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Biomolecular Phase Separation: From Molecular Driving Forces to Macroscopic Properties

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Abstract

Biological phase separation is known to be important for cellular organization, which has recently been extended to a new class of biomolecules that form liquid-like droplets coexisting with the surrounding cellular or extracellular environment. These droplets are termed membraneless organelles, as they lack a dividing lipid membrane, and are formed through liquid-liquid phase separation (LLPS). Elucidating the molecular determinants of phase separation is a critical challenge for the field, as we are still at the early stages of understanding how cells may promote and regulate functions that are driven by LLPS. In this review, we discuss the role that disorder, perturbations to molecular interactions resulting from sequence, posttranslational modifications, and various regulatory stimuli play on protein LLPS, with a particular focus on insights that may be obtained from simulation and theory. We finally discuss how these molecular driving forces alter multicomponent phase separation and selectivity.

1. INTRODUCTION

MLO: membraneless organelle

LLPS: liquid-liquid phase separation

IDP: intrinsically disordered protein

IDR: intrinsically disordered region

Membraneless organelles (MLOs) have recently been shown to occur in a variety of biological contexts, facilitating a wide array of functions requiring compartmentalization (1). These organelles, which lack a surrounding lipid membrane, have demonstrated liquid-like properties (2) and are characterized by a region of highly concentrated proteins and frequently also nucleic acids, coexisting with the surroundings through the process of liquid-liquid phase separation (LLPS) (3). MLOs differ from membrane-bound organelles in their ability to spontaneously form and dissipate (2, 4) and their permeability (5, 6). MLOs have also been linked to the formation of pathological aggregates associated with neurodegenerative diseases such as amyotrophic lateral sclerosis, frontotemporal dementia, and Alzheimer's disease (1, 7–9). Gaining a greater understanding of the normal and pathological functions of MLOs requires a clear view of the molecular interactions underlying LLPS and of how different biomolecules may contribute to the process of phase separation.

In this review, we discuss the different types of biomolecules that participate in LLPS and the formation of MLOs, and we provide some perspective on what interactions contribute to phase separation, how these interactions may be altered by environmental conditions, and how overall interactions between components promote phase separation of multiple components into two or more phases. Elucidating such interactions by experiment is challenging owing to the heterogeneous structure of components within MLOs, such that any observables are always averaged over a broad distribution of structures. Molecular simulations can play an extremely valuable role in this situation, providing detailed information on the driving forces behind phase separation (which can of course subsequently be tested against experiment); they can also be used for developing and testing new analytical theories. In addition, simulations allow rapid screening of sequence changes or other modifications, which may be more costly to do experimentally. We therefore place particular emphasis on the role that simulation can play in exploring the space of sequence, structure, and phase properties of intrinsically disordered proteins (IDPs).

2. STRUCTURE VERSUS DISORDER

2.1. Role of Intrinsic Disorder in Phase Separation

Recent studies have linked protein intrinsic disorder to MLOs, showing that the proteome for MLOs has a significantly greater fraction of proteins containing intrinsically disordered regions (IDRs) than the overall proteome (10). IDPs are proteins that do not adopt a stable folded structure, yet are able to carry out biological functions (11). They are also highly abundant, composing a large fraction of the eukaryotic proteome (11). Different classes of proteins generally tend toward being disordered, such as typical IDPs, which are rich in charged amino acids (12); elastin-like polypeptides (ELPs), which are more enriched in hydrophobic amino acids (13); and prion-like domains (14, 15), which generally have a simple repetitive sequence and are composed largely of only a few different amino acid types. Each of these are generally enriched in glycine or proline residues that disfavor formation of normal secondary structures.

2.1.1. Why are intrinsically disordered proteins important to liquid-liquid phase separation? IDPs have previously been suggested as being important to LLPS because of their ability to form many contacts with one another simultaneously, due to their high multivalency (16). Indeed, the length of an IDP has been shown to correlate with its ability to phase separate (6, 17, 18), with the trend being consistent with polymer theories such as Flory-Huggins (19). In vitro studies have shown that a fully disordered protein may undergo LLPS, and that it remains disordered

while in the phase-separated state (19, 20), forming weak interactions promiscuously between all types of amino acids (21). Another advantage of IDPs is that the amino acids are more exposed and therefore more accessible to posttranslational modification (PTM) (22), which is a major regulator of biomolecular phase separation (23–27). Simulations have shown that inclusion of folded domains may drastically slow down dynamics within phase-separated compartments, indicating that intrinsic disorder may also be important for the liquid-like properties of MLOs (18).

2.1.2. Disordered chain dimensions as an indicator of phase separation propensity. When studying IDPs, researchers may infer characteristics of phase behavior from simple theory, simulation, or experiment using the properties of single chains (4, 28, 29). This is because the same interactions driving compaction (or otherwise) of a single chain tend to also stabilize the protein-rich phase in LLPS; thus, the degree of collapse is expected to be correlated with the propensity to phase separate. Single-molecule experiments, scattering experiments, or simulations of IDPs can be used to identify the average size of an IDP in solution (30–32). This is related to the backbone flexibility of the protein (33), as well as the overall strength of interactions between its amino acids (18) and of course the overall chain length. Lin & Chan (28) used random phase approximation theory to study the relationship between the critical temperature of phase separation (T_c) and the radius of gyration (R_g) of a series of synthetic polyampholytic protein sequences of the same length, and they found that they are highly correlated.

To approximately remove the effects of chain length, the size of an IDP in solution can be quantified by its Flory scaling exponent ν (34); for example, the end-end distance R of a disordered polymer should approximately scale with the number of residues as $R = bN^{\nu}$ (for proteins, $b \approx 5$ Å is frequently a good approximation). In this description, the degree of collapse of the protein is entirely captured by the scaling exponent ν, which takes on distinct values in a poor solvent when the protein is collapsed ($\nu \approx 1/3$), in a good solvent where the protein interactions are essentially repulsive (self-avoiding random walk) ($\nu \approx 3/5$), or if attractive and repulsive interactions are exactly balanced ($\nu \approx 1/2$), also known as Θ-solvent conditions (34). In simulations, ν is often estimated by fitting the scaling of internal distances in the protein with sequence separation (35). In FRET experiments, ν can be estimated from studying different labelings, as pioneered by Hofmann et al. (30), or from fitting polymer model distributions (32). In scattering experiments ν can in principle be obtained directly from the mass fractal dimension from the raw scattering data, although in practice this is very challenging (36). More practical alternatives are the use of ensemble fitting (36, 37) from a simple extended Guinier analysis (38) or from the molecular form factor fitting procedure (4). This scaling exponent may be used to estimate the Θ-solvent temperature (T_Θ) (or Θ conditions for control variables other than temperature). This has been shown to be nearly equivalent to T_c (29), in agreement with previous studies showing the same relationship for homopolymers in the limit of infinite chain length (39). Simulation studies benefit greatly from this approach, as systems containing a large number of polymeric chains may become computationally intractable (40, 41). Simulation studies have made use of this relationship and have been able to demonstrate the collapse (42) and phase separation of IDPs with increasing temperature (43) due to temperature-dependent, solvent-mediated interactions.

2.2. Role of Folded Domains in Phase Separation

In addition to IDPs and IDRs, folded proteins and domains contribute to MLOs in various ways. Folded domains involved in LLPS include RNA-recognition motifs (RRMs), which bind to specific sequences of RNA (4, 44); oligomerization domains (6, 45, 46); and other domains that carry out the intended function of the MLO, such as facilitating metabolic catalysis (47), promoting

PTM: posttranslational modification

T_c: critical temperature of phase separation

R_g: radius of gyration

ν: Flory scaling exponent

T_Θ: Θ-solvent temperature; the temperature at which a polymer chain behaves as in an ideal solvent

RRM: RNA-recognition motif

Oligomerization: assembly of a finite number of components into a single cluster

gene expression (48), and recruiting specific cargo molecules (6, 25). In some cases IDRs have even been shown to inhibit LLPS, while folded domains operate as the main factor driving phase separation (4).

2.2.1. Phase separation of folded proteins. Going back many decades, X-ray crystallography studies have observed LLPS at some conditions during screening for protein crystallization (49). This, however, generally requires very high protein concentration and rather extreme conditions, unlike many IDRs that may phase separate at much lower concentrations (17, 26). Folded proteins may aggregate or crystallize, leaving only a small window of conditions in which LLPS may occur (50). However, IDRs, including those involved in LLPS, have also been known to be prone to aggregation and formation of disease-causing inclusions (7, 11, 51).

2.2.2. Folded domains facilitate phase separation with intrinsically disordered regions. Many proteins involved in LLPS, such as ribonucleoproteins (RNPs), include multiple folded domains tethered together with disordered linkers (15). These folded domains may contribute significantly to phase separation by oligomerizing multiple protein molecules and effectively increasing the multivalency and number of interactions a single particle is able to form (45). Taking advantage of this, researchers have engineered proteins including a light-activated oligomerization domain (52), thus enabling induction of phase separation in a controlled manner inside living cells (48, 53). RNPs even more commonly include RRMs, which selectively bind to particular regions of RNA and can promote LLPS in the presence of these particular RNA sequences (4, 44, 54). Partially folded structures may also contribute to phase separation through folding upon binding to specific binding partners (55, 56) or promotion of secondary structure upon self-association (8, 57). Inclusion of short helical motifs within an ELP also contributes to phase separation with significant hysteresis (58), having a considerably higher saturation temperature (T_{sat}) upon heating compared to cooling.

2.2.3. Folded domains carry out orthogonal functions. Some folded domains in phase-separating proteins are relatively passive and do not contribute appreciably to the ability of the protein to phase separate. Such domains may have an orthogonal function, such as enzymes (47, 59) and RNA-remodeling helicase domains (60, 61). Many studies use protein constructs containing green fluorescent protein or other similar fluorescent protein domains in order to visualize LLPS within cells (23, 44). Importantly, different fluorescent tags have been shown to incorporate into droplets of the LAF-1 RGG with different preferences (6), indicating that in some contexts the inclusion of fluorescent tags may alter LLPS.

2.3. Interactions Between Folded and Disordered Domains

Another advantage of IDPs that makes them preferable for driving LLPS is that they can interact promiscuously with a large number of binding partners [this may be why they occur at protein interaction network hubs with a significantly higher frequency than folded proteins (62, 63)]. IDPs may interact with other IDPs, sometimes with very high affinity, while remaining fully disordered and having no specific bound complex (36). Many IDPs also interact with folded domains in a specific manner, by adopting a folded structure, usually via an induced fit mechanism (11, 55, 56), though they may simply interact with folded domains and remain disordered (64, 65). Self-complementary RNA structures also play a role in LLPS by imparting an identity to the MLO which the RNA is incorporated in and by preventing merges with other MLOs containing different folded RNAs (66, 67).

Aggregation: formation of solid inclusions composed largely of misfolded protein, commonly associated with cell death and disease

RNP: ribonucleoprotein

Hysteresis: a history-dependent phase behavior, such as displaying different saturation temperatures when heating versus cooling

T_{sat}: saturation temperature, or cloud-point temperature

Helicase: a specific class of enzymes whose function is to unwind structured or double-stranded nucleic acids

Interaction network: a map that shows connections between each pair of proteins that are known to interact with each other

Induced fit: binding of an intrinsically disordered protein to a partner that promotes folding into specific folded structure

3. SEQUENCE-LEVEL DRIVING FORCES

3.1. Amino Acid Interaction Modes

Since weak multivalent interactions between disordered and folded protein domains are the major driving forces of phase separation, it is important to understand exactly what are the different modes of interaction that cause proteins to assemble, demixing from their normal solvated state. Biology has provided proteins with a diverse set of amino acids with differing side-chain chemistries and an even more extensive library of PTMs (68). The result is hundreds of different types of amino acid derivatives, in addition to the 20 canonical amino acids, and many different possible interaction modes arising from these (15, 69, 70). A full understanding of each of the interaction modes and of how they contribute to or detract from a system's ability to phase separate is needed to appreciate the implications of protein composition and sequence.

sp^2-hybridized: arrangement of electron orbitals into a planar triangular shape, present in carbon atoms having three σ-bonds

π-bond: additional bond between sp^2-hybridized atoms involving out-of-plane electron density

3.1.1. Charge-charge interactions. IDPs are commonly enriched in charged amino acids (12), and thus LLPS usually involves interactions between charged amino acid side chains and termini (**Figure 1**). Charge-driven LLPS can take place as a single sequence containing both cationic and anionic amino acids, such as the disordered domains of LAF-1 (5, 71) and Ddx4 (19, 72, 73), or as cophase separation of two oppositely charged biomolecules through a complex coacervation (74–76). A high net charge may allow a single protein to exist at high concentrations without undergoing LLPS (24). Depending on the overall charge composition, IDPs display salt-out or salt-in behavior due to screening of electrostatic interactions as well as Hofmeister effects (19, 71, 77).

Several factors may alter the charge state of amino acids, such as pH, which has a significant effect on histidine (side-chain pKa $\sim$ 6) but must usually be very high or low to have an appreciable effect on other amino acid side chains. Charge state may also be modified by various PTMs, such as phosphorylation (9, 24, 78) or acetylation (27). The polarity of the environment may also have an impact on the formal charge of ionizable side chains, which can shift their effective pKa when buried within the hydrophobic core of a protein (79) or in the proximity of other charged residues.

3.1.2. π-Interactions. Another mode of interaction that has been suggested as an important driver of biomolecular phase separation is planar interactions between sp^2-hybridized atoms, commonly referred to as π-π interactions (**Figure 1**). π-bonds occur within sp^2-hybridized groups, which are most predominant in aromatic amino acids (80, 81). Since the aromatic groups of phenylalanine, tyrosine, and tryptophan are electron rich, they would most likely interact in off-center parallel or edge-to-face perpendicular configurations (82), though aromatic rings may also become polarized and prefer face-centered stacking (83, 84). Vernon et al. (85) suggested that interactions between all sp^2-hybridized groups, and not just between aromatic side chains, contribute to phase separation of proteins. Since all amino acids contain a backbone peptide bond with a partial π-bond, each may contribute to the planar sp^2 interactions driving phase separation. This would result in weak multivalent interactions throughout the full protein sequence and with the nucleotide bases in single-stranded nucleic acids.

In addition to interaction between two sp^2-hybridized groups, there are also interactions between aromatic rings and charged amino acids, particularly cationic residues (84, 86). Cation-π interactions are demonstrably important to phase separation in many commonly studied proteins (87), particularly between arginine and tyrosine residues. Dimethylation of arginine residues significantly reduces phase separation propensity in the hnRNPA2 protein (26), even though this PTM does not alter the net charge of the arginine residue. It is likely that the methylation of arginine disrupts multiple modes of arginine interactions with all other amino acids, including

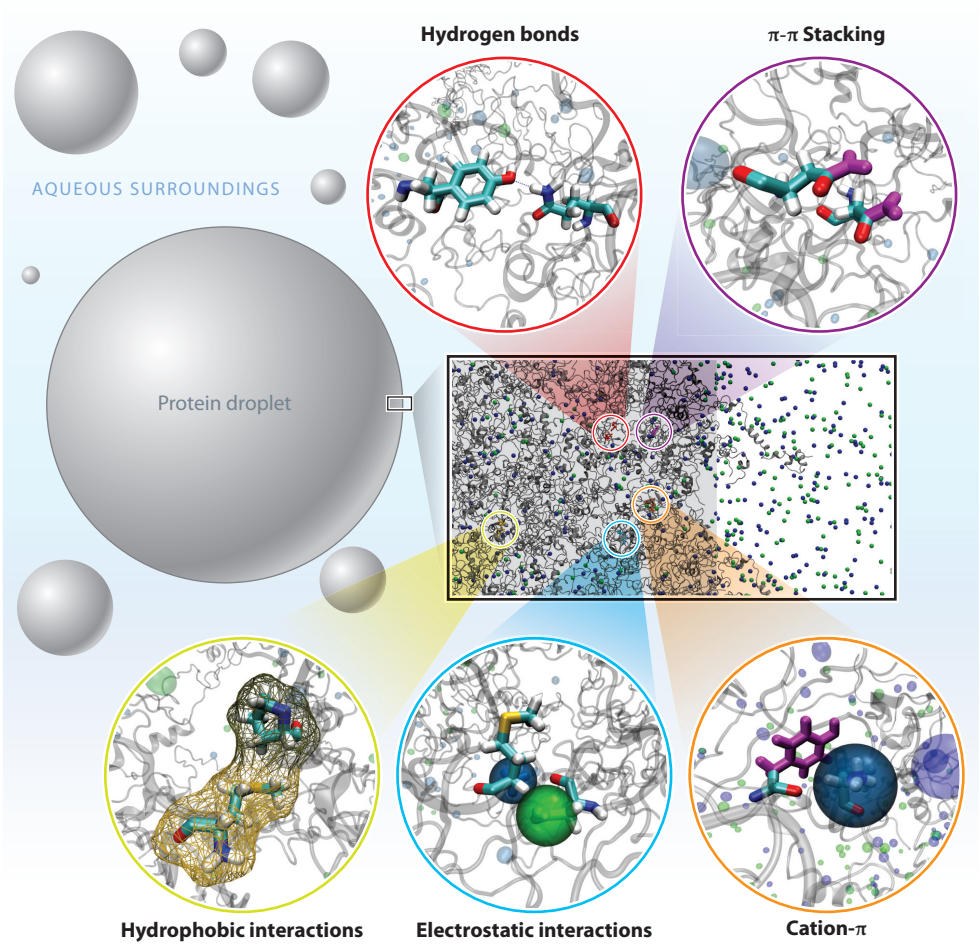

Figure 1

Schematic of a single-component droplet in phase coexistence with the surrounding aqueous environment. The box shows a molecular configuration of proteins stabilizing the condensed phase at the interface. The colored side chains and zoomed insets highlight the different interaction modes occurring between protein molecules.

tyrosine, which may suggest that Arg-Tyr interactions are stabilized by multiple interaction modes in addition to cation-π. Cation-π interactions may also result in apparent non-Fickian diffusion within condensates where groups get trapped and move slowly on short length scales while diffusing more quickly at longer length scales, suggesting that cation-π interactions are also important for tuning the material and transport properties of condensates (88).

Importantly, these interactions are prominent within both proteins and nucleic acids, particularly single-stranded, unfolded nucleic acids that have their aromatic nucleotide bases exposed, which is consistent with studies showing that ssDNA, but not dsDNA, is incorporated into IDP-rich droplets (72, 73, 88). Thus, proteins and nucleic acids may colocalize through many interactions with sp^2-hybridized groups, and folding of proteins or nucleic acids may function as a regulator of such interactions and phase separation. Residues most prone to sp^2-hybridized interactions include the aromatic residues Tyr, Phe, Trp, and His; carboxyl and carboxamide groups of

Asn, Asp, Gln, and Glu; the guanidine group in Arg; and the exposed backbone peptide bond of Gly and other amino acids with small side chains (85).

3.1.3. Hydrophobic contacts. In the context of LLPS, hydrophobic interactions may be less predominant than in folded proteins (89), but they are likely to be still important due to the appreciable content of hydrophobic amino acids in some cases (**Figure 1**). The lower fraction of hydrophobic residues likely allows for the chains to remain disordered and for the assemblies to be liquid-like rather than solid. Rauscher & Pomès (40) performed simulations of a large liquid-like assembly of ELPs and were also able to observe hydrophobic contacts between valine residues that largely stabilize the condensed phase. NMR studies on a minimal model of the processing body MLO highlight the role of hydrophobic leucine-rich helices in its formation and stabilization as a condensate (90). Importantly, hydrophobic amino acids may also interact strongly with aromatic amino acids, which tend to be quite prevalent in phase-separating proteins. Even amino acids that are generally considered polar may contain nonpolar groups that participate in hydrophobic interactions, such as glutamine, which contains two methylene groups and associates with other hydrophobic atoms in glutamine and tyrosine residues within FUS (21). Hydrophobic interactions are also a major driving force of protein folding, promoting the formation and stability of various folded domains and oligomers that may additionally promote phase separation (45, 91), and hydrophobic interactions often play an important role in specific binding of ligands to incorporate them preferentially into the condensed phase (56, 92).

3.1.4. Hydrogen bonds. While hydrogen bonds are largely considered to be responsible for solvation of polar amino acids, they also contribute much to the self-association of biomolecules and are also driving forces of biomolecular phase separation. Most amino acids contain both hydrogen bond donors and acceptors, suggesting that hydrogen bonding could be very common within the densely packed proteinaceous condensates. A combination of NMR and simulation techniques has shown that glutamine residues in the low-complexity region of FUS are important drivers of phase separation, and that hydrogen bonding is highly prevalent (21). Other studies have used atomistic molecular dynamics simulations to highlight this contribution of hydrogen bonds to phase separation (40, 42, 93). Hydrogen bonding is also a major factor in recognition of nucleotide bases (94), and it is likely important for incorporation of RNA and DNA into condensates (95).

Hydrogen bonds may also contribute to biomolecular phase separation more indirectly through the formation of secondary structure in proteins (8), the hybridization and secondary structure of nucleic acids (67, 73, 88), and the incorporation of water into the condensate (21, 93, 96). Hydrogen bonds also contribute to the formation of fibrillar structures in FUS, which are similar to amyloids but are stabilized by hydrogen bonds rather than a hydrophobic core (97), and to the transient formation of small fibrillar structures such as LARKS (low-complexity aromatic-rich kinked segments) (98) or other transiently interacting structures (99). All amino acid types may participate in hydrogen bonding; however, the most susceptible are generally those with polar or charged side chains.

3.1.5. Summary of interactions. Proteins and nucleic acids make use of diverse chemistries to drive self-association and incorporation or exclusion of other molecules and to control dynamical and transport properties of phase-separated assemblies (15, 21, 68, 81, 85, 87, 88, 100). Some amino acids may interact through several different interaction modes, which may work cooperatively to provide even stronger binding (21). Since the components of MLOs are highly dynamic and usually disordered, it is a major challenge to directly determine which interaction modes are

contributing and the relative importance of each to LLPS (20, 24). Atomic-resolution simulations provide a promising path toward a better understanding of this, as they can be used to observe all of the different interaction modes directly (21, 26, 40, 42, 78). The current challenge in using simulations is the cost of running atomic-resolution simulation on a large assembly of many proteins, and so far this has only been achieved in one study (40). Studies identifying interactions within proteins may be useful in identifying which interactions contribute most significantly, how small perturbations can be made to the sequences to alter the macroscopic phase behavior, and how naturally occurring mutations may have significant physiological and biophysical repercussions (7, 14, 57).

Block copolymer: a polymer containing two or more types of monomer units with the sequence being arranged in blocks, each consisting of only one type of monomer

SCD: sequence charge decoration

3.2. Sequence and Arrangement of Interactions

In addition to amino acid composition, the arrangement and sequence of amino acids also has an important role in IDP properties, as well as in the phase separation of biomolecules. Many decades of research have been devoted to relating protein sequence to structure, but for disordered proteins, the role of sequence is not as well understood (12, 101). This is largely due to a lack of structural information from experiment, which thus requires alternative measurements of IDPs such as size measurements (R_g, R_h, and ν), and to their propensity to self-associate, aggregate, or phase separate. Indeed, phase separation may serve as an excellent descriptor for studying the sequence determinants of disordered proteins, nucleic acids, and their interactions (18, 74, 95).

3.2.1. Charge patterning. To explore the effects of the arrangement of charged amino acids, Das & Pappu (101) used all-atom implicit solvent simulations to demonstrate the wide range of single-molecule behaviors for a set of proteins having identical composition. The specifically designed sequences are composed of 25 positively charged lysine residues and 25 negatively charged glutamate residues arranged differently throughout the sequence, with the extremes being a strictly alternating dipeptide repeat ($[KE]_{25}$) and a block copolymer ($[K]_{25}[E]_{25}$) (101). To quantify the degree of charge segregation, they devised a parameter, κ, defined as

$$\kappa = \left(\frac{\delta}{\delta_{max}}\right), \quad 1.$$

where

$$\delta = \frac{1}{N_{blob}} \sum_{i=1}^{N_{blob}} (\sigma_i - \sigma)^2, \quad 2.$$

$$\sigma_i = \frac{(f_+ - f_-)_i^2}{(f_+ + f_-)_i}, \quad 3.$$

N_{blob} is the number of "blobs" or segments of five or six residues of the sequence in a moving window, $f_{+/-}$ is the fraction of positive or negative residues within the blob, σ is calculated over the full sequence, and δ_{max} is the maximum possible δ value for all sequences of the given composition. A κ value near 0 indicates the strictly alternating sequence, and a value near 1 indicates the block copolymer. Das & Pappu (101) found that sequences with low κ values are more extended, while sequences that are more blocky (i.e., charge segregated) are more collapsed. Sawle & Ghosh (102) then used mean field theory to support this assessment and proposed an alternative charge patterning metric termed sequence charge decoration (SCD),

$$\mathrm{SCD} = \frac{1}{N}\left[\sum_{m=2}^{N}\sum_{n=1}^{m-1} q_m q_n (m-n)^{1/2}\right], \quad 4.$$

where N is the number of amino acids in the sequence and q is the formal charge on each residue. A large negative value of SCD indicates a block copolymer, while a value near 0 would indicate an alternating sequence. SCD may also be positive for sequences having a nonzero net charge. Since the single-chain size of the protein is generally correlated with its phase separation propensity, it also follows that the charge patterning values of these polyampholytic sequences are highly correlated with their critical temperatures as calculated by theory (80) and simulation (103). Importantly, charge patterning has been demonstrated to have a significant impact on the phase separation of biological IDPs such as Ddx4, which contains 25% charged amino acids (19, 72). While these two charge patterning metrics are highly correlated with each other, some sequences may be designed in which one metric predicts collapse while the other predicts extended configurations. For such a sequence, neither metric is effective at predicting its single-chain or LLPS behavior (103), demonstrating that both may be limited in their predictive capabilities in special circumstances. Other metrics have also been used to quantify degree of charge patterning such as charge fluctuations (77) and the average number of consecutive residues with the same charge (76).

3.2.2. Hydrophobic/aromatic patterning. There is also evidence that the effects of patterning are nonnegligible for other types of interactions, particularly involving hydrophobic and aromatic amino acids. Previous studies have looked at the arrangement of hydrophobic and hydrophilic amino acids to find that folded proteins need to have particular patterning in order to collapse properly (104). Computational work has also shown how sequence correlation of hydrophobic residues can be used to design disordered proteins of identical hydrophobic and hydrophilic composition with very different single-chain behaviors (105), which should also result in differences in phase separation (29). A hydrophobic correlation parameter may be used similarly to the SCD and κ metrics to make predictions about IDP single-chain behavior and phase separation, though when comparing, it will also be important to take into account the distance dependence of such interactions (106, 153). Some disordered proteins with a very blocky nature may even function as biological surfactants, promoting mixing of polar and nonpolar molecules (107).

It is curious how many LLPS-enabled IDPs, particularly prion-like domains, have aromatic residues that are widely distributed rather than clustered together (80, 108). It is possible that the dispersion of these amino acids prevents hydrophobic collapse or folding. Since cation-π interactions (87) and π-π interactions (85) are known to be particularly competent at promoting phase separation, a patterning metric that considers cations and aromatic residues could also prove useful to predicting phase behavior directly from sequences.

3.2.3. Patterning as mode of recognition. Patterning of amino acids is very important to a protein's ability to phase separate, and it is also important to biological functions through molecular recognition and condensate selectivity. Lin et al. (109) have shown that differences in charge patterning between polyampholytic sequences result in significant differences in partitioning of the two components into two or three distinct phases, where similar sequences phase separate cooperatively, while very different patterning results in separation of the two components into separate phases. In addition, sequence patterning may also serve as a mode of recognition between disordered proteins and folded proteins with patchy surfaces (64), which could result in additional selectivity of condensates for both disordered and folded domains.

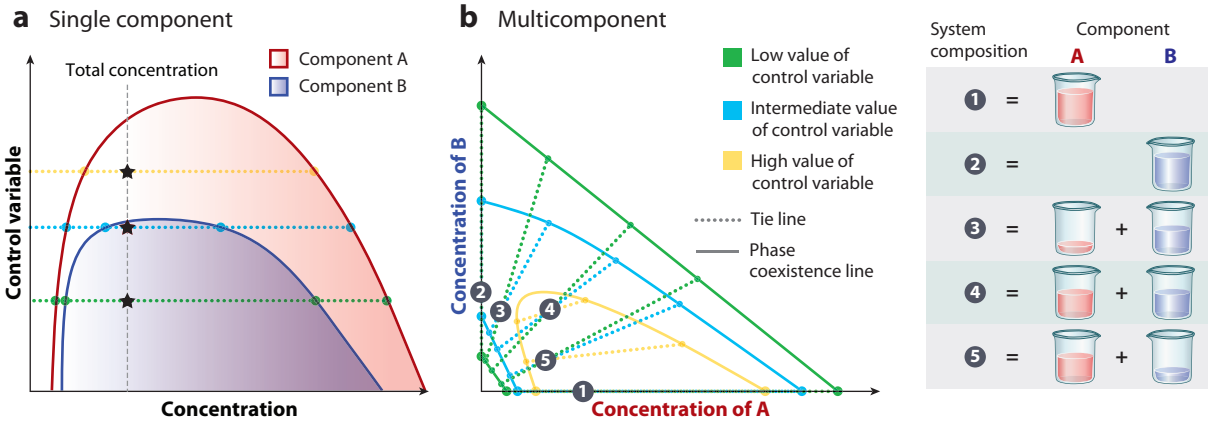

Figure 2

Cophase separation of two species with similar self- and cross-interactions. (*a*) Single-component phase diagrams for component A and component B, with tie lines at three values of the control variable (e.g., temperature, pH, ionic strength): At the lowest value of the control variable, both components may phase separate (*green*); at an intermediate value, both A and B may phase separate, but B is nearing its critical point (*blue*); and at the highest value of the control variable, only A is able to form a condensed phase (*yellow*). (*b*) Multicomponent phase diagram of mixtures of component A and component B, with the control variable indicated by color. Numbers in panels *b* and *c* indicate different experiments conducted at different relative total compositions of the two components, where 1 and 2 only contain a single component (these compositions are also indicated by the asterisks in panel *a*), and 3–5 contain a mixture of the two. Tie lines show the resulting concentrations within the two phases for each value of the control variable (color scheme as above). We observe a cooperative condensation of both components into a single condensed phase for the two lowest values of the control variable (*green*, *blue*); at conditions where only A may phase separate, we observe a scaffold-client phase diagram (*yellow*).

3.3. Stimulus Responses of Different Interaction Modes

Many perturbations have been shown to alter the phase separation of diverse proteins (21, 44, 48, 110, 111). Depending on the amino acid sequence, different stimuli may either promote or disrupt phase separation and may change the way in which multiple components mutually or exclusively phase separate. In **Figure 2** we show two different components at different values of a control variable that could be temperature, salt concentration, pH, etc. The two components have different propensity to phase separate as a function of the control variable, where component A may phase separate over a wider range of conditions than B; the self-interactions A-A and B-B are also taken to be similar in strength to the cross (A-B) interactions. **Figure 2*a*** illustrates the single-component phase diagrams of each of the two components, showing a region where LLPS is permitted at low values of the control variable and a region above the critical point where the system is in a single continuous phase. At conditions where both may phase separate, it is likely that condensates may form that contain both components; however, in the region between the two critical points, it is unclear whether component B will be able to incorporate into droplets of A. To visualize this further, **Figure 2*b*** shows a two-component phase diagram at three conditions highlighted in **Figure 2*a***. As seen in the figure, varying the control variable causes a qualitative change in the two-component phase diagram from a cooperative condensation to a scaffold-client phase diagram. This example illustrates how altering conditions in solution may lead to different phase behaviors, and it shows that some components may still be incorporated into condensates even if they would not undergo LLPS in isolation.

3.3.1. Temperature. While cells generally exist in a narrow range of temperatures, changes to interactions in response to temperature are still very important to understanding MLOs, and

particularly the role of different interaction modes due to each one's distinct temperature response. An increase in temperature may induce phase separation of proteins through active processes that accelerate at higher temperatures or through thermodynamically driven and reversible LLPS (110). The thermodynamically driven phase separation that is promoted at higher temperatures results in a lower critical solution temperature (LCST)-type phase transition. García Quiroz & Chilkoti (17) have provided a comprehensive characterization of the composition-dependent phase behavior of IDPs, demonstrating that sequences containing many polar and aromatic amino acids generally follow an upper critical solution temperature (UCST) phase transition, while those containing more hydrophobic amino acids follow LCST transitions. Some protein sequences such as An16-resilin even show a reentrant phase behavior, where LLPS occurs at both low and high temperatures with a region of miscibility in between (112), typically referred to as having an hourglass phase diagram (43, 113).

LCST: lower critical solution temperature

UCST: upper critical solution temperature

Cold denaturation: the unfolding of proteins with decreasing temperatures

With increasing temperature, the loss of chain entropy accompanying IDP collapse or phase separation will increase (29), which is sufficient to fully explain the UCST phase transitions. However, to observe an LCST phase transition, it is important to consider the temperature dependence of solvent-mediated interactions, particularly with the different types of amino acids (114). Privalov & Makhatadze (115) conducted solvent transfer experiments on small molecule analogs of the 20 different amino acid side chains and backbones to show that the free energy of solvation is temperature dependent and increases with temperature. Amino acids becoming more insoluble with increasing temperature could, in principle, overcome the effect of chain entropy and allow for LCST phase transitions. The difference in temperature dependence between different types of amino acids based on how different interaction modes are strengthened or weakened by temperature would explain the ability to switch between UCST and LCST phase transitions based on overall composition.

Indeed, the temperature-dependent solvation free energy of hydrophobic molecules is also nonmonotonic, having an initial increase of hydrophobicity up to a turnover point and subsequent decrease (116), which may be explained by the dominance of enthalpy at low temperatures and entropy at high temperatures (117, 118). By fitting this temperature dependence to a thermodynamic equation, Dill et al. (119) developed a theoretical model to explain protein folding and thermal stability, showing that temperature-dependent interactions can be used to explain increasing stability with increasing temperature, as well as cold denaturation. By using a temperature-dependent interaction potential calculated from solved solution NMR structures (120), Dignon et al. (43) developed a coarse-grained simulation model, which is successful in predicting the composition dependence of UCST and LCST phase transitions from García Quiroz & Chilkoti (17). Models such as this one can be quite helpful in elucidating the sequence determinants of temperature-controlled phase behavior.

3.3.2. Salt. Salt is an important solution additive that can be used to tune phase separation as it is easily controlled in vitro, and it is perhaps more physiologically relevant than large changes in temperature. Early studies have shown that for some proteins, increasing salt concentration may induce (salt-out) phase separation (8, 20) or prevent (salt-in) phase separation (19, 71). An obvious effect of increasing ionic strength is the screening of electrostatic interactions (121). However, for a sequence such as the low-complexity domain of FUS, which is nearly devoid of charged amino acids, it is unlikely that charge screening is the only factor contributing to the large effect that salt concentration has on its phase separation. In our previous work, we suggest the possibility of salting-in and salting-out effects on the solubility of all amino acids by shifting effective pairwise interactions to be more or less attractive (18). Brangwynne et al. (106) also discussed the possibility that several other interaction modes are affected differently by salt concentration.

Hofmeister series: ranking of anions and cations by their ability to precipitate proteins

c_{sat}: saturation concentration; the concentration above which a solution becomes turbid and begins to phase separate

The identity of the salt ions also plays an important role in the effects on phase separation. The Hofmeister series ranks different anions and cations by their ability to solubilize or precipitate proteins (122), and the effect of these ions on the phase separation of FUS has been shown to follow a similar trend (21). The kosmotropic salts were shown to promote phase separation of FUS at considerably lower saturation concentrations (c_{sat}) than the standard NaCl, and the chaotropic salts did the opposite by greatly increasing the required FUS concentration to observe LLPS (21). Ions with higher valency such as transition metals will also facilitate phase separation for biomolecules having an abundance of the opposite charge (50, 123). With all of these considerations, salt may present one of the most tunable handles to perturb phase separation.

3.3.3. pH. Another stimulus likely to induce changes to phase behavior is the solution's pH. Kroschwald et al. (44) showed that reduced pH as a result of cellular starvation causes the formation of stress granules (SGs) in vivo and in vitro. Interestingly, they found that pH-induced phase separation results in SGs that are more dynamic than those induced by heat shock, implying that different assembly mechanisms may be occurring (44). Changes to a system's pH will undoubtedly alter the charges within a protein, and depending on the types and arrangement of charged residues within the sequence, this may also have a strong impact on the charge patterning of the sequence. Thus, pH might also be used as a powerful tool for tuning LLPS and selectivity through altered net charge and charge patterning (113).

3.3.4. Other stimuli. Other factors that the cell uses to control LLPS include ATP (111, 124) and poly ADP-ribose (PAR) (125). One process by which ATP may modulate phase separation is by acting as a hydrotrope, thus solubilizing nonpolar groups of the proteins and preventing, or reducing, phase separation when driven by hydrophobic interactions (126). Another consideration is that ATP-driven reactions, particularly phosphorylation of amino acid side chains, may also drive or prevent phase separation (127). PAR may seed phase separation, particularly in cases where phase separation is required to aid in repair of damaged DNA (125).

LLPS may also be mediated by various small molecules such as 1,6-hexanediol (9, 48, 81, 108, 128), chemical chaperones (129), and large molecular crowders, including polyethylene glycol (PEG) (130–132). Braun et al. (50) looked at the effects of solvent isotope content and found that D_2O promotes hydrophobicity-driven phase separation of BSA (bovine serum albumin) more strongly than H_2O, which has important implications for NMR experiments that commonly use D_2O as a solvent. Oxidation also plays an important role in LLPS in various ways. Reed & Hammer (133) showed that oxidative cross-linking of a cysteine residue within a designed oleosin protein can facilitate LLPS by promoting dimerization and increasing the multivalency of the protein. They also find that the position of the cysteine residue within the sequence can control the degree to which LLPS is promoted (133). In contrast, oxidation of methionine side chains has been shown to prevent LLPS of the yeast ataxin-2 protein (134). Thus, oxidizing and reducing environments are able to promote or abrogate LLPS depending on protein sequence and composition.

3.3.5. Summary. The stimulus response of biomolecular condensates may currently be somewhat unpredictable, but an understanding of the molecular interactions underlying LLPS, and of how such interactions are perturbed by various stimuli, will go a long way in making it more predictable. This, however, is very challenging, and it is not clear what the best way to quantify interactions is. Solvation free energy is a useful metric, but it considers the interactions of the amino acid only with solvent and not with other amino acids (119), and use of small molecule analogs may also neglect the polymeric effect (115). Alternative strategies that may provide the field with much-needed insights include bioinformatics (120, 135) and experimental (136) or computational

(137, 138) characterization of binding energies of all amino acid pairs and of their dependencies on relevant stimuli.

4. MULTICOMPONENT PHASE SEPARATION

4.1. Modes of Two-Phase Systems with Two Components

Most MLOs exist not as a single-component condensate but as a mixture of dozens to hundreds of different components, including proteins, nucleic acids, solvent, ions, and other cargo molecules (110, 124, 128, 139, 140). Many of these organelles have redundancy built in, such that no single component is solely responsible for the formation of the MLO (140). To characterize the relationship between different components and their relative abilities to phase separate, as well as their mode of phase separation (i.e., cophase separation, scaffold/client, exclusivity, core-shell, etc.), one may make use of the relative interaction strengths between different components. The interaction strength between two molecules may be quantified using metrics such as the osmotic second virial coefficient (B_{22}) (5, 29). Interaction strengths can be altered with changes in temperature (or other stimuli discussed in Section 3.3), and the temperature at which B_{22} becomes zero is known as the Boyle temperature (T_B), which has been shown to correlate well with the critical temperature of phase separation for a single-component system (29). For a two-component system, one may consider four possible cases involving phase separation into two distinct phases.

B_{22}: osmotic second virial coefficient of self-interaction

T_B: Boyle temperature, where B_{22} disappears

4.1.1. Cooperative cophase separation. The first case we consider is one in which both components have strong self-interaction affinity, as well as strong cross-interactions (**Figure 3*a***). For such a system, one may expect formation of a heterotypic droplet enriched in both components and a surrounding aqueous phase with both components at low concentration, also known as a condensation phase transition (141). This is most likely to occur with droplets in which the constituent proteins are very similar to one another, such as cophase separation of a wild-type protein with a fraction of the same protein with a fluorescent tag or NMR-active label (26); however, it could also occur between sequences that are quite different from one another. Depending on the relative total concentrations of the components or relative interaction strengths, the droplet may be more enriched in one component than the other (**Figure 3*a***). The slopes of the tie lines can also be used to determine which component is contributing more to the phase separation of the system.

4.1.2. Scaffold-client cophase separation. The second case we consider deals with systems where one component is able to phase separate as a single component, and the second is not but has a strong attraction for the first (**Figure 3*b***). The shape of the scaffold-client phase diagram may change rather significantly but should always have a continuous phase along one of the axes, and thus the phase lines should never intercept one of the two component axes. An example of such a system would be an RNA-binding protein that is prone to phase separation and an RNA molecule that generally would be incapable of phase separation on its own. Here, the protein functions as a scaffold molecule, promoting phase separation, and the RNA functions as a client, being incorporated into the droplet (8, 71).

One way in which the shape of the phase diagram may vary is if the cross-interaction is considerably weaker than the self-interaction of the scaffold and the client is more excluded from the condensate than recruited, which would be indicated by negatively sloped tie lines in the phase diagram. Another possible difference is whether c_{sat} increases or decreases with the initial addition of client concentration. If the client molecule occupies the interaction sites of the scaffold, it may reduce the propensity of the scaffold to phase separate (142, 143), thus increasing c_{sat}, as in **Figure 3*b***. In contrast, if the client molecule additionally promotes phase

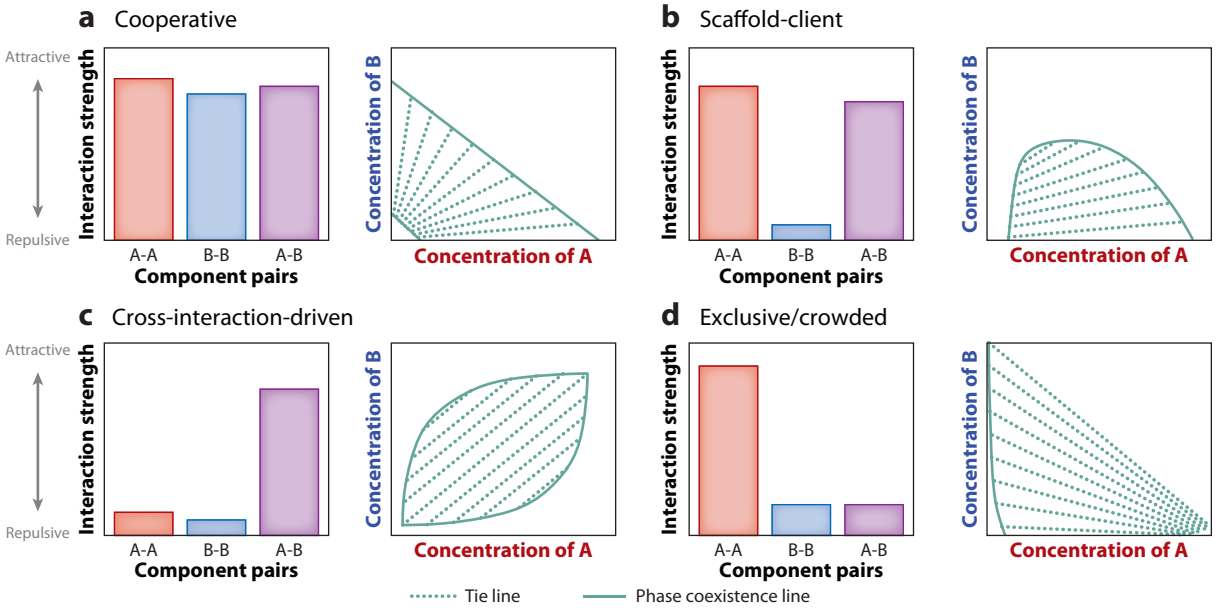

Figure 3

Possible shapes of multicomponent phase diagrams and the relative self- and cross-interaction strengths of component A and component B. (*a*) A cooperative condensation phase transition occurs when both components have strong self-attraction as well as a mutual attraction. This is characterized by phase coexistence lines (*thick lines*) running from one axis to the other at low and high concentrations, and tie lines (*dotted lines*) having a positive slope in between the two phase lines. (*b*) A scaffold-client relationship occurs when one component (here, component A) has strong self-attraction and can undergo liquid-liquid phase separation (LLPS), while the second (component B) cannot but has some attraction for the first component. This results in a single phase line that intercepts the scaffold concentration axis twice. Tie lines may be positively or negatively sloped, indicating recruitment or exclusion, respectively. (*c*) Cross-interaction-driven phase separation occurs when neither component has an attractive self-interaction, but both components have strongly attractive mutual interactions. The phase lines do not intercept either axis, since neither pure component is able to undergo LLPS. Tie lines are always positive because both components must be present in sufficient quantities and associate cooperatively for the condensed phase to be stabilized. (*d*) Exclusive/crowded phase separation occurs when one component has strongly attractive self-interactions, while the other has no attractive interaction with itself or the first component. In this case, the first component (here, component A) forms a condensed phase, while the second (component B) preferentially occupies the other phase, indicated by negatively sloped tie lines.

separation, the saturation concentration of the scaffold will decrease initially, as in **Figure 2*b***. In this second case, there will eventually be a turnover, and c_{sat} will begin to increase again, which has been observed as a reentrant phase separation upon increasing RNA concentration (144). In this case, the turnover occurs roughly at the charge-inversion point, when the phase-separating components balance each other out in net charge (144).

4.1.3. Cross-interaction-driven cophase separation. Cross-interaction-driven phase separation is the phase separation of two mutually attractive species, which are each incapable of phase separating in the absence of the other. The phase diagram of such a system will not intersect either component axis, since neither pure component may phase separate. The coexistence region occurs in the middle, and it always has positively sloped tie lines to indicate cooperativity between the components (**Figure 3*c***). Complex coacervation is a common example of this, where the cross-attraction and self-repulsion are driven by electrostatics (76) and may occur between oppositely charged proteins (74) or positively charged proteins with RNA (145).

Similar phase diagrams may also be achieved through the use of chains of heterospecific binding partners—such as the SRC homology 3 (SH3) domain with proline-rich motifs (3) or SUMO and SUMO interaction motifs (139)—attached by linkers. For systems with specific binding partners such as these, the phase diagram can be tuned through modifying the number of repeats of each of the interaction sites (3, 139, 146). It should be noted that due to the specificity of these interactions, the recruitment of molecules into condensates can be done with a particular stoichiometric ratio (146). Modeling studies on multivalent proteins with specific binding sites also predict a "magic number" relationship whereby saturation of the binding sites may actually reduce the phase-separation propensity by forming tightly bound oligomers that do not interact strongly with each other (70, 147).

4.1.4. Exclusive phase separation. The final two-phase system is one in which a single component is prone to phase separate and a second component is not, nor does it have attractive interactions with the first component (**Figure 3*d***). Model systems may include a phase-separation-prone protein and inert crowding agents such as ribosomes (148) or PEG (131, 132). In many of these cases, increasing concentration of the inert crowding agent allows the protein to phase separate at a lower c_{sat}, likely due to excluded volume (132). The phase diagram of such systems shows negatively sloped tie lines, indicating that one component is excluded from the other. In the case shown, component A may phase separate in the absence of component B, but it will form condensates at lower concentration as the concentration of the second component is increased (**Figure 3*d***). However, one must also consider the possibility that even inert crowders may have some level of interaction with proteins and may be incorporated into the condensates at low concentrations. This may have effects on the condensate itself, such as altering its material properties (132).

4.2. Multiple Condensed Phases

In addition to systems that segregate into two coexisting phases, there are also cases involving more complex phase behaviors that have been observed in biology. Interestingly, some MLOs may be characterized as multilayered core-shell assemblies containing condensates within larger condensates, such as the P granule, which is characterized by a gel-like phase rich in the disordered MEG-3 protein, surrounded by a liquid-like phase rich in the PGL-3 protein (128); the related Z granule (149); and the nucleolus, which consists of three distinct layered compartments (150). Biophysical studies of the nucleolus have suggested the cause for the core-shell structure as differences in surface tension between the droplets of different components, with the component having the highest surface tension at the center (150). This type of architecture would be most likely to occur when both components have strong self-interaction strength and significant, yet weaker, cross-interaction strength. Strong self-interactions and weaker cross-interactions could also result in the formation of two distinct types of droplets that do not mix or fuse, which can also be represented using two-component phase diagrams with regions of two-phase and three-phase coexistence (109). Recent studies have also suggested the possibility that some MLOs may form concentration gradients between components (151).

4.3. Phase Separation with Many Components

Studying two components forming distinct phases can be useful for characterizing the different relationships between phase-separating proteins and nucleic acids. However, most MLOs contain dozens or hundreds of different components, making in vivo condensates drastically different from in vitro reconstituted droplets or simplified theoretical and computational models. However, useful information may arise from considering the relative interaction strengths of each component

with itself and all other components, for example, through an interaction matrix (141, 150, 152). Jacobs & Frenkel (141) undertook the challenge of reducing systems with many different components to a couple of parameters, looking at the effects of the number of components and the interactions between the components. They find that in general, systems with many components having a large distribution of interaction strengths tend toward demixing of fewer components, while systems with more homogeneous interaction strengths result in a condensation phase transition, with most of the components being incorporated into the condensed phase. As the number of components increases, systems tend toward forming a single condensed phase with the many components.

SUMMARY POINTS

1. Both folded and unfolded domains are important to phase separation and function.
2. Liquid-liquid phase separation (LLPS) is driven by weak, multivalent interactions of different types.
3. The roles of disordered regions, folded regions, and different interaction modes are highly context specific.
4. Different interaction modes respond to stimuli in different ways, making biomolecular phase separation highly tunable.
5. Multicomponent systems can display multiple cophase separation scenarios, depending on protein/RNA composition and sequence, and can be tuned by external stimuli.
6. Molecular simulations are a powerful tool for relating the molecular properties of the components undergoing LLPS to the phase separation.

FUTURE ISSUES

1. How applicable is the study of equilibrium phase behavior to the highly nonequilibrium processes occurring in biology?
2. Can the responses to different stimuli be tuned independently within a sequence?
3. Can robust selectivity be achieved by designing proteins with different interaction modes driving phase separation?
4. Can two-body interaction strengths be used to infer entire two-component phase diagrams?
5. Can the interaction matrix between a large library of components predict the concentration-dependent phase separation, or are higher-order terms necessary?

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