doi:10.1093/jmcb/mjab010
Published online February 3, 2021

Perspective

Membrane-associated phase separation: organization and function emerge from a two-dimensional milieu

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Liquid–liquid phase separation (LLPS) of biomolecules has emerged as an important mechanism that contributes to cellular organization. Phase-separated biomolecular condensates, or membraneless organelles, are compartments composed of specific biomolecules without a surrounding membrane in the nucleus and cytoplasm. LLPS also occurs at membranes, where both lipids and membrane-associated proteins can de-mix to form phase-separated compartments. Investigation of these membrane-associated condensates using *in vitro* biochemical reconstitution and cell biology has provided key insights into the role of phase separation in membrane domain formation and function. However, these studies have generally been limited by available technology to study LLPS on model membranes and the complex cellular environment that regulates condensate formation, composition, and function. Here, I briefly review our current understanding of membrane-associated condensates, establish why LLPS can be advantageous for certain membrane-associated condensates, and offer a perspective for how these condensates may be studied in the future.

LLPS underlies the formation of cellular compartments that are enriched in specific proteins, nucleic acids, and small molecules without a surrounding membrane. LLPS of biomolecules into mesoscale structures readily occurs in three-dimensional space in the nucleus and cytoplasm and can be constrained to two-dimensional membranes (Banani et al., 2017). Transient multivalent interactions can drive LLPS when the concentration of components is above the critical concentration for phase separation (Li et al., 2012; Sanders et al., 2020). Numerous *in vitro* and live-cell investigations of three-dimensional condensates have sought to understand their formation and function (Lyon et al., 2021). Several tools, such as optodroplets where blue light is used to induce the formation of condensates, have also been developed to study cellular condensates in a controlled manner (Shin et al., 2017). Many *in vitro* studies focus their attention on the minimal components necessary for LLPS. In such simplified *in vitro* experimental assays, specific biophysical characteristics, such as the critical concentration for phase separation, is fixed. However, in cells, the thermodynamics governing LLPS of complex condensates containing dozens or hundreds of components, such as stress granules, is not singular values, but rather changes depending on the concentration of phase-separating components (Riback et al., 2020). These results demonstrate the need for carefully designed experiments to understand the role of biological phase separation in cells (McSwiggen et al., 2019). Recently, protein–protein phase separation that is constrained to a two-dimensional membrane surface has gained traction as an important mechanism by which cell membranes can be organized.

Two-dimensional, membrane-associated LLPS is governed by the same thermodynamics as three-dimensional phase separation; above the critical concentration, components will de-mix into two phases to organize cellular compartments, concentrate specific components, and spatially regulate biochemical reactions. One major difference is that the critical concentration at which LLPS occurs when constrained to a membrane is an order of magnitude lower than in solution. For instance, using components of T cell signaling clusters phospho-LAT (pLAT), Grb2, and Sos1, where pLAT is attached to the membrane and the other components are in solution, phase separation is readily observable in the nM concentration range of Grb2 and Sos1. When all three components are in solution, phase separation occurs in the μM concentration range of Grb2 and Sos1 (Figure 1). One caveat of this comparison is that pLAT is not in comparable units; in two-dimensions, the density of pLAT is ∼300 molecules/μm^2, while in three-dimensions, the concentration of pLAT is 1 μM or ∼600 molecules/μm^3. However, the data suggest that by localizing one

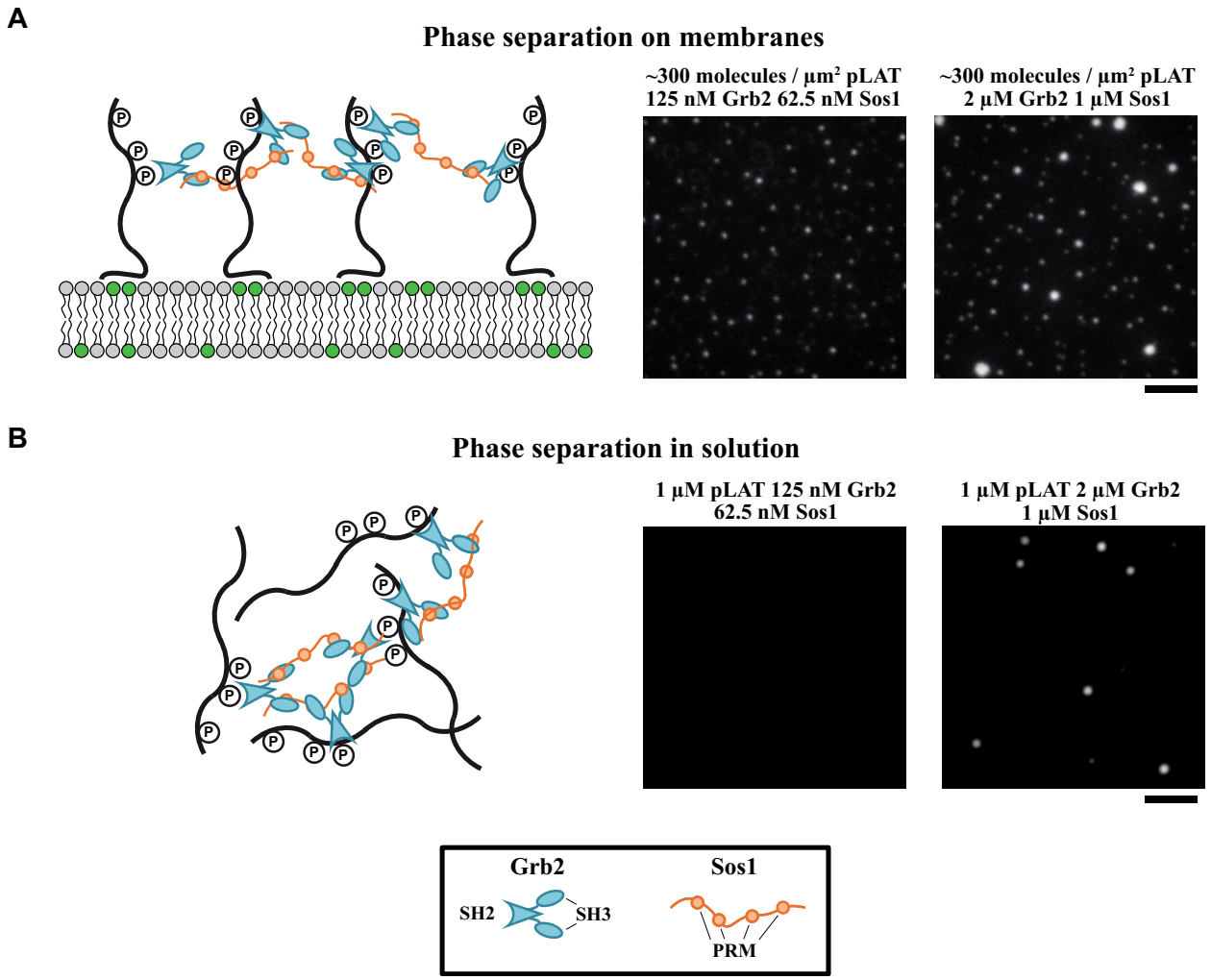

Figure 1 Phase separation of pLAT, Grb2, and Sos1 on membranes and in solution. (**A**) Phase separation of pLAT on SLBs. AlexaFluor 488-labelled pLAT attached to SLBs at ~300 molecules/μm^2 phase-separates with 125 nM Grb2 and 62.5 nM Sos1. Phase separation increases as Grb2 and Sos1 concentrations are increased to 2 μM and 1 μM, respectively. Images were captured using TIRFM. Brightness and contrast are consistent between images in **A**. (**B**) AlexaFluor 488-labelled pLAT in solution at 1 μM does not detectably phase-separate with 125 nM Grb2 and 62.5 nM Sos1. Phase separation is observed at 1 μM pLAT, 2 μM Grb2, and 1 μM Sos1. Images were captured using spinning disk confocal microscopy. Brightness and contrast are consistent between images in **B**. Grb2 consists of a single Src homology 2 (SH2) domain that binds to phospho-tyrosine residues on pLAT as well as N- and C-terminal SH3 domains. Sos1 consists of four specific proline-rich motifs (PRM) that bind to Grb2 SH3 domains. Scale bar, 5 μm.

component of phase-separating systems to membranes, cells can take advantage of lower critical concentrations to drive condensate formation and function in specific locations at membranes. Given the difference in both critical concentration of molecules required for phase separation and geometric constraints (being limited to a two-dimensional surface) of membrane-associated condensates, *in vitro* experiments should be designed to enable membrane association of components that naturally exist in cellular membranes.

Much of the work to understand protein–protein phase separation at membranes initially focused on clusters that form on the plasma membrane (Banjade and Rosen, 2014; Su et al., 2016). However, condensates likely form at membranes throughout cells including at the Golgi (Rebane et al., 2020), endoplasmic reticulum (Ma and Mayr, 2018; King et al., 2020), lysosomes (King et al., 2020), peroxisomes (King et al., 2020), and membrane–membrane or membrane–liposome junctions (Hariri et al., 2018). Many of these membrane-associated condensates are smaller than the resolution limit of light microscopy, making experiments in cells challenging. Recently, King et al. (2020) used osmotic gradients to induce the formation of large intracellular vesicles (LICVs) to study organelles in their native environments. The development of new technologies to study intracellular condensates provides an opportunity to understand whether two-dimensionally constrained phase separation regulates membranous organelle function and localization.

To date, several membrane-associated condensates have been experimentally shown to phase-separate *in vitro* (Case et al., 2019a). T cell condensates composed of LAT and its binding partners (Su et al., 2016; Ditlev et al., 2019; Huang et al., 2019), the podocyte filtration barrier protein nephrin (Banjade and Rosen, 2014; Case et al., 2019b), postsynaptic density proteins PSD-95, Homer3, Shank3, and DLGAP (Zeng et al., 2018), tight junction protein ZO-1 (Beutel et al., 2019), and cell polarity proteins Par3/Par6/aPKC and Numb/Pon (Shan et al., 2018; Liu et al., 2020) all undergo LLPS on membranes above their critical concentrations. Several other membrane-associated clusters of proteins also likely phase-separate but require additional experimental evidence to support this prediction. Focal adhesions, growth factor receptor clusters, chimeric antigen receptor clusters in engineered T cells, organelle–organelle tethers or contacts, and Golgi contacts are composed of multivalent proteins that likely interact with each other in an analogous manner to membrane-associated proteins shown to phase-separate.

Classically, membrane clusters have been considered to be oligomers or networks of proteins (Kortum et al., 2013). Therefore, an important question needs to be asked: are there advantages for membrane-associated condensates being phase-separated entities rather than oligomers or networks? The phase separation of proteins results in the formation of condensates with the following features: (i) consisting of unique biochemical environments that promote or inhibit specific biochemical reactions (Rauscher and Pomès, 2017; Kim et al., 2019), (ii) having electrostatic properties that modulate their composition (Su et al., 2016), (iii) capable of interacting with structural elements of cells to regulate their location in the nucleoplasm, cytoplasm, or on membranes (Ditlev et al., 2019), (iv) having surface tension that enables them to exert forces on membranes, cytoplasm, and nucleoplasm (Woodruff et al., 2018), and (v) having material properties that can be altered over time or by phosphorylation (Patel et al., 2015; Kim et al., 2019). These features enable phase-separated condensates to perform functions that are not normally conferred on oligomers or networks of proteins. In addition to unique functional consequences of phase separation, the formation of phase-separated condensates can be influenced by factors that do not contribute to oligomer or network formation. For instance, the properties of intrinsically disordered regions (IDRs) that connect structured domains can alter the concentration at which condensates form without altering the affinity of binding interactions. In systems that undergo LLPS, extension or compaction of IDRs, compared to IDRs in an intermediate state, will raise the critical concentration at which condensates form (Harmon et al., 2017), thus altering the ability of proteins to undergo LLPS. Phosphorylation of IDRs can regulate the physical state of the IDR (whether it is compact, intermediate, or extended) to alter condensate formation without changing binding affinities between interacting partners. Indeed, experimental evidence suggests that the phase separation of ZO-1 is regulated in this manner (Beutel et al., 2019). This is not the case for protein oligomers or networks that depend solely on binding affinities to drive their formation. In summary, there are distinct functional and formational advantages for membrane clusters to be phase-separated condensates rather than oligomers or networks of proteins. However, at this time, it is unclear which membrane-associated proteins undergo LLPS to form clusters. Therefore, developing and using appropriate technology and assays to evaluate whether membrane clusters are indeed phase-separated condensates and the functional consequences of membrane protein LLPS is essential for determining the role of LLPS in membrane signaling and cellular function.

Reconstitution of condensates that are constrained to a two-dimensional membrane requires experimental techniques that enable the evaluation of LLPS on membranes. A popular technique used to study membrane-associated condensation is the supported lipid bilayer (SLB) (Figure 2A; Su et al., 2017). SLBs are model membranes that usually contain phosphatidyl choline, either 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) or 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC), and a low percentage of a modified lipid, such as 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] nickel salt (DGS-NTA(Ni)), that enables specific His-tagged protein association with the SLB. A benefit of using SLBs to study membrane-associated condensation is that total internal reflection fluorescence microscopy (TIRFM) can be used to observe condensation and its functional consequences on a two-dimensional surface in real time (Su et al., 2016; Case et al., 2019b; Ditlev et al., 2019; Huang et al., 2019). However, SLBs are also limited in that they likely retain a strict two-dimensional geometry due to their interactions with the glass surface of the microscope slide. Thus, any potential geometric changes to the membrane structure caused by phase separation will likely not occur using SLBs as model membranes. The glass–lipid interaction also limits the complexity of model membranes that can be studied using SLBs; when additional, often charged, lipids are added to the SLB, the fluidity of the SLB decreases, preventing diffusion of lipids and attached proteins. Finally, because SLBs interact directly with the glass of the microscope slide, studying the contribution of transmembrane domains, which would protrude from the glass-facing side of the SLB and interact with the glass, to phase separation on membranes is not possible.

Giant vesicles are another experimental platform that can be used to study two-dimensional phase separation (Figure 2B). Two types of commonly used giant vesicles are giant unilamellar vesicles (GUVs) and giant plasma membrane vesicles (GPMVs) (Baumgart et al., 2007). GUVs are often formed using electroformation or microfluidic jetting and are composed of a research-controlled lipid mixture (Stachowiak et al., 2008). GPMVs are formed by

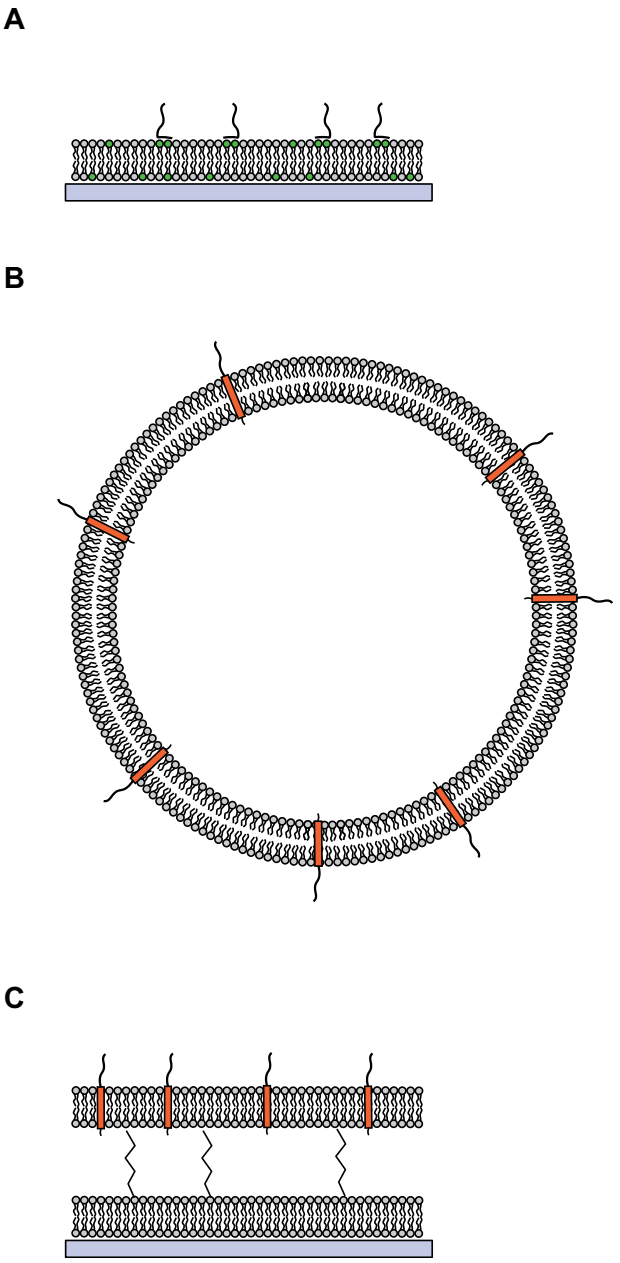

Figure 2 Experimental platforms used to study membrane-associated phase separation. (**A**) SLBs are placed directly on the glass surface used for TIRFM. SLBs are composed of a low percentage of modified lipids (green) that can be used to attach membrane protein tails to study phase separation. (**B**) GUVs can be composed of complex lipid mixtures and transmembrane domains (orange). Phase separation of lipids and transmembrane proteins on GUVs can be observed using fluorescence confocal microscopy. (**C**) DSLBs are a system of two lipid bilayers, an experimental bilayer (top) and a support bilayer (bottom). In the illustrated DSLB system, the support bilayer contacts the glass and is doped with a PEG-modified lipid that spaces both bilayers on the glass surface. Like GUVs, the experimental bilayer can be composed of a complex lipid mixture and transmembrane proteins. Phase separation can be observed using TIRFM.

blebbing membrane from live cells and are composed of a cell-determined mixture of lipids (Baumgart et al., 2007). A benefit of using giant vesicles to study protein phase separation is that the membrane environment is not limited by being attached directly to a substrate, as with SLBs. For instance, GUVs can be used to study phase separation-induced membrane deformation (Last et al., 2020). Transmembrane domains can also be incorporated into the membrane, and their contribution to phase separation can be studied (Richmond et al., 2011). GPMVs largely retain the cellular membrane composition, allowing for the study of LLPS of physiological densities of proteins and lipid membrane components in a controlled, biophysical experimental setting. Thus, depending on the needs of the researcher, both GUVs and GPMVs offer useful platforms for studying protein and lipid phase separation in membranes. However, because of the mobility of both lipids and proteins in GUVs and GPMVs and the three-dimensional geometry of giant vesicles, obtaining time-resolved, three-dimensional, fluorescence microscopy images in real time is experimentally challenging.

One potential technology that could overcome both the limitations of SLBs and GUVs/GPMVs is the 'double' supported lipid bilayer (DSLB) (Figure 2C). In a DSLB experimental setup, two bilayers, a top 'experimental' bilayer and a bottom 'support' bilayer, are laid on top of one another on a glass slide. This allows for the top 'experimental' bilayer to be essentially independent from the glass support while maintaining a two-dimensional geometry near the surface of the glass slide and can be imaged using TIRFM. By suspending the experimental bilayer above the support bilayer, the lipid composition of the experimental bilayer is not limited, and transmembrane domains can be incorporated because the bilayer does not contact or interact with the glass slide. LLPS-driven membrane deformation may also be evaluated using DSLBs because the 'experimental' bilayer is not tethered to a solid substrate. Techniques to produce DSLBs are under development and offer an exciting possibility for a new experimental platform that can be used for the future study of membrane-associated LLPS.

Another consideration for two-dimensional phase separation is the

contribution of lipids and other membrane components to protein–protein phase separation. Considerable effort has been made to understand two-dimensional lipid-driven phase separation in membranes (Sezgin et al., 2017). A recent study showed that membrane-associated LAT, Grb2, and Sos1 condensation in phase-separated lipid domains resulted in the localization of KRas, a substrate of Sos1, with LAT condensates (Chung et al., 2021). It is important to note that coupled phase separation in this study did not include the transmembrane domain of LAT. Transmembrane domains of membrane-associated proteins are predicted to localize to either liquid ordered (L_O) or liquid disordered (L_D) membrane domains depending on their residue composition and intrinsic biochemical properties of those residues or modifications, such as palmitoylation (Lorent et al., 2017; Sezgin et al., 2017). Thus, it remains unclear how the transmembrane domain of proteins contributes to protein–lipid coupled phase separation. A complete understanding of cellular two-dimensional phase separation will rely on an experimental understanding of the contribution of protein–protein, protein–lipid, and lipid–lipid interactions.

Many questions remain regarding the nature and function of membrane-associated condensates, both *in vitro* and in cells. What is the minimum number of molecules that are needed for a cluster to be a phase-separated condensate? Computational simulations of elastin, an extracellular protein that undergoes LLPS to impart elasticity to tissues, provide insight to this question. Simulations predict that a combination of 945 residues in disordered chains or ~83 kDa of disordered proteins are capable of forming a phase-separated compartment (Rauscher and Pomès, 2017). It is important to note that these simulations do not establish a lower limit of material for phase separation; rather, they establish that systems of this size are physically capable of undergoing LLPS. Considering that many membrane-associated systems consist of proteins with large IDRs, it is conceivable that the interaction of only a few molecules would provide enough material to form a phase-separated condensate with a unique internal biochemical environment. What is the structure of the condensate? Are specific condensates effectively two-dimensional, i.e. a single layer of molecules on a membrane, or are they three-dimensional condensates that are constrained to a membrane, like the postsynaptic density? What is the interplay between protein–protein phase separation, protein–lipid phase separation, and lipid–lipid phase separation in membrane-associated condensates? Does the size of membrane-associated condensates regulate some aspect of their function? Are there functional consequences for non-phase-separating proteins localized to membrane-associated condensates through lipid-controlled localization, like KRas localized to LAT clusters? Changes in the specific function of proteins localized to condensates have been observed in multiple phase-separating systems (Case et al., 2019b; Huang et al., 2019; Kim et al., 2019). What is the contribution of phase separation to the function of membrane-associated condensates? Is increased or decreased specific activity of proteins due to changes in the density of the protein, phase separation of the protein, or changes in protein localization on membranes? What is the role of aberrant membrane-associated phase separation in disease? Finally, are the biophysical characteristics and functional outputs observed in biochemical reconstitution assays indicative of cellular condensate behavior? Because many membrane-associated condensates are near or smaller than the resolution limit of light microscopes, correlating specific biophysical characteristics and functional outputs from microscopy-based biochemical reconstitution assays can be difficult. This experimental limitation must be taken into account as more research is performed to understand the role of phase separation in membrane-associated condensate formation and function. With the development of new technologies and continued progress in our understanding of biological phase separation, it is exciting to consider the future study of membrane-associated phase-separated condensates.

[I thank Michael Rosen and Julie Forman-Kay for stimulating discussions regarding liquid–liquid phase separation and Lindsay Case and Salman Banani for their critical reading of the manuscript. This work was supported by The Hospital for Sick Children Research Institute to J.A.D.]

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