Descriptive title: The membrane phase transition gives rise to responsive plasma membrane structure and function

Short title: The plasma membrane as a susceptible fluid

Sarah A. Shelby[1] and Sarah L. Veatch[2]
[1]Biochemistry & Cellular and Molecular Biology, University of Tennessee Knoxville, Knoxville, TN, USA sshelby4@utk.edu

[2]Program in Biophysics, University of Michigan, Ann Arbor MI, USA sveatch@umich.edu

Abstract

Several groups have recently reported evidence for the emergence of domains in cell plasma membranes when membrane proteins are organized by ligand binding or assembly of membrane proximal scaffolds. These domains recruit and retain components that favor the liquid-ordered phase, adding to a decades-old literature interrogating the contribution of membrane phase separation in plasma membrane organization and function. Here we propose that both past and present observations are consistent with a model in which membranes have a high compositional susceptibility, arising from their thermodynamic state in a single phase that is close to a miscibility phase transition. This rigorous framework naturally allows for both transient structure in the form of composition fluctuations and long-lived structure in the form of induced domains. In this way, the biological tuning of plasma membrane composition enables a responsive compositional landscape that facilitates and augments cellular biochemistry vital to plasma membrane functions.

Introduction

For decades it has been argued that membrane phase separation plays a role in the organization and function of plasma membrane proteins, facilitating broad processes including signaling, trafficking, and polarization (Simons and Van Meer 1988; Simons and Ikonen 1997; Brown and London 1998; Anderson and Jacobson 2002; Parton and Hancock 2004; Sengupta et al. 2007a; Lingwood and Simons 2010; Kusumi et al. 2011, 2020; Sezgin et al. 2017). Analogous to the condensed liquid droplets formed by protein and nucleic acid biopolymers in three dimensions (3D) (Hyman et al. 2014; Banani et al. 2017; Shin and Brangwynne 2017), membranes can organize into coexisting liquid phases in two dimensions (2D) (Veatch and Keller 2005). The two phases that form, called liquid-disordered (Ld) and liquid-ordered (Lo), have different membrane compositions and other physical properties that have been characterized by a myriad of different biophysical methods (Chapman and Wallach 1968; Oldfield and Chapman 1972; Mouritsen and Zuckermann 2004; Silvius 2005; Chiantia et al. 2009; Marsh 2009; Giocondi et al. 2010; Heberle and Feigenson 2011; Shaw et al. 2021).

While phase separation is gaining broad acceptance as a mechanism to functionally organize certain proteins and nucleic acids within cells (Banani et al. 2017), the role of phase separation in biomembrane structure and function has remained controversial (Munro 2003; Kenworthy 2008). Membrane domains associated with Lo or Ld phases in cells are often described as small and dynamic (Hancock 2006; Pike 2006), challenging both their experimental observation and their mechanistic connection to phase separation, which applies in the thermodynamic limit of large and stable domains. We recently proposed a framework that reconciles this apparent contradiction, in which living cell membranes reside in a single phase biologically tuned close to phase separation (Shelby et al. 2023; Veatch et al. 2023). This tuning confers membranes with some remarkable material properties that impact plasma membrane biochemistry and signaling functions. Importantly, these phenomena can be rigorously modeled and interrogated experimentally, connecting plasma membrane thermodynamics, structure, and function.

In this review we will introduce liquid-liquid phase separation in membranes as well as several features that distinguish this transition from the demixing of protein and nucleic acid biopolymers in solution. We then describe characteristics of single-phase membranes with compositions tuned near phase separation, and present recent evidence supporting the view that cell plasma

membranes share these characteristics. We will then reflect on how this new framework aligns with the decades old “lipid raft” hypothesis, which proposed that lipids can drive functional heterogeneity of cell membranes.

The membrane phase transition and its relation to biomolecular condensates.

Purified model membranes and isolated plasma membranes can demix into coexisting Ld and Lo phases. Phase separation can be detected experimentally by imaging the organization of a membrane probe that preferentially partitions into one phase (Figure 1A). At temperatures and compositions outside of phase separation, probes are uniformly distributed across the membrane surface on the micron-scale. Lowering temperature or changing composition can drive membranes through the phase transition, leading to the formation of domains that sort membrane components (Veatch and Keller 2003). Phase separated membrane domains typically have circular boundaries, are mobile, and can coarsen via domain coalescence (Stanich et al. 2013). The Ld phase typically enriches lipids containing unsaturated acyl chains while the Lo phase typically enriches lipids with saturated chains (Veatch et al. 2004). The Lo phase also incorporates a sterol such as cholesterol, which interacts favorably with saturated lipids to promote lipid packing and chain ordering while maintaining fluidity (Hjort Ipsen et al. 1987; Vist and Davis 1990; Sodt et al. 2014). Proteins are also sorted between phases, with most single-pass transmembrane proteins favoring the Ld phase (Lorent et al. 2017). Peripheral and transmembrane proteins that are post translationally modified with saturated palmitoyl groups can gain access to the Lo phase (Shelby et al. 2023), as can glycosylphosphatidylinositol (GPI) linked proteins and toxins that bind to multiple ganglioside lipids on the extracellular leaflet (Sengupta et al. 2008).

Both simple polymers and more structurally complex proteins and nucleic acids can separate into dense and dilute liquid phases in 3D, both through *in vitro* reconstitution and within the complex environment of the cell cytoplasm or nucleus (Brangwynne et al. 2015; Srivastava and Tirrell 2016) (Figure 1B). Like membrane phases, biopolymer rich liquid phases have round boundaries, are mobile, can coarsen via domain coalescence, and enrich or deplete other system components. In both systems, domains form at equilibrium because demixing lowers the free energy. The free energy contains enthalpic contributions that reflect interactions between components and entropic contributions that reflect the number of configurations available to the system. Attractive interactions between molecules contribute negative values to the enthalpy, lowering the free energy. Clustering components into domains reduces the number of possible configurations of the system, reducing entropy and increasing the free energy. Phase separation occurs when the enthalpic benefit to clustering overcomes this entropic cost.

In order to maintain fluidity, the energetic barriers to rearranging bonds must be small enough to allow molecules to explore many configurations. Large biopolymers distribute favorable interactions across many sites (Li et al. 2012; Brangwynne et al. 2015), allowing individual bonds to break and rearrange on reasonable time-scales while providing enough enthalpy to overcome the entropic cost of demixing. Lipids, in contrast, are small molecules and interactions are distributed over fewer individual sites. As a consequence, strongly interacting lipids are prone to form solid phases with low mobility (Nagle 1980). Liquid-liquid coexistence in membranes is enabled by the presence of cholesterol or a related sterol (Beattie et al. 2005). These sterols have the remarkable ability to fluidize strongly interacting lipids while maintaining attractive interactions, and are a required component of the Lo phase (Mouritsen and Zuckermann 2004).

The molecular complexity of large biopolymers, including the ability to fold into secondary structures, allows for many different types of specific interactions (Peran and Mittag 2020). Lipids, while chemically diverse, have conserved structural elements (Harayama and Riezman 2018). Chemical differences across lipid species alter the magnitude of interactions but generally do not encode new interaction types, limiting their specificity. As a potential consequence of these specificity differences (Jacobs and Frenkel 2017; Graf and Machta 2022), only two liquid phases are detected in membranes while many different classes of liquid phases can be assembled from biopolymers (Mountain and Keating 2020; Lafontaine et al. 2021).

Membrane and biopolymer phase transitions in cells also differ in their biological tuning (Figure 1C). Many proteins and lipids are uniformly distributed on the micron-scale in unperturbed and intact cell membranes at growth temperatures, consistent with being in a single liquid phase (Singer and Nicolson 1972). Vesicles isolated from living plasma membranes are also in a single liquid phase at growth temperature but separate into Lo and Ld phases at low temperature (Baumgart et al. 2007). Moreover, plasma membrane vesicles pass near a critical point (Veatch et al. 2008), a special region of the phase boundary where tie-lines merge into a single point and large composition fluctuations are observed (Honerkamp-Smith et al. 2009). Additional measurements support the conclusion that this thermodynamic state is biologically tuned, as cells grown at different temperatures tune their membrane composition to maintain proximity to this phase transition (Burns et al. 2017). In contrast, protein and nucleic acid condensates typically form high contrast puncta within cells (Hyman et al. 2014; Banani et al. 2017; Shin and Brangwynne 2017), indicating that these transitions are biologically tuned to one edge of a long tie-line connecting phases with very different compositions. There are many examples of condensates being constitutively present in cells (e.g. (Feric et al. 2016)), and also examples where high contrast droplets form upon some stimulus, for example the introduction of an environmental stress (e.g. (Molliex et al. 2015)). In both cases, it appears that this 3D phase transition is biologically tuned to be at or near phase separation, but in a part of the phase diagram far from the miscibility critical point.

The differences between membrane and biopolymer phase transitions likely reflect their different functional roles. Condensates of proteins and nucleic acids sequester components in times of stress, organize specific classes of biomolecules into discrete organelles, or gather components of signaling cascades (Hyman et al. 2014; Banani et al. 2017; Shin and Brangwynne 2017). While it has been proposed that the membrane phase transition can serve a similar purpose (e.g. (Simons and Ikonen 1997)), the lack of specificity and near-critical biological tuning are less compatible with all-or-nothing functional mechanisms, especially those that require the recruitment of specific components. Instead, cells likely utilize the unique material properties of this biological tuning. This could take the form of, for example, modulating effective interactions between membrane proteins involved in biochemical networks (Reynwar and Deserno 2008; Machta et al. 2012), impacting the diffusion of membrane bound proteins (Machta et al. 2011), allosterically regulating internal states of proteins (Kimchi et al. 2018; Levental and Lyman 2023), or, as discussed below, adjusting the local membrane composition in response to membrane-proximal cues.

A susceptible membrane links nano- and macro- scale membrane heterogeneity.

One feature of single phase systems that can separate into coexisting liquid phases near a critical point is that they have a high compositional susceptibility (Goldenfeld 1992). A susceptibility is the thermodynamic quantity that describes how an extensive property of the system is dependent on changes in its intensive conjugate force. One familiar example is compressibility, which describes how the volume of a 3D system changes with an applied

pressure. Another example is the magnetic susceptibility, which describes how robustly dipoles in a material align when placed within a magnetic field. The compositional susceptibility describes how responsive the local composition is to forces that bias those compositions, and its magnitude depends strongly on proximity to the critical point.

One consequence of a large compositional susceptibility is that weak thermal forces can drive the spontaneous assembly and dissolution of composition fluctuations that are much larger than individual molecules (Figure 2A). Over time, these transient fluctuations uniformly sample the membrane area, producing a homogenous membrane on time average. In the same membrane, macroscopic domains can be induced when a subset of components are organized by an external force or when weak forces bias the localization of bulk components (Figure 2B). On the molecular scale, these weak forces act to gather and retain spontaneous fluctuations, producing a heterogeneous membrane on time-average. The contrast of induced domains depends on both the magnitude of the applied force and on the magnitude of the compositional susceptibility (Figure 2C), while the size and lifetime of induced domains is determined by the extent and longevity of the applied force.

Very close to the critical point, the compositional susceptibility is so large that spontaneous composition fluctuations can be imaged by conventional fluorescence microscopy (Veatch et al. 2006; Honerkamp-Smith et al. 2008). The size and lifetime of spontaneous fluctuations decrease along with the susceptibility as thermodynamic parameters are tuned away from the critical point (Honerkamp-Smith et al. 2012), but are still detected by methods sensitive to these time and distance scales. Spontaneous composition fluctuations with dimensions between 10 and 100nm are detected by Atomic Force Microscopy (AFM) and Nuclear Magnetic Resonance (NMR) near known critical points (Veatch et al. 2007; Connell et al. 2013; Khadka et al. 2015). Heterogeneities with dimensions closer to the molecular-scale (<5nm) have been reported on extensively using spectroscopic methods (reviewed in e.g. (Chapman and Wallach 1968; Oldfield and Chapman 1972; Mouritsen and Zuckermann 2004)). In many cases, nanoscale structure is detected in single phase membranes that can undergo the membrane phase transition with modest changes in temperature or composition (e.g. (de Almeida et al. 2003; Heberle et al. 2010; Pathak and London 2015)). These studies documenting nanoscale heterogeneity do not explore the role of susceptibility directly, but most theoretical mechanisms developed to describe this nanoscale structure have roots in the Lo-Ld phase transition (Schmid 2017).

Experiments have documented induced domains within single phase model membranes in vesicles stabilized through adhesion (Zhao et al. 2013), when protein or DNA meshworks are assembled on a subset of a vesicle surface (Liu and Fletcher 2006; Manley et al. 2008; Chung et al. 2021; Kanwa et al. 2023) or when a supported membrane is coupled to a minimal actin cytoskeleton (Honigmann et al. 2014). Lipid sorting is also observed when near-critical membranes are subjected to the mechanical perturbation of pulling a tether (Sorre et al. 2009), demonstrating that membrane domains can assemble in response to a broad array of applied forces beyond simply clustering components. In all of these cases, where investigated, the contrast of induced domains varies with proximity to the phase transition (e.g., by varying temperature) and the magnitude and type of the applied force (e.g., how clustered proteins are anchored or how taut a tether is pulled).

The plasma membrane as a susceptible fluid

An examination of past literature suggests that plasma membranes exhibit many hallmarks of a fluid with a high compositional susceptibility. Beyond appearing as a single phase on the

micrometer scale, there is evidence that these membranes are heterogeneous on the nanometer length-scale with structures that share properties with Lo and Ld phases (Varma and Mayor 1998; Sharma et al. 2004; Rao and Mayor 2005; Swamy et al. 2006; Sengupta et al. 2007b; Bagheri et al. 2022). Past work indicates that domains reminiscent of Lo and Ld phases are induced by coupling to cortical actin (Gowrishankar et al. 2012; Raghupathy et al. 2015), when cell signaling is activated (Gaus et al. 2005), or when membrane components are clustered (Harder et al. 1998). As mentioned above, isolated plasma membrane vesicles are in a single phase at growth temperature and phase separate at lower temperature, exhibiting large composition fluctuations near the phase transition temperature (Baumgart et al. 2007; Veatch et al. 2008). Analogous to reports in purified model membranes, several studies document nanoscale heterogeneity in isolated plasma membrane vesicles above their transition temperature (Ge et al. 2003; Heberle et al. 2020; Li et al. 2020) and extended domains are induced from single phase membranes when a subset of membrane components are organized through adhesion or protein clustering (Zhao et al. 2013; Urbančič et al. 2021; Wang et al. 2023).

We recently probed induced domains within intact, living cells by combining live cell super-resolution fluorescence localization imaging with B cell receptor (BCR) clustering (Shelby et al. 2023). We found that clustering BCR produced membrane domains capable of sorting and retaining membrane components based on their Lo phase preference (Figure 3A-C). Because our measurements were quantitative, we established that the magnitude of sorting at receptor clusters was reduced in cells compared to phase separated Lo domains in vesicles (Figure 3D), consistent with the behavior of induced domains in a highly susceptible membrane. We further demonstrated that domain contrast could be tuned (Figure 3E,F), either by changing the magnitude of the domain-organizing force by incorporating minimal co-receptors in BCR clusters or by altering the magnitude of susceptibility by incorporating components that shift the membrane phase transition temperature.

Several recent studies from other groups are also consistent with the plasma membrane being a highly susceptible fluid. For example, T cell receptor clustering leads to the stabilization of extended membrane domains that resemble the Lo phase in their chain ordering (Urbančič et al. 2021) and in their ability to sort membrane probes that label Lo and Ld phases (Wang et al. 2023). A separate study showed that clustering the ganglioside GM1 led to the formation of a membrane domain that recruited GPI-linked proteins, but only when GM1 contained fully saturated acyl chains (Arumugam et al. 2021). Protein assemblies that form at contacts between the membrane and the actin cytoskeleton provide another mechanism for proteins to induce structure at membranes. Recent experiments combining homo-FRET with dyes that sense lipid chain ordering showed that actin-membrane contacts cluster both PS lipids via PS-binding proteins and GPI-linked proteins in the outer leaflet, and that this clustering induced a local membrane environment that resembles the Lo phase (Saha et al. 2022).

There are also recent reports demonstrating that probe mobility is impacted by protein clustering in a manner dependent on probe phase partitioning. Single particle tracking experiments observed local dwelling of minimal membrane anchor probes upon protein clustering (Koyama-Honda et al. 2020). In this case, clustering Lo phase markers induced a local retention of Lo membrane probes but not Ld probes. This change in mobility was observed near clustered proteins and not monomeric proteins, indicating that clustering was required for formation of domains. Similarly, recent imaging fluorescence correlation spectroscopy (iFCS) detected Lo/Ld partitioning-dependent changes in diffusion of minimal membrane anchors upon clustering the IgE receptor FcεRI in mast cells, supporting the idea that receptor clustering can stabilize ordered domains that differentially affect the mobility of Lo vs. Ld probes (Bag et al. 2021).

The raft hypothesis and its connection to a susceptible membrane

Our proposal that the plasma membrane is a susceptible fluid shares many commonalities with the classic model of lipid raft structure and function (Simons and Ikonen 1997). Lipid rafts are described as microdomains formed through the favorable packing of sphingolipids, cholesterol, and saturated phospholipids, into which membrane proteins can be selectively incorporated or excluded. In both models, lipid interactions that give rise to Lo/Ld phase separation are a key physical driver for membrane organization, and the tendency of proteins and lipids to preferentially partition with Lo or Ld phases determines their association with or exclusion from domains. Formation of larger, stable domains in the membrane in response to some activation event is another feature shared by both models, and in both cases, these stabilized domains promote local signaling activity.

A primary distinction between the raft and susceptible fluid models concerns the physical nature of small domains in the absence of any stimulus or external force that would incorporate them into a larger platform. In the classic interpretation of the raft hypothesis, small and mobile domains that confine Lo components pre-exist in the plasma membrane. In contrast, a single-phase but highly susceptible membrane does not contain pre-existing Lo domains, but instead supports small and transient composition fluctuations that sample Lo and Ld compositions (Figure 2A). While fluctuations have compositions similar to those of Lo and Ld phases, individual components can diffuse across boundaries, and individual fluctuations have finite lifetimes.

A second distinction between the raft and susceptible fluid models involves the mechanisms for stabilizing larger domains. In early versions of the raft model, activation signals were transduced through translocation of receptors into raft domains upon ligand binding, or by clustering rafts into more robust signaling platforms. In a highly susceptible membrane, forces acting on the membrane plane need not alter the intrinsic phase partitioning of individual components, nor do they merge or coalesce pre-existing domains. Clustering components is expected to enhance partitioning by reducing the entropic cost to organization while maintaining interactions with neighboring lipids (Putzel and Schick 2009). On the molecular scale, scaffolds that organize membrane components locally bias the formation of composition fluctuations. The extent of this bias is dependent on the density of organized components, their Lo/Ld partitioning, and on the proximity of the membrane to the phase transition (Figure 2B). Biasing the probability that a fluctuation will occur at a specific location impacts the concentration of components when averaged over time. Therefore, a single membrane can support many different types of induced domains if different types of forces are used to organize membrane components (Figure 2C). For example, induced domains within this model can have ordered or disordered average character given by the propensity of the organizing force to bias ordered or disordered composition fluctuations. This contrasts with conventional lipid rafts, which are typically thought of only as ordered membrane domains.

Proteins within induced domains can be recruited based on their phase partitioning, or can be further retained through specific interactions with other proteins or ligands, giving them access to local environments that regulate biochemical activities (Stone et al. 2017). Moreover, effective interactions between membrane components mediated by a highly susceptible membrane can also contribute to their clustering even when specific interactions are not sufficient to drive oligomerization (Reynwar and Deserno 2008; Machta et al. 2012; Rouches et

al. 2021). Combining a susceptible membrane with specific protein interactions has the potential to produce a diverse and responsive compositional landscape.

Interpreting results of classical raft assays within the framework of a susceptible membrane.

The early framing of the raft hypothesis was largely informed by methods thought to isolate or perturb raft domains (Simons and Ikonen 1997) and there is an extensive literature correlating the results of these assays with readouts of cell function. Over time, several studies demonstrated pitfalls with many of these techniques (Heerklotz 2002; Kwik et al. 2003; Douglass and Vale 2005), and a healthy skepticism emerged regarding the interpretation of these experimental findings (Munro 2003; Kenworthy 2008). Our proposed framework of a highly susceptible membrane provides a new perspective to re-interpret the findings of this valuable literature.

Detergent resistance: Much of the older raft literature utilized the biochemical isolation of rafts using nonionic detergents at low temperature, followed by separation on a sucrose gradient to isolate soluble and membrane bound fractions (Brown and Rose 1992). These detergent-resistant membrane (DRM) isolations documented the enrichment of palmitoylated proteins and specific lipid types suggestive of the Lo phase (Ahmed et al. 1997; Foster et al. 2003), supporting the interpretation that this procedure purified existing membrane domains. Subsequent work found that the composition of DRMs was dependent on the type of detergents used (Magee and Parmryd 2003), and that detergents themselves could induce ordered domains in otherwise single-phase membranes (Heerklotz 2002).

The results of this assay can be revisited by considering that the membrane is in a single phase but close to a miscibility transition. In this context, addition of a detergent and lowering temperature likely drives the membrane through its phase transition (Figure 4A) prior to solubilizing proteins and lipids in the Ld phase (Figure 4B). The phases that emerge in response to these perturbations broadly resemble the spontaneous nano-scale fluctuations present prior to the perturbation, since these fluctuations sample phases of the near-by phase transition. Different detergents or protocols are expected to drive the membrane through phase separation along distinct trajectories, giving rise to DRM isolations with different compositions (Figure 4B). Induced ordered domains in cell membranes would also isolate in DRMs, although the addition of detergent at low temperature would enhance the compositional contrast of these domains, since they would be driven to phase separate (Figure 4C). For this reason, the composition of DRMs with induced domains may be indicative of the type of domain (ordered or disordered) without necessarily reflecting the composition of that domain. This may be why the distributions of some proteins shift to more exclusively favor DRMs after they are clustered.

Cholesterol depletion: Cholesterol or a similar sterol is a required component of the Lo phase (Hjort Ipsen et al. 1987), and is needed to support coexisting Lo and Ld domains in model membranes (Beattie et al. 2005). Removing cholesterol from model membranes results in a decreased fraction of Lo domains and eventual loss of liquid-liquid phase separation or emergence of a more rigid solid phase (Veatch and Keller 2003). In cells, observing a change in function upon cholesterol removal has long suggested a role for rafts in the cellular process in question (Simons and Ikonen 1997). Often, the reasoning is that removing cholesterol also removes pre-existing ordered domains because fewer proteins are found in DRMs under this condition (e.g. (Sheets et al. 1999a)) (Figure 4D). This simple interpretation has been

questioned in several cases. For example, some past work suggests that cholesterol depletion can alter membrane protein mobility, membrane tension, and/or cortical actin integrity (Kwik et al. 2003; Biswas et al. 2019), which may themselves be the drivers of functional modulation rather than a direct effect of membrane domains. Also, cholesterol modulation can have non-monotonic impacts on functional outputs (e.g. (Sooksawate and Simmonds 2001)), suggesting more complex phenomena are involved.

In the context of an adaptable membrane, cholesterol depletion would lower the fraction of the membrane occupied by ordered fluctuations and also the stability and life-time of those fluctuations (Levental et al. 2009; Stone et al. 2017). Membrane perturbations that shift conditions away from phase separation will tend to reduce the contrast and therefore functional impact of membrane domains. Membrane perturbations that reduce the fraction of the membrane occupied by ordered fluctuations can have less straight-forward effects. For example, a moderate reduction in the fraction of ordered membrane fluctuations might produce an enhancement of ordered domain-mediated interactions, as components that partition with ordered fluctuations find fewer ordered regions to occupy within the membrane. Under these conditions, induced domains might also have higher contrast, giving rise to enhanced function (Stone et al. 2017). Drastic reduction of ordered domains would be expected to diminish their effectiveness due to their small number, reducing their functional impact. Most likely, acute cholesterol removal from cell membranes results in some combination of these two effects. An alternative to cholesterol modulation could be acute treatment with compounds that shift phase boundaries without dramatically altering the fraction of phases, such as n-alcohols or bile acids (Zhou et al. 2013; Machta et al. 2016). Chronic cholesterol manipulation is expected to have even more complicated consequences, as cells can adapt the compositions of other components as well.

Solvatochromic dyes. LAURDAN and other solvatochromic dyes undergo a spectral shift that is dependent on the solvent properties of the probe microenvironment. This read-out is often a proxy for local lipid chain order, since tighter packing of lipid chains presents greater barriers to water access into the hydrophobic core of the bilayer (Bagatolli et al. 1999). Local polarity is also often connected to “fluidity,” which has implications for single molecule mobility, since these physical properties are often correlated with each other. In many cases, a quantity called the generalized polarization (GP) is extracted, which is a ratio of the fluorescence intensity between two regions of the emission spectrum. This GP value exhibits large differences between Lo and Ld phases in purified vesicles and smaller differences between phases in isolated plasma membrane vesicles (Kaiser et al. 2009; Owen et al. 2012).

In adaptable membranes, a broadened emission spectrum is expected, reflecting contributions from dyes found within ordered and disordered domains as well as at the boundary between domains. Induced ordered domains would give rise to emission spectra and GP that is more biased towards the one phase, where the magnitude and sign of the shift reflects the contrast of the domain. Interpreting GP changes upon membrane perturbations is more complicated, since a shift in average GP could mean an increase in the fraction of one type of domain, a change that impacts both ordered and disordered domains, or some combination of the two. More sophisticated detection and analysis of solvatochromic dye spectra could in principle distinguish these alternatives (Golfetto et al. 2013).

Spectroscopic methods: Spectroscopic methods such as Förster resonance energy transfer (FRET) and Electron spin resonance (ESR) are sensitive to very small length-scales and very short time-scales. These techniques have been used to detect nanoscale heterogeneity in model and cell membranes both in the presence and absence of stimulating factors that induce

larger raft platforms (Swamy et al. 2006; Sengupta et al. 2007b). ESR, similar to solvatochromic dyes, is sensitive to the molecular motions in the direct vicinity of probes (~1nm), reporting the local environment on the time-scale of the spin probe relaxation (0.1-100ns) (Chiang et al. 2005). Similarly, FRET reports on the local density of acceptor probes within a Förster radius of a donor probe (≤ ~5nm), on a timescale given by the fluorescent decay, which is typically several ns (Loura and Prieto 2011). These timescales are fast compared to lipid translational motion, therefore these techniques report on particular configurations of probe local environments and not environments averaged over time. Results from these spectroscopic methods reflect a compositional landscape decorated by spontaneous composition fluctuations, rather than the more uniform picture that appears when these configurations are averaged over time.

Revisiting a membrane mediated model of immune receptor activation.

Studies using techniques like DRMs and cholesterol depletion have proposed that rafts modulate protein-protein interactions between immune receptors and their signaling partners. Particularly well studied is the Multichain Immune Recognition Receptor (MIRR) family, which includes T cell receptor, B cell receptor, and the high-affinity IgE receptor FcεRI (Langlet et al. 2000). The translocation of receptors into rafts upon receptor ligation was proposed as a general mechanism for initiation of receptor phosphorylation by promoting receptor interactions with raft-resident positive regulators while segregating receptors from raft-excluded negative regulators. The coalescence of rafts after receptor translocation establishes a larger raft platform, promoting formation of stable signaling complexes (Sheets et al. 1999b; Viola 2001; Dykstra et al. 2003). For the specific case of the B cell receptor, DRMs were shown to concentrate Lyn kinase while excluding the phosphatase CD45 as well as the majority of BCR itself in resting cells (Rodgers and Rose 1996; Cheng et al. 1999, 2001; Aman and Ravichandran 2000). Upon receptor crosslinking with antigen, phosphorylated BCR is associated with DRMs. BCR crosslinking also reduced the detergent solubility of some downstream signaling proteins, including additional kinases and key adaptor proteins (Guo et al. 2000). Depletion of membrane cholesterol resulted in the redistribution of BCR and signaling partners between detergent resistant and detergent soluble fractions, which was interpreted to be a result of the dispersal of raft domains in cells. Cholesterol depletion attenuated enrichment of Lyn in DRMs and blocked translocation of BCR to DRMs (Petrie et al. 2000; Awasthi-Kalia et al. 2001; Cheng et al. 2001).

Our recent work in B cells is fully consistent with these past experimental results, with some differences in interpretation. Instead of isolating pre-existing ordered raft domains from the membrane, the DRM protocol itself induced domains in the membrane, preserving the membrane association of proteins such as Lyn, which strongly partitions with the Lo phase (as in Figure 4B). Cholesterol depletion reduces the quantity of Lo phase membrane assembled during the DRM preparation, resulting in fewer isolated Lyn proteins (as in Figure 4D).

BCR clustering induces a membrane domain that sorts protein regulators based on their partitioning into the Lo phase, resulting in a local change in the concentration of kinases and phosphatases (Stone et al. 2017). Even subtle local changes can be sufficient to support sustained receptor phosphorylation where kinase/phosphatase activity is balanced to be near a tipping point in resting cells, as has been suggested for the T cell receptor (Hui and Vale 2014). After receptor phosphorylation, downstream adaptor and signaling proteins are recruited to BCR via protein-protein interactions (Dal Porto et al. 2004). Some of these proteins are themselves palmitoylated, and these additional proteins likely contribute to the induction of a larger and higher-contrast ordered domain. Upon DRM preparation, these induced domains would

transition to Lo phase separated domains that incorporate signalosome components (as in Figure 4C).

Raft assays have also been used to interrogate signal modulation by co-receptors and in B cells in different developmental stages or disease states, and similar re-interpretations can be applied to these past results. For example, when BCR is co-ligated with the CD21/CD19/CD81 C3d complement co-receptor complex, BCR becomes more enriched in DRMs and this enrichment persists to longer times after BCR clustering (Cherukuri et al. 2001). Engagement of this co-receptor complex enhances the immune response, and both this signaling and the DRM effect on BCR is dependent on palmitoylation of CD81 (Cherukuri et al. 2004b, 2004a). Within a susceptible membrane, co-ligation of BCR with a Lo partitioning protein would enhance the compositional contrast of the induced ordered domain favoring both receptor phosphorylation and possibly recruitment of downstream components needed to sustain the domain. In another example, co-ligation of BCR with the down-regulatory receptor FcγRIIB results in suppressed BCR clustering, signaling, and disruption of BCR association with DRMs (Aman et al. 2001; Liu et al. 2010). Most transmembrane proteins lacking acylations tend to partition with Ld domains. In the case that this applies to FcγRIIB, co-ligation with BCR would give the BCR cluster a more Ld character, suppressing receptor phosphorylation and the recruitment of downstream adapters.

Concluding remarks

In this review we have put forth an updated model for functional heterogeneity in the plasma membranes of living cells. We propose that the mammalian plasma membrane can exist in a single phase, but one with high compositional susceptibility. This view allows us to rationalize a broad array of past results and reconcile them with an evolving picture of membrane organization with increasing spatiotemporal resolution. Having a high compositional susceptibility means that small and dynamic domains are spontaneously present in the plasma membrane. This unique physical state also means that the membrane can locally adapt to forces acting in and on membranes by remodeling to form long-lived domains of variable composition. In many cases, these applied forces take the form of protein scaffolds whose stability is dominated by relatively strong and specific protein-protein interactions, potentially in synergy with lipid-driven forces that sort membrane components based on Lo/Ld phase preference. In either case, induced domains can provide local hot spots of functional activity, by altering the local concentration and therefore effective interactions between proteins. In this way, collective properties of membrane lipids work together with membrane proteins to regulate and perform a broad range of functions at the plasma membranes of mammalian cells.

While most recent work focuses on membrane domains induced by protein cluster scaffolds, any structure that contacts the membrane can in principle couple to a specific membrane compartment, for example, at ER-plasma membrane contact sites, within clathrin- or caveolin-mediated invaginations, at neuronal synapses, during viral assembly, or within microvilli that enrich curvature sensitive proteins. At the same time, regulatory mechanisms that impact the phase partitioning of membrane proteins, such as palmitoylation, oligomerization, or conformational changes could impact the composition of domains induced by these proteins, or the ability of proteins to access certain domains. Extended structures, such as the cortical cytoskeleton, could in principle inhibit the induction of large domains by spreading interactions over a large area, and this could have a negative regulatory effect on domain mediated functions.

Another implication of our updated model is that the membrane itself, through its compositional susceptibility, can act as a master regulator of diverse plasma membrane functions. This framework predicts that cellular changes that tune the susceptibility will lead to global changes in membrane domains, altering the interactions and functions of proteins and protein networks. The susceptibility could be acutely modified to augment signaling responses, for example through the enzymatic conversion between lipid species, the fusion of secretory vesicles, or the rapid activity of scramblases to disrupt transbilayer asymmetry. Longer-term changes in lipid metabolism or cholesterol homeostasis could also adjust the compositional susceptibility, impacting the sensitivity of cells to stimuli, or shifting biochemical networks to favor different types of signaling outcomes. These concepts invite new questions – how is compositional susceptibility biologically tuned? Does feedback between signaling cascades and lipid metabolism play roles in shaping cell fate and development landscapes? Does dysfunction in these feedback loops lead to disease? In general, we expect this new framework will help to draw connections between plasma membrane composition, structure, and function and will initiate new and exciting areas for future study.

Acknowledgments

We thank Ben Machta, Thomas Shaw, and Ilya Levental for helpful conversations related to the topics covered in this review, and Yousef Bagheri for sharing his micrographs of supported membranes and polymer droplets for Figure 1. Work was supported by research grants from the NIH (GM129347 and GM110052) and the NSF (1552439 and 1905600).

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Figures and captions

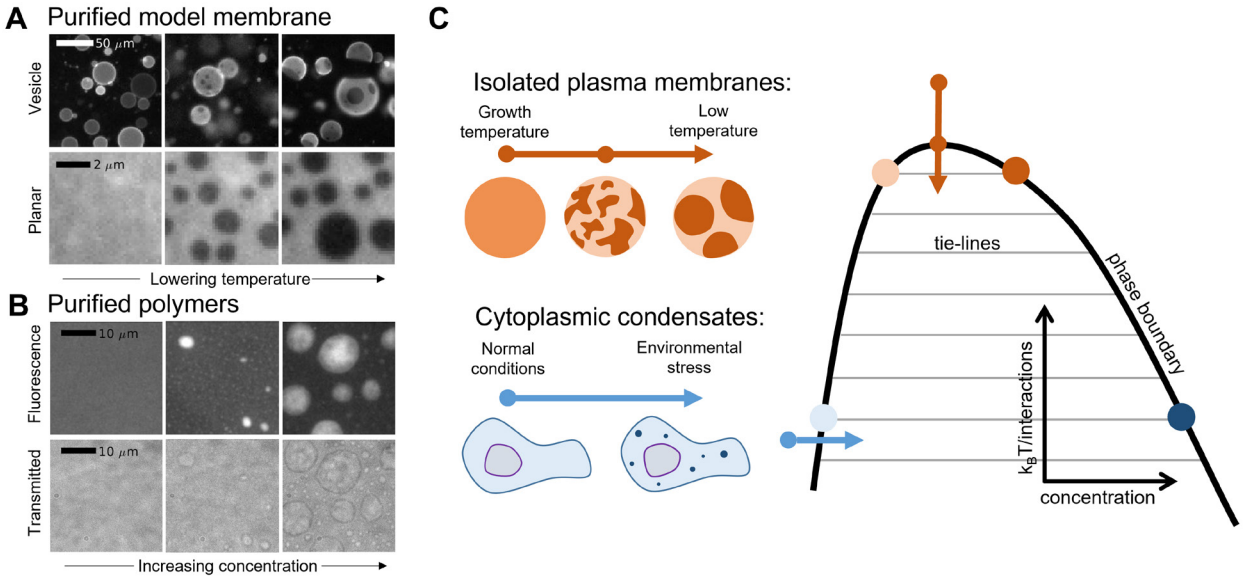

Figure 1: Phase separation in membranes and biopolymers. (A) Micrographs of giant unilamellar vesicles (top) and a planar supported multilayer (bottom) showing purified model membranes in a single liquid phase (left) and coexisting Lo and Ld liquid phases (middle and right). In these examples, phase separation is initiated by lowering temperature. (B) Micrographs of solutions containing poly lysine and poly glutamic acid imaged using a fluorescently tagged poly lysine (top) or using transmitted white light microscopy (bottom) showing solutions in a single dilute phase (left) and with coexisting dilute and condensed phases (middle and right). In these examples, phase separation is initiated by increasing polymer concentration. (C) Biological tuning of membrane and biopolymer phase transitions in the context of a schematic phase diagram. Isolated plasma membranes (schematically presented in orange) are in a single phase at growth temperature, but phase separate at lower temperatures and pass close to a critical point at the phase transition (orange arrow on diagram and schematic). Cytoplasmic condensates (schematically presented in blue) are typically sparse, high contrast droplets, and sometimes assemble in response to stimuli such as environmental stresses. This suggests that they cross the phase boundary far from a critical point (blue arrow drawn on diagram and schematic). Blue and orange dots on the phase boundary represent compositions of resulting coexisting phases, with light and dark shades corresponding to light and dark regions in the vesicle and cell schematics.

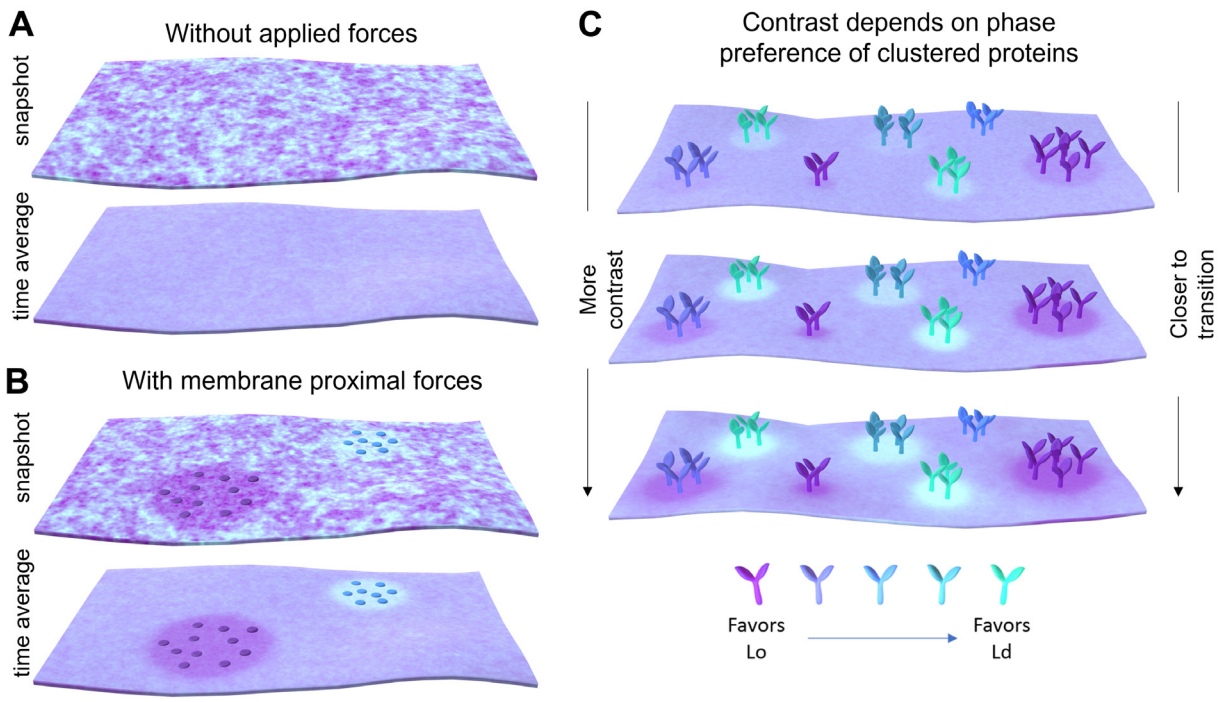

Figure 2: Spontaneous composition fluctuations and induced domains in single phase membranes. (A) (Top) Schematic showing an instantaneous snapshot of spontaneous fluctuations within a single-phase membrane in the absence of applied forces that bias local composition. (Bottom) This membrane appears uniform when many snapshots are averaged together, since spontaneous fluctuations are transient. (B) (Top) Schematic showing an instantaneous snapshot of spontaneous fluctuations within in a single-phase membrane in the presence of a spatially heterogeneous applied force. Here, the applied force is represented schematically as a subset of components organized into clusters. (Bottom) This membrane remains heterogeneous when many snapshots are averaged together, because the applied forces impact the likelihood that spontaneous fluctuations will occur at a given location. We refer to this as an induced domain. (C) The contrast of induced domains depends on the phase preference of the clustered proteins and the magnitude of the compositional susceptibility, which increases when the phase transition is approached. Schematics are prepared from simulations as described in (Shelby et al. 2023).

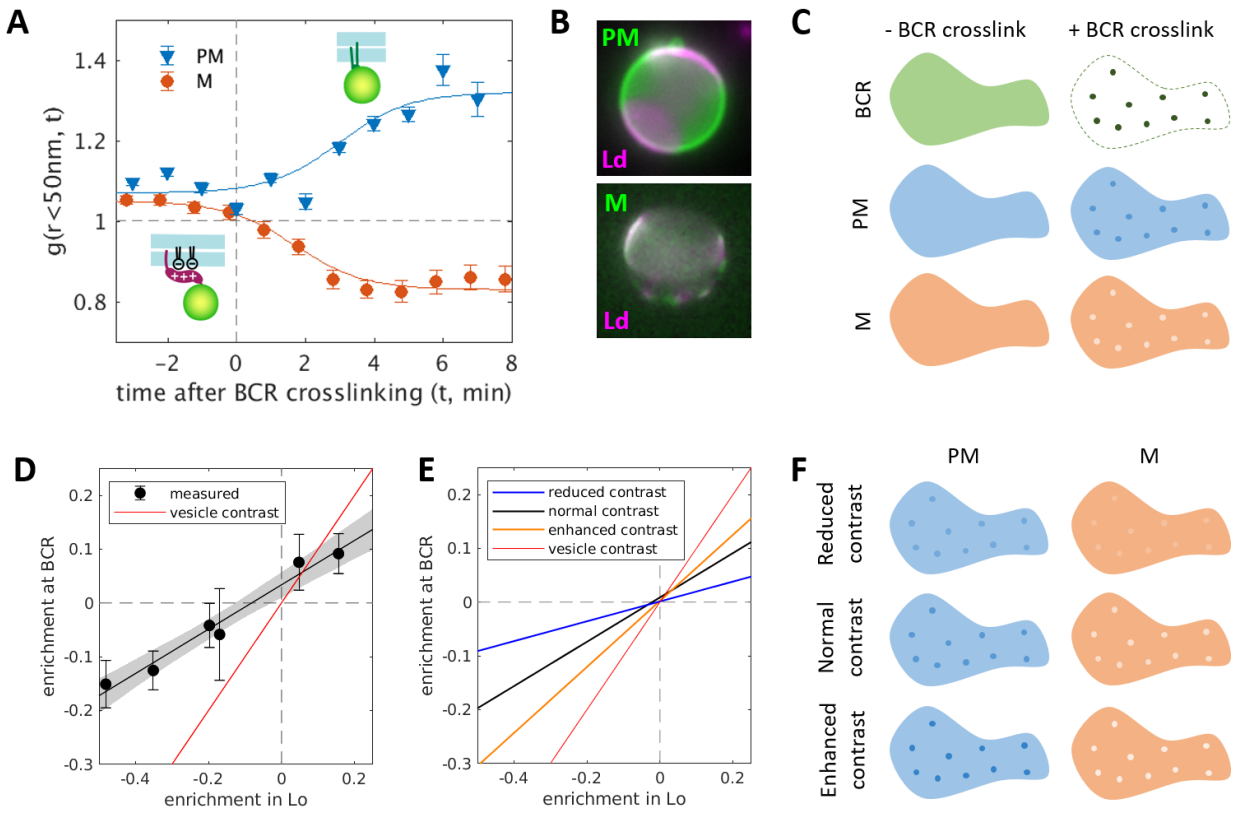

Figure 3: Sorting of membrane-anchored probes at BCR clusters. (A) Upon BCR clustering, a membrane domain emerges that sorts two probes anchored to the inner leaflet of the plasma membrane. One probe, (PM) contains palmitoyl and myristoyl post-translational modifications while the second (M) contains a myristoyl modification and a stretch of basic amino acids. Both are truncations of Src family kinases (Lyn and c-Src respectively). (B) PM and M probes partition with Lo and Ld phases, respectively, in isolated plasma membrane vesicles imaged at low temperature. (C) Schematic representation membrane organization of BCR, PM, and M both before and after BCR crosslinking. (D) The partitioning of probes with respect to BCR clusters is quantitatively predicted by their enrichment in the Lo phase in vesicles for a series of probes that anchor to the inner leaflet (black points). The black line is a linear fit to probe partitioning values with the 95% confidence interval of the fit shown in gray. The slope of this line indicates the relative contrast of domains in cells vs. vesicles. For reference, the red line has a slope of 1 and passes through the point (0,0), indicating the contrast of vesicles in these units. (E) Schematic representation of the effect on probe partitioning when domain contrast is modulated. Contrast is enhanced when domains more effectively sort probes, while contrast is reduced when domains are less effective at sorting probes. (F) Schematic representation of membrane organization of PM and M in cells after BCR crosslinking showing domains with variable contrast. Parts A,B,D, and E are adapted from (Shelby et al. 2023).

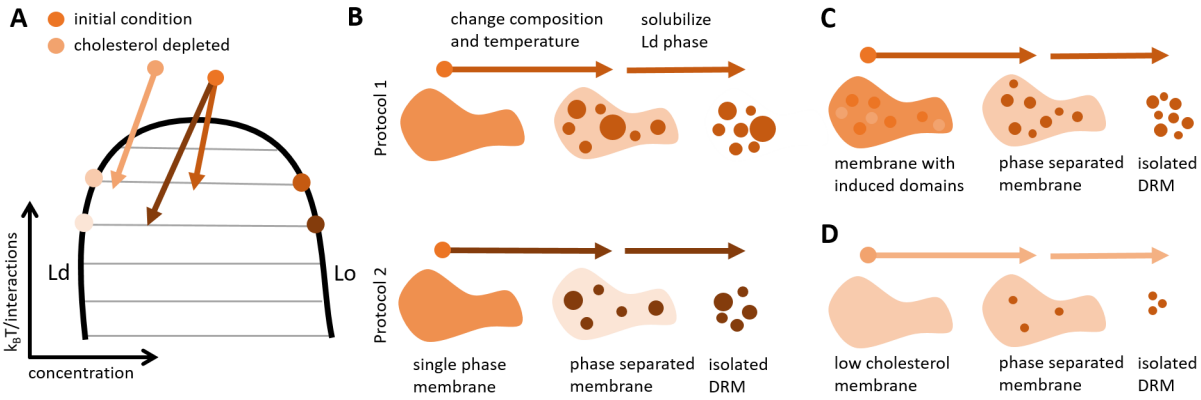

Figure 4: Interpretation of DRMs isolated from membranes with a high compositional susceptibility. (A) Schematic phase diagram with arrows representing possible simplified trajectories followed when membranes are exposed to detergents at low temperature. (B) (Top) Over-simplified schematic of the actions of a DRM protocol on membrane organization and isolation. First, detergent entering the membrane and low temperature induces Lo-Ld phase separation of plasma membrane lipids. Then, more disordered regions are solubilized, producing isolated DRMs. (Bottom) Different detergents or isolation protocols could alter the trajectory of the membrane through the phase diagram, producing Lo and Ld domains with different compositions and with different relative abundance. (C) Induced domains with Lo phase properties would be expected to partition with Lo phase domains within the DRM protocol, while induced domains with Ld properties would partition to Ld and become solubilized. (D) Removing cholesterol is expected to reduce the surface fraction of Lo phase in membranes, therefore a smaller quantity of DRMs would be isolated.