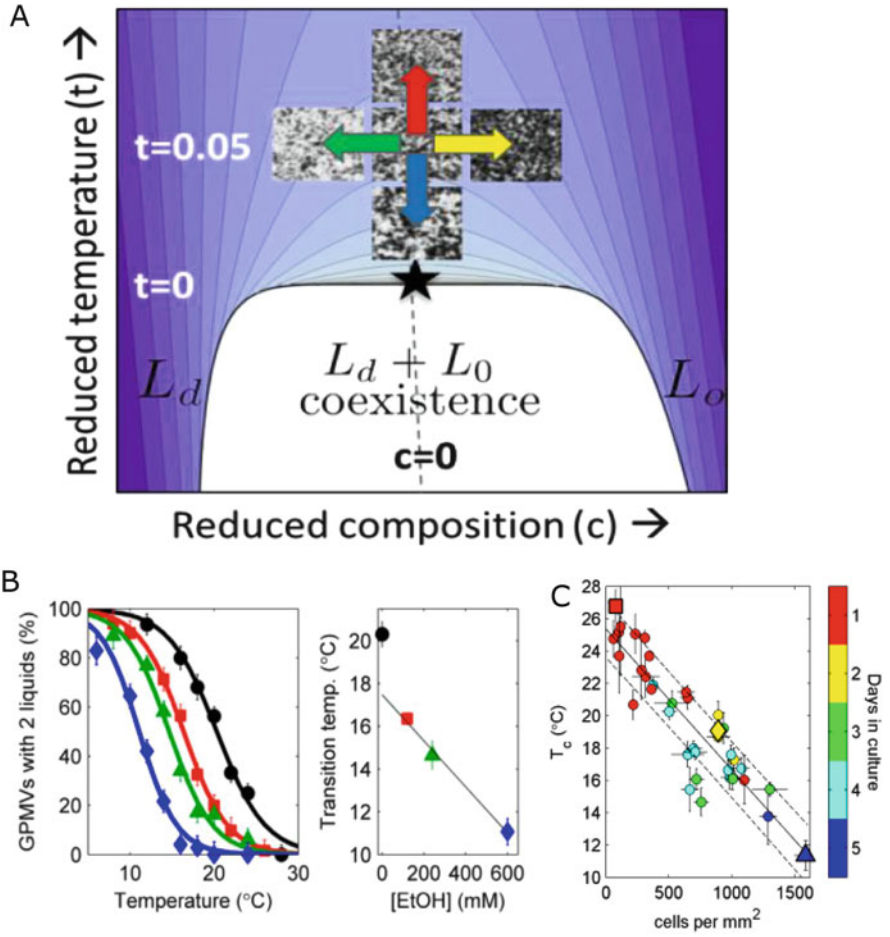

Fig. 3 There are now many cases where changes in lipidomics have been shown to correspond to varying distance to the critical point. (**a**) Schematic phase diagram of the PM, illustrating what different types of perturbations might do to lipid membrane structure. The data in (**b**) show that cell exposure to ethanol leads to composition changes that move the membrane away from criticality, as measured in isolated plasma membrane vesicles. Cells also can alter their own critical temperatures, panel (**c**) shows the case of cells grown up to different densities [77]

or enter different regulatory states, or in the context of disease [116]. We present here a few examples, and describe them in the eye of critical lipid behavior (Fig. 3).

More broadly, it is very likely that the regulatory and functional system coming from criticality underpins many "raft" dependent functional processes, ranging from immune, growth factor, and other signaling systems in other cell types to cellular processes where lipids are thought to play a role such as apoptosis, endocytosis, polarization, and cell division [112, 117–120]. Targeting biochemical modulators of membrane heterogeneity could be considered as a novel therapeutic strategy against

diseases characterized by altered lipid homeostasis, such as diabetes, inflammation, and some cancers [103, 121].

5.1 Lipid Criticality in Cancer

Cholesterol and phospholipid homeostasis is significantly altered in many cancers [103], this acts to suppress apoptotic signaling [122] and promote growth pathways [123] leading to cellular proliferation. For example, in human prostate cancer, there is an increased ratio of monosaturated to saturated fatty acids and these changes have been shown to affect the Akt pathway [124]. Frequently cholesterol levels are increased in tumors compared to normal tissues [125], and sphingolipid levels are reduced in many cancers, especially those resistant to traditional cancer therapies [126]. Interestingly, cancer cells primarily synthesize lipids directly [127], and the ABCA1 transporter is down-regulated in many cancers, resulting in decreased efflux of excess cellular cholesterol into LDL particles circulating in the blood [122]. In sum, there is a vast literature indicating that cancer cells actively alter their lipid composition while isolating themselves from regulation by other tissues. Numerous studies have implicated plasma membrane lipids in the maintenance and regulation of signaling pathways frequently disrupted in cancer. For example, modulations of growth factor signaling cascades are major hallmarks of cancer, and numerous studies have shown that the tyrosine kinase activity of these receptors is influenced by the local lipid environment. Both cholesterol and gangliosides are implicated in modulating the activity of receptors [128, 129], and have been shown to alter cellular responses to drugs that act to directly target receptor phosphorylation using kinase inhibitors [120]. Commonly mutated proteins in cancer downstream of growth factor receptors, such as Ras, are regulated in part through plasma membrane lipids [130]. In apoptosis, sphingomyelin lipids are converted to ceramides, and it is thought that this acts to cluster proteins involved in apoptosis pathways. Interestingly, cholesterol removal can initiate apoptotic signaling pathways even in the absence of ligands [131]. Alteration of plasma membrane lipids also recruits the tumor suppressor PTEN to the plasma membrane, which is vital for its roles in maintaining control of cell growth [132]. Thus, modified lipid compositions plays an important role in cellular proliferation beyond providing the additional biological material required for rapid cell growth.

Modulation of lipids and lipid metabolism provides a significant therapeutic effect in a variety of cancers, and is an emerging target of cancer therapies. For example, acute lowering of membrane cholesterol levels in cell culture can lead to apoptosis [133] or make cells more sensitive to cancer drugs [120]. Rates of cancer in the US population have been attributed in part to the wide use of cholesterol lowering drugs [134]. Exposure of cancer cells to lipid soluble small molecules can directly lead to apoptosis or can synergize with other drugs to promote cell death. Some examples include edelfosine [117] and 2-Hydroxyoleate [135] which are both in clinical trials, EGCG [136], and short chain ceramides [126].

Further, many drugs are thought to have a secondary mode of action linked to modulation of lipids. For instance, cisplatin which acts primarily by intercalating into and damaging DNA also induces apoptosis by clustering membrane receptors in a cholesterol dependent manner [137]. Also, the histone deacetylase inhibitor depsipeptide induces apoptosis in human prostate cancer cells by clustering death receptor ligands at the plasma membrane [138].

5.2 Lipid Criticality in Immunity

While the concept that lipids and lipid domains (sometimes referred to as "lipid rafts") contribute to the organization of immune receptors and downstream signaling partners has been around for decades [28, 139–142], we still lack a basic mechanistic understanding of how lipids influence the key signaling functions [143, 144]. In naive B cell lymphocytes, there are a large number of proteins known to modulate immune signaling cascades, most of which are anchored to the membrane through motifs that preferentially partition into either liquid-ordered (L_o) or liquid-disordered (L_d) phases at low temperatures (e.g., BCR, Lyn, and PAG1/CBP into L_o and CD45 and FcγRIIB into L_d) either in isolated plasma membranes or in membranes partially solubilized with detergent [145–149]. Acute changes in temperature or plasma membrane lipid composition, factors expected to impact the size and stability of membrane heterogeneities [52], also modulate signaling functions such as receptor phosphorylation, calcium mobilization, and antibody production [148, 150, 151]. It is likely that plasma membrane lipid heterogeneity modulates the detailed interactions between proteins involved in early steps of the B cell receptor (BCR) mediated signaling cascade. Confirming this would provide a novel and quantitative framework to understand how lipids influence immune signaling processes, enabling new strategies for the treatment of immune related disease through specific targeting of membrane physical state.

Many transmembrane and peripheral proteins are involved in initiating and modulating signaling responses that occur after the BCR is clustered through soluble or surface presented antigen [152–154]. The majority of these proteins interact with the BCR and other signaling partners primarily through direct binding, but it is also accepted that significant interactions likely arise from motifs that anchor these proteins or their adaptor proteins to the plasma membrane [149, 152, 155–157]. For example, Lyn kinase is thought to be primarily responsible for initial BCR phosphorylation after ligand binding. When activated, Lyn is known to bind the BCR weakly through direct interactions with the unique domain, or more strongly when at least one BCR ITAM tyrosine is phosphorylated [152]. Lyn is also anchored to the inner leaflet of the plasma membrane through palmitoyl and myristyl posttranslational modifications which favor more ordered lipid domains. This anchoring motif is shown to play important roles in localizing Lyn to sites of BCR clustering and for protecting Lyn from deactivation by phosphatases that prefer more disordered lipids [154, 158].

A large body of work exists supporting the concept that lipids play an important role in regulating signaling through the BCR [155, 157, 159]. Lowering cholesterol levels has been shown to lead to a reduction in the protein content found in detergent resistant membrane fractions, as well as decrease receptor and kinase phosphorylation. Other proteins involved in the BCR activation pathway are not affected or show increased activation in cells with reduced cholesterol content [148, 151]. This suggests that lipids also play important roles in down-regulating activated responses. However complications in singling out the role of sterols on specific processes, along with the lack of direct methods to probe the effects of membrane perturbations, have probably led the B cell signaling field to shift its focus from lipids to other key aspects of this signaling pathway, such as actin remodeling and the important roles of co-receptors [160, 161], perhaps missing an important element.

In innate immunity, there is clear evidence of large systematic changes in the "lipidomics" occurring together with "activation" (the pro-inflammatory set of changes in genetic regulation that neutrophil and macrophage cells make in response to sensing, for example, a bacterial infection) [162]. As part of this cell activation, the activity of various receptors is upregulated; one can imagine that changes in the lipid composition, such that the cell is moved close to the critical point, would automatically lead to a more heterogeneous membrane and hence directly to a higher fraction of dimerized receptors and signaling complexes [163], and hence a regulated higher activity (see schematic in Fig. 2a).

6 Progress Towards Direct Evidence of Lipid Criticality in Living Systems

Recent advances in single and super-resolution imaging are opening doors to a deeper understanding and characterization of heterogeneity in membranes [164]. But what do we expect to see using these tools if indeed the plasma membrane is a super-critical fluid? This in itself is a difficult question to answer. There are only subtle indications of criticality evident when monitoring single molecule mobility, especially when monitoring components like lipids or simple anchored peptides which only have direct interactions with a few lipid neighbors at any given instant. This is a well-known feature of critical systems, which can have slow dynamics of the average composition while maintaining fast dynamics of single molecules. As such, methods sensitive to single molecule motions such as FCS or single particle tracking are not expected to observe significant signatures of this type of membrane heterogeneity [165]. Super-resolution imaging methods may have a better chance of directly observing evidence of criticality in intact cells [166] (Fig. 4).

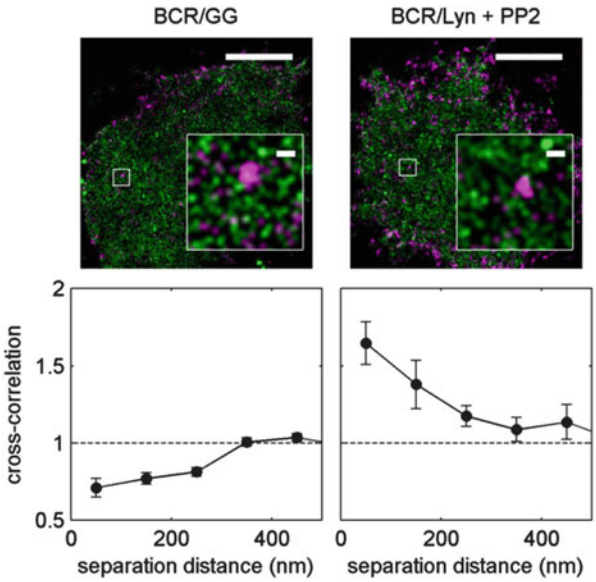

Fig. 4 Super-resolution fluorescence localization is beginning to be able to probe lipid-mediated sorting in live cells. A current challenge is to quantify the degree of lipid and protein co-localization, and then to determine the causal relation in specific situations. Imaging is likely to be an essential tool for further progress in this area. Reconstructed images show clustered B cell receptors (magenta) along with markers of disordered (GG) and ordered (Lyn) phases (green). The GG peptide is geranylgeranylated, prefers a disordered local lipid environment, and is excluded from BCR clusters. The full length Lyn protein is anchored to the plasma membrane with two saturated acyl modifications giving it a preference for more ordered lipids. This protein is recruited to BCR clusters even when cells are pre-treated with the SRC kinase inhibitor PP2 to block direct interactions between Lyn and the BCR. This is quantified using the steady state cross-correlation functions shown, where a value of 1 indicates a random distribution, less than 1 indicates exclusion, and greater than one indicates enrichment [166]

6.1 Super-Resolution Direct Imaging

One would expect various consequences in living cells based on the proximity of membrane compositions to phase separation, and specifically to critical points in the composition phase diagram:

(a) Subtle correlated densities of membrane components that partition into the same phases over short (<100 nm) length-scales .
(b) Correlated densities across-membrane leaflets, since the membrane acts as a single 2D fluid.
(c) Relatively long-lived structure in the average composition (the fundamental physics in model systems was characterized in [53]).
(d) It should be relatively easy to template changes in the average composition by coupling to structures or processes adjacent to the membrane (e.g., adhesion [167], cytoskeleton [168, 169], receptor clustering [44], etc.).

(e) Weak but long range potentials acting on components, through composition [170] or curvature effects [171], possibly coupled together (see Fig. 4).
(f) Only subtle effects on single molecule diffusion for most membrane components, as shown in [172].

Super-resolution imaging can provide direct evidence for these proposed regulatory mechanisms based on the lipid behavior. In principle, these methods have the lateral resolution and sensitivity to detect the small (<100 nm) and subtle heterogeneity expected from fluctuations. In practice, experimental details of probe over-counting, statistics, and subtle bleed-through make experiments challenging even in fixed cells. In live cells, fast single molecule mobility complicates things further [173]. Over-counting [174] and multiple observations of the same fluorophore (or antibody labeling the same protein) [175] lead to a signature in the auto-correlation of a membrane component being imaged. This is frequently much larger than the auto-correlation expected from the heterogeneity itself. Uncertainty in the magnitude of this contribution reduces the sensitivity of a single color measurement. Problems with over-counting can be addressed by co-localizing two distinct components, although problems can arise due to bleed-through between imaging channels, but can be mitigated with the use of the correct probes and imaging conditions. Even still, the predicted structures are on the edge of current resolution limits and statistics can be limiting. An easier measurement is one where one component is structured, e.g., through explicit clustering or through adhesion to a surface. In this case a second component can be probed to determine if its localization is affected by the structuring of the first component. In fixed cells there is always the concern that fixation leads to the observed heterogeneity. In live cells, single molecules diffuse orders of magnitude farther than the size of the structures being probed even when fast acquisition conditions are used.

6.2 Challenges, Controversy in Live Cells

Right now the research in this area is very active. Alongside various papers discussed and cited so far, which build on or are consistent with the idea of lipid criticality playing a significant role, it is fair to cite here a few very recent reports that challenge this view, claiming to see no evidence for "rafts" or "phase like segregation." The absence of discontinuous changes in diffusion coefficients on intact live cells, as a function of temperature, was taken as evidence that the membrane remains homogeneous [176]. We note however that crossing a second order phase transition one would not expect to observe discontinuities in the physical parameters. Also, we expect the presence of the critical point to induce effects (see section above for which effects) also in the one-fluid phase above the critical temperature. In another very recent report [177]), data is presented where live cells are grown on micropatterned substrates, whereby GPI proteins are anchored; no co-clustering of other proteins is seen in the cells, concluding against point (d) from

the list in the section above. It is very questionable however whether that experiment has the required precision to sustain this conclusion. A similar experiment, in model membranes, did observe a weak enrichment or depletion of a lipid probe at sites where a second membrane component was bound to a surface: a 20% effect [167], which is well within the error bars of [177].

7 Conclusion

Lipid composition is critical to many biochemical processes, and lipid homeostasis is important to enable cell functions in general. For example, liquid general anesthetics lower critical temperatures of the plasma membrane [77]. In particular, there is growing evidence that in living cells the lipid composition is regulated to maintain a certain distance to the critical point [65, 90], a fact that is being noted in the biological literature [178] in connection to the concept of lipid rafts. Lipid rafts represent the well-known fact that biological membranes present domains enriched with particular lipids, and that this heterogeneity couples to partitioning or adhesion of specific proteins to those regions. It is also clear that protein components of the cytoskeleton, in particular the cortical cytoskeleton, can couple to the lipid composition fluctuations [106]. The phase behavior of the lipid components seems to us a very important consideration in rationalizing complex lipidomics data, although connecting the lipidome to knowing lipid heterogeneity is itself a non-trivial task. Functional consequences upon changes in membrane organization are known for specific systems, and we have discussed various general ways in which protein function can couple to effects of lipid composition criticality (for example, some membrane receptors are known to cluster in lipid domains, affecting signaling [179]); experiments so far provide many intriguing instances of correlation, but this does not yet prove that lipidomics is acting as a regulatory mechanism: more direct evidence of causality is required. What is beyond doubt is that physiological proximity of the membrane composition and temperature to the critical point allows composition fluctuations to occur spontaneously or with very low energy cost; elucidating the biological consequences of this, and looking for general principles of membrane protein regulation by lipid composition, remain active areas of research. These questions are ripe for investigation with newly developed experimental methods capable of quantifying interactions between proteins in their native environment.

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