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Phase separation: Bridging polymer physics and biology

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Abstract

Significant parallels exist between the phase separation behavior of polymers in solution and the types of biomolecular condensates, or 'membraneless organelles,' that are of increasing interest in living systems. Liquid–liquid phase separation allows for compartmentalization and the sequestration of materials and can be harnessed as a sensitive strategy for responding to small changes in the environment. Here, I review many of the parallels and synergies between ongoing efforts to study and take advantage of phase separation in living versus synthetic materials.

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Introduction

The scientific community has long looked to nature for inspiration. This is particularly true in the case of self-assembling materials, where living cells have developed extremely complex systems that achieve exquisite levels of control over chemical reactions, signaling, and data transfer based purely on the complex interactions between molecules. In looking to understand and learn from these systems, there is a natural tension between more reductionist, physics-based approaches that look to distill the inherent complexity of a living system down to an elegant set of fundamentals and biochemical approaches that work to explore the network from within.

Over the last decade, there has been an explosion of research demonstrating the importance of self-assembling, liquid phase–separated domains in both eukaryotic cells [1–8] and bacteria [9,10]. These droplets of RNA and protein, commonly referred to as 'membraneless organelles,' take advantage of liquid–liquid phase separation to achieve intracellular compartmentalization while enabling rapid exchange of their contents with the surroundings. Tremendous work has been carried out to identify the biological components that drive the formation of these biomolecular condensates [11], and ongoing efforts are exploring their functional role in the cell.

At the same time, there have been large-scale efforts in the materials science community focused on studying liquid–liquid phase separation of polymers in water. This work includes investigations of various aqueous two-phase systems, such as the segregative phase separation observed for systems of poly(ethylene glycol)/dextran [12], polymers that undergo either a lower critical solution temperature (LCST) transition or an upper critical solution temperature (UCST) transition based on interactions with water [8], such as elastin-like polypeptides (ELPs) [13,14], and complex coacervation as a form of associative phase separation involving multiple species [15–20] or via self-coacervation of polyampholytes [21–24]. Various aspects of these phase separating systems have relevance to the formation and properties of membraneless organelles. In particular, the formation of these condensates is most closely associated with the types of molecular interactions implicated in complex coacervation and LCST/UCST behavior [5,6,8,25,26]. Here, I will discuss parallels, challenges, and potential synergies associated with extending the results of elegant, reductionist studies of simplified materials systems to the levels of molecular, chemical, and conformational diversity present in living systems.

The benefits of phase separation

In the context of a complex network of reactions, compartmentalization allows for the segregation of incompatible processes as well as providing a means for imposing spatiotemporal control over the system. The critical feature of any compartment is the defining interface. Although cells achieve some of this organization through the use of more traditional membrane-bound organelles, the benefits of such a robust and

relatively impermeable boundary can also be a weakness, as the flow of material across a lipid bilayer can require energy-consuming transport mechanisms and specialized machinery.

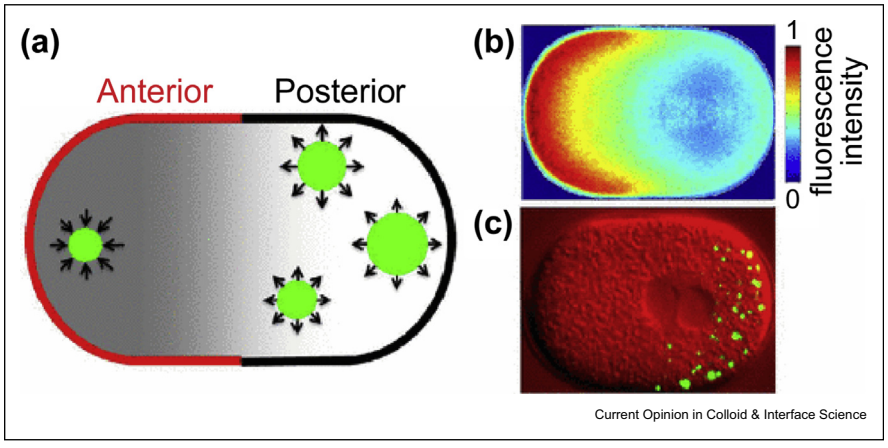
In contrast, liquid–liquid phase separation allows for the creation of an interface that does not inhibit the easy transport of materials into and out of the compartment. Diffusion occurs along the direction of a gradient in chemical potential. Typically, this means that diffusion in solution occurs from areas of high to low concentration. However, at an interface between two equilibrium phases, such as those present in a liquid–liquid phase separated system, the difference in the chemical potential is zero. Simultaneously, the materials that define the two phases might have different affinities for the solute molecules. As a consequence, liquid–liquid phase–separated materials can facilitate and maintain a step-jump in the concentration of a given species without limiting transport across the interface. This natural partitioning of materials can then be used to locally generate high concentrations of reactants or spatially colocalize a cascade of reactions for efficient chemistry. A detailed discussion of diffusive behavior across interfaces in the context of liquid–liquid phase separation in biology is given in a recent review by Hyman *et al.* [3].

Life exists near tipping points

In addition to the benefits of an existing compartment, the formation and dissolution of liquid–liquid phase separated, or otherwise reversibly aggregated, materials can be triggered by small variations in concentration and/or solution conditions [26]. Living systems have harnessed this kind of tunability, using liquid–liquid phase separation to respond to environmental and/or metabolic stresses and sequester enzymes and potentially RNA components related to the affected pathway [3,25,27–29]. In one example, all six enzymes related to purine biosynthesis in HeLa cells were shown to cluster into punctate 'purinosomes' when cells were grown in purine-depleted media but were spread diffusely through the cytoplasm in the absence of the metabolic stress [30–33]. It has been hypothesized that this ability of the cell to cluster closely related metabolic enzymes could provide an efficient means for regulating the flux of product species in response to variations in environmental conditions. Thus, when clustered, more efficient biosynthesis can take place along the multienzyme cascade with the low levels of material that are present in the cell, whereas the dissolution of the clusters slows production when levels are higher. However, the exact mechanisms whereby the cell triggers purinosome formation remain under investigation.

In a more dynamic example, a recent work by Brangwynne and coworkers [34] demonstrated how weak gradients in the concentration of a protein Mex-5 are used to regulate position-dependent formation of P granules to achieve asymmetric cell division (Figure 1). Furthermore, experimental evidence indicates that this phase separation takes advantage of many weak, multivalent interactions to facilitate the formation of P granules while maintaining a low overall concentration of protein within the droplet [35]. These results are particularly exciting because they highlight how the cell is able to achieve dramatic structural rearrangements in a near-passive way, with minimal energy and material requirements.

Figure 1



(a) Schematic representation of the polarized, one-cell stage *Caenorhabditis elegans* embryo prior to asymmetric cell division. Cell polarity is established by asymmetric localization of proteins in the anterior (red) and posterior (black) cell membranes. This asymmetry is required to generate a cytoplasmic gradient of Mex-5 protein (gray). P granules (green) segregate to the posterior side where they grow, whereas they disappear in the anterior side. **(b)** False color representation of fluorescence intensity indicating the gradient of Mex-5 in the cell. **(c)** Composite micrograph highlighting the presence of fluorescently labeled P granules localized to the posterior of the *C. elegans* embryo. Reprinted figure with permission from Ref. [34] [Lee, Brangwynne, Gharakhani, Hyman, Julicher. *Phys Rev Lett.* 2013, **111**:088101] Copyright 2013 by the American Physical Society.

In contrast to the ways in which cells are able to respond to very subtle environmental cues, the majority of synthetic phase-separating systems require a more dramatic response. This is not to say that biopolymers somehow possess different types of interactions than those synthesized in the laboratory. However, the inherent polydispersity in length and/or the lack of control over the sequential presentation of chemistry means that the phase behavior and material properties of synthetic polymers are the averaged result of a more diverse population of molecules than in many living tissues.

Interestingly, while there are an increasing number of examples where cells use liquid–liquid phase separation in response to a specific stimulus, compartmentalization also provides a way for the cell to passively filter noise and avoid responding to small variations in a particular signal [36]. Thus, compartmentalization is key in both identifying the important stimuli and then acting upon them. However, the ability of either living or biomimetic synthetic systems to distinguish between and then take advantage of varying low-level signals represents a challenge for future experiments.

Crowding and entropy versus 'soft interactions' and enthalpy

The intracellular environment, regardless of compartmentalization, is extremely crowded (~20–40% protein by mass) [37–39]. Although the concentration of an individual species might not be high, the total concentration of biomacromolecules is enough to decrease entropy because of crowding and volume exclusion. The study of entropic effects has focused mainly on reactions, or equilibria, where conformational changes occur, as crowding favors smaller, more compact states [37,39–65]. For instance, crowding increases the renaturation rate of DNA by 1–2 orders of magnitude, drives the association of 70S ribosomes from 30S and 50S particles, favors actin filament growth, and has been used to explain the function of molecular chaperones [40,49,64,66]. In addition, Pielak and coworkers [67] recently performed elegant experiments demonstrating that the shape of a protein can determine whether a crowded environment is stabilizing or destabilizing using two different dimeric variants of the B1 domain of protein G.

While the potential importance of crowding is very intuitive, there are also many cases where crowding has not produced the expected result. For instance, despite favoring the more compact, folded conformation of a protein, high levels of crowding were insufficient to drive folding of a variant of protein L [68]. Data from in-cell nuclear magnetic resonance spectroscopy (NMR) experiments demonstrated that enthalpic protein–protein interactions helped to stabilize the unfolded form of protein L against the entropic effects of crowding. In a landmark article, McGuffee and Elcock used Brownian dynamics simulations of a molecular model of the *Escherichia coli* cytoplasm to demonstrate that macromolecular crowding alone cannot account for the observed diffusive behavior of proteins *in vivo* and the subtle electrostatic and hydrophobic interactions between protein molecules must be taken into account [69].

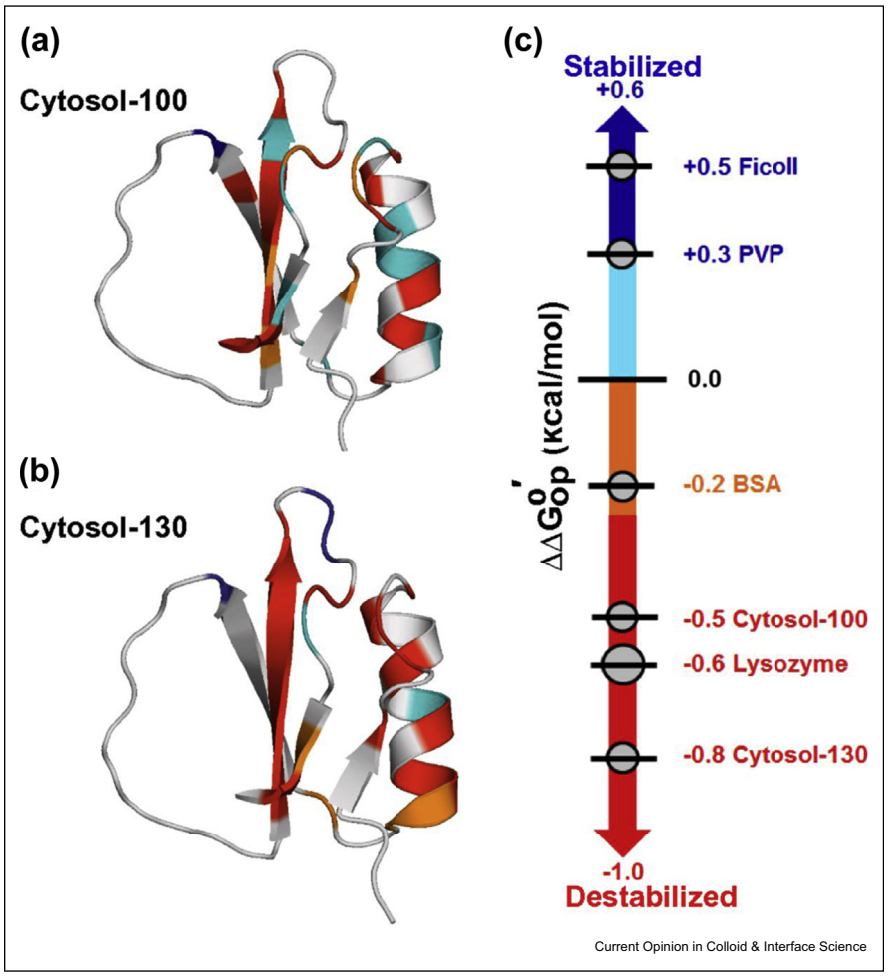
While a number of excellent studies have taken advantage of *in situ* measurements such as in-cell NMR to study aspects of biomolecules in their native environment [33,71], one of the most significant limitations associated with efforts to study the effect of the material environment on the stability, activity, and/or structure of a given protein or other biomolecule is the challenge of reconstituting the complexity of the cellular microenvironment in solution. Commonly used crowding agents, such as polyethylene glycol (PEG), dextran, and Ficoll, as well as related aqueous two-phase system strategies [12,72], are accessible but do not recapitulate either the chemical composition of the cell interior or the specific interactions that are at play and can potentially give misleading results [73,74]. In a dramatic example, Pielak and coworkers used proton-exchange NMR experiments to quantify changes in the stability of chymotrypsin inhibitor 2 in the presence of crowding agents ranging from polymers to purified proteins to reconstituted cytosol. Stability, quantified as a relative change in the free energy required to expose amide protons to solvent ($\Delta\Delta G^{o}_{op}$), varied dramatically over a range of +0.5 kcal/mol to −0.8 kcal/mol (it should be noted that the overall stability of the protein is greater than 6.0 kcal/mol) [75], with minimally interacting crowding agents such as Ficoll providing stabilization, whereas the reconstituted cytosolic environment proved to be destabilizing (Figure 2) [70,71,75].

Liquid–liquid phase separation: segregation, purification, and stabilization

While the use of in-cell studies and reconstituted cytosol have utility in the context of biophysical experimentation, proteins are involved in an increasing number of real-world applications that could benefit from bioinspired stabilization strategies. For instance, NMR-based studies have shown that charge–charge interactions between a protein and a crowding molecule can be stabilizing if they are repulsive (*i.e.*, effectively increasing the excluded volume). In contrast, attractive charge-based or hydrogen bonding interactions tend to be destabilizing because unfolding of the protein tends to expose more surface to interact with [71,76]. These results raise the question of whether tailored materials that undergo liquid–liquid phase separation could be used as a purification strategy and/or to create formulations with enhanced stability and/or activity.

In terms of purification, ELPs have been used as a purification tag on recombinantly expressed proteins using

Figure 2



Impact of different crowding agents on the stability of chymotrypsin inhibitor 2 (CI2). The backbone of CI2 colored based on the relative change in free energy ($\Delta\Delta G^{o'}_{op}$) in the presence of **(a)** 100.0 g/L and **(b)** 130.0 g/L dry weight reconstituted cytosol. **(c)** Changes in the average global stability of CI2 in the presence of a range of different crowding materials. The colors in **(a)** and **(b)** correspond to the color scale shown in **(c)**. Figure adapted with permission from Ref. [70] Sarkar, Smith, and Pielak *Proc. Natl. Acad. Sci. U.S.A.* 2013, **110**:19342–19347.

a protocol termed the inverse transition cycle (ITC) [13,77,78]. ELPs are artificial polypeptides, derived from the pentapeptide repeats (VPGXG) found in human tropoelastin, where X can be any amino acid other than proline [13]. ELPs typically undergo a reversible phase transition when heated above a critical temperature (*i.e.*, LCST behavior), which can be tuned through judicious choice of the variable amino acid X, the length of the peptide, and the ionic strength of the solution. In this way, cycles of salt concentration and/or temperature have been shown to produce equivalent protein purity as immobilized metal ion affinity chromatographic methods, but in less time and with fewer equipment requirements [78].

Dubin and coworkers have also demonstrated the utility of direct coacervation for protein purification [79–82]. In fact, the charge–charge interactions that drive coacervation were sufficient to enable separation of two isoforms of β-lactoglobulin that differ only by two amino acids [81]. A recent work carried out in our laboratory has highlighted the potential for using polymers with patterns of charge to enhance the uptake of specific proteins into the coacervate phase [83]. While the use of complex coacervation requires the addition of additional polymers to facilitate phase separation, judicious polymer selection should facilitate straightforward downstream purification, if needed. However, for applications such as drug delivery, sensing, or biocatalysis, the associating polymer could potentially be used in the subsequent formulation or device fabrication.

While most of the reports on the complex coacervation of proteins take advantage of the charge nature of the protein itself, Kapelner and Obermeyer [84] recently reported a strategy that takes advantage of

recombinantly expressed short ionic peptide tags (6–18 amino acids) to facilitate the phase separation of tagged proteins via complex coacervation. Of particular note was the result that proteins with the same total amount of charge distributed isotropically on the protein surface showed lower stability against dissolution by salt than proteins where coacervation with a strong polyelectrolyte was driven by the presence of an ionic tag. These results suggest a range of interesting potential applications, from protein purification to the colocalization of enzymes and more.

Complex coacervation has also been used to enhance the stability, and potentially the activity, of an incorporated protein. In fact, many examples have taken advantage of block copolymer architecture to facilitate the uptake of proteins into nanometer-scale coacervate-core micelles. Examples include the stabilization of the fluorescent protein mCherry [85], improvements in the activity of encapsulated lipase [86], and stabilization of an organophosphate hydrolase for use in the enzymatic decomposition of nerve agents [87]. The strategy associated with incorporating organophosphate hydrolase into a micellar coacervate structure is particularly noteworthy because of the need to perform the reaction in the presence of both water and an organic solvent. The phase-separated nature of the coacervate core protected the protein against denaturation, while simultaneously maintaining sufficient water in the coacervate matrix to facilitate the decontamination reaction.

Although it is easy to draw parallels between the use of liquid–liquid phase separation in the abovementioned examples and the types of biomolecular condensates observed in cells, one important difference is the sequence specificity of the 'polymers' present in living systems. Given the sheer diversity of folded protein structure and surface chemistry, the idea of designing a specific interaction can be daunting. However, in a very exciting report, Xu and coworkers [88] demonstrated that control over the statistical monomer distribution in a random heteropolymers allowed for creation of a chaperone-like polymer shell that improved protein stability in both water and organic solvents. The statistical design of the heteropolymers was informed by analysis of the size and spacing between clusters of residues with the same properties (*i.e.*, positive charge, negative charge, hydrophobic, and neutral hydrophilic) (Figure 3). In fact, the ability of these random heteropolymers to flexibly adapt to stabilize the folded state of a protein enabled the cell-free synthesis and proper folding of the membrane protein aquaporin at levels an order of magnitude higher than with traditional liposome-based technologies. The elegance of this approach comes from the way in which it took inspiration from biology, while taking advantage of the flexibility and scalability of traditional polymer synthesis.

Liquid–liquid versus liquid–solid phase separation

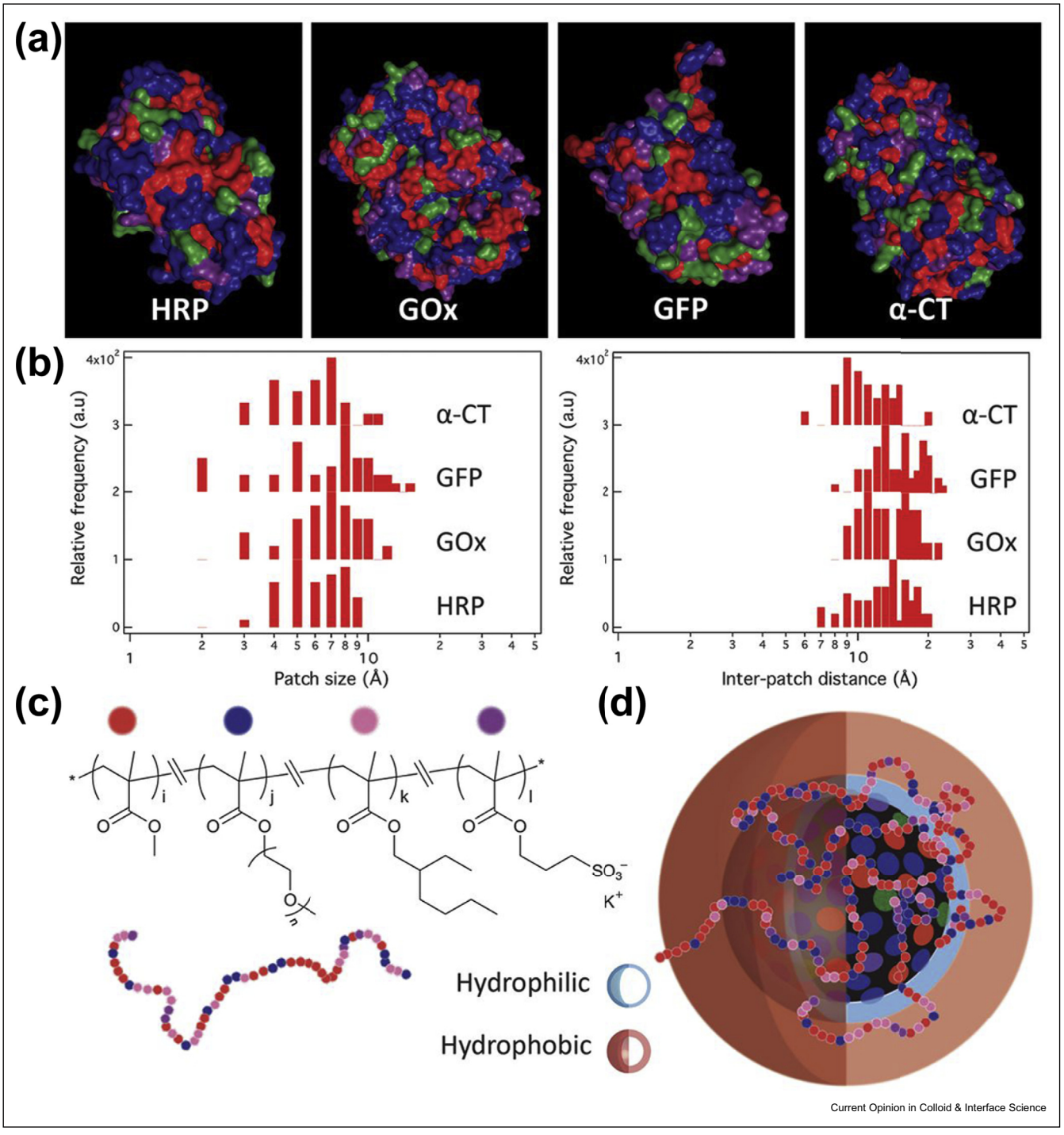
In addition to a wide variety of liquid–liquid phase separated materials in cells, there is also evidence that self-assembling biomolecular condensates can form solids [6,29,89–91]. This range of behaviors allows for the presentation of a spectrum of properties. For instance, one current hypothesis is that liquid-like condensates serve to facilitate reactions by concentrating enzymes and substrates, whereas solid-like granules can more significantly sequester enzymes away from their targets [6,29] and/or potentially serve as a filter to prevent the passage of certain molecules, as in the case of the nuclear pore complex [92,93]. However, some condensed liquid states have shown metastability and will age over time to form an aggregated, amyloid-like, or even crystalline state [90,94–97]. Thus, although the formation of more solid-like structures can drive cell function, it has been proposed that misregulation of the pathways associated with these materials may be relevant to neurodegenerative and other protein aggregation-related diseases [4,29,35,89,90,97–99].

The formation of liquid versus solid condensates is typically linked to the strength of the interactions that drive the phase change. For instance, in simple peptide systems that undergo complex coacervation, such as mixtures of poly(lysine) and poly(glutamate), electrostatic interactions alone drive liquid–liquid phase separation. However, if hydrogen bonding interactions between the peptide backbones are also allowed to occur, the resulting increase in interaction between the peptides leads to the exclusion of solvent and the formation of solid precipitates with a β-sheet structure [100–105]. By the same token, the synthetic polymers poly(styrene sulfonate) and poly(-diallyldimethylammonium chloride) form solid precipitates at low ionic strength. However, increasing the salt concentration helps to weaken the interpolymer interactions and allows for the formation of a liquid coacervate phase [106,107]. In both of these systems, the transition from liquid to solid is a gradual continuum that can be effectively described as physical gelation [107]. For synthetic systems, this tunability of properties has been harnessed in a range of applications to facilitate processing of materials in the liquid state, followed by solidification [108–113].

The challenge of understanding a diversity of interactions among a diversity of components

The growing number of examples where phase separation drives biological function or could inspire an analogous materials system, strongly advocates for the need to better understand the underlying molecular interactions to either elucidate phenomena or allow for

Figure 3



Design of random heteropolymers based on protein surface pattern for protein solubilization and stabilization in organic solvents. **(a)** Space-filling protein structures for horseradish peroxidase (HRP), glucose oxidase (GOx), green fluorescent protein (GFP), and α-chymotrypsin (α-CT) showing the chemical heterogeneity of the surface. Colors indicate different chemistries: neutral hydrophilic, blue; hydrophobic, red; positively charged, green; negatively charged, purple. **(b)** The histograms of the diameter and the interpatch distance for hydrophobic groups. **(c)** Designed random heteropolymer with statistical distribution of monomers with varied hydrophobicity matching that of the protein. **(d)** The random heteropolymer can coassemble with protein (schematically shown as a patchy particle) in organic media and adjust its local conformation to maximize protein-heteropolymer interactions without denaturing the protein's local structure. Figure adapted from Ref. [88] Panganiban , Qiao, Jiang, DelRe, Obadia, Nguyen, Smith, Hall, Sit, Crosby, Dennis, Drockenmuller, de la Cruz, and Xu, *Science* 2018, **359**:1239–1243. Reprinted with permission from AAAS.

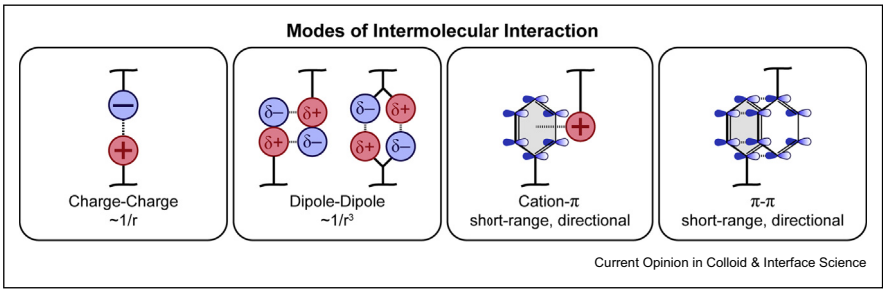
their tailored design. However, one of the most significant challenges in looking to nature for inspiration is the sheer diversity of molecular level interactions and the number of components that are in play. The intrinsically disordered proteins associated with phase separating domains in cells are typically low-complexity sequences, characterized by long stretches with a low overall diversity of amino acids. These proteins often involve repetitive sequences that are enriched in polar amino acids such as glycine (G), glutamine (Q), asparagine (N), and serine (S); positively charged amino acids arginine (R) and lysine (K); negatively charged aspartic acid (D) and glutamic acid (E); or aromatic phenylalanine (F) and tyrosine (Y) [5,114]. Still, even in the limit if only this subset of amino acids was present, there exists an incredibly diverse array of interactions and combinatorial arrangements of chemistry that can drive the behavior of these materials.

The types of interactions that have been implicated in the self-assembly and phase separation of membraneless organelles in cells range from long-range electrostatic effects to shorter-range dipolar contacts to short-range and directional cation−π and π−π interactions (Figure 4) [5,8,27,29,97,115−117]. From a modeling perspective, short−range interactions can often be treated at the phenomenological level through the use of an 'effective χ', as is common in polymer physics [118]. However, difficulties still exist in linking the details of a specific chemistry to the value of χ because of the complicated interplay between molecular packing and quantum effects. In contrast, electrostatic interactions are long-range and often compete with molecular interactions at shorter-length scales [18,119]. Furthermore, intuition regarding electrostatics is typically based on theories that are not valid at relevant salt concentrations and may not be valid for polymers. The result is that the competition between steric, electrostatic, short-range, and entropic considerations are challenging to describe both theoretically and conceptually [18].

One recent example that has achieved a level of success in combing theory and experiment used extensive mutagenesis on the Fused in Sarcoma (FUS) family of proteins to demonstrate that phase separation for these proteins is primarily governed by multivalent cation−π interactions, specifically between tyrosine and arginine residues [97]. The authors then use the theory of associative polymers to interpret their results [120,121]. This theory predicts that phase behavior is dictated through the interaction of associative 'stickers' that are separated from one another along a polymer chain by 'spacers.' As shown in both experiments and theory, the number of stickers present in the system scales inversely with the saturation concentration of phase separation but is not impacted by the flexibility of the spacer groups. Instead, the authors demonstrated that switching out highly flexible glycine residues in the spacer regions for alanine, glutamine, or serine decreased the fluidity of the resulting condensates. Despite the elegance of this explanation, the authors acknowledge that they cannot rule out effects, such as increases in hydrophobicity, also affecting the properties of their materials.

This difficulty in describing the competition between the various modes and length-scales of interactions is further exacerbated by the need to validate theoretical and computational approaches via comparison with experimental data, which can be similarly difficult to obtain and/or interpret because of the diversity of interactions, conformational states, and species present. Fortunately, a combination of experimental efforts using model systems, as well as the development of new analytical techniques, is helping to address this issue. For instance, a combination of experimental and computational approaches was recently used to highlight the importance of entropic effects in driving the complex coacervation of polypeptides with different patterns of charge [122]. In particular, this insight into the entropic nature of complex coacervation highlights the importance of sequence effects, rather than overall composition, and provides intuition regarding the design of such systems. These results were supported by mutagenesis studies of the nephrin intracellular domain (NICD), which highlighted a hierarchy of interactions that drive phase separation [117]. Complex coacervation

Figure 4



Molecular interactions underlying intracellular phase transitions.

with a positively charged partner was necessary to weaken electrostatic repulsion, and the dense phase was further stabilized by shorter-range interactions involving aromatic and hydrophobic residues. Most interestingly, the authors demonstrated that this hierarchy of interactions meant that the distribution of charged amino acids had a significant effect on phase separation, while the sequence of more hydrophobic residues was not as critical.

The overall architecture of the molecules can also have important consequences on phase behavior. A series of reports have investigated how the differences in the flexibility and charge density of single- versus double-stranded DNA affects its ability to undergo liquid–liquid phase separation (*i.e.*, complex coacervation) versus liquid–solid precipitation [123,124]. Similarly, the secondary structure of mRNA has been shown to dramatically affect recruitment into a phase-separated domain [125]. Branching or a comb polymer architecture has also been shown to have effects similar to those of charge patterning [105].

One of the biggest challenges associated with the characterization of both model synthetic and biological systems is the quantity of material required. In a very exciting development, the Brangwynne laboratory recently reported the use of a novel method based on fluorescence correlation spectroscopy and confocal microscopy to infer information on species concentration (*i.e.*, phase behavior) and the material properties (*e.g.*, diffusivity and second virial coefficients) [35]. This development has the potential to enable the collection of phase diagram information on a much wider range of materials than had been previously accessible.

Conclusion/outlook

There is a growing wealth of knowledge associated with how unstructured proteins and/or polymers can take advantage of many weak, multivalent interactions to drive complexation, phase separation, and/or other functional behaviors. Fundamental efforts focused on understanding the full palette of molecular interactions available are ongoing, as are biochemical and biophysical studies focused on illuminating specific biological phenomena. In particular, the ways in which cells take advantage of the complexity of intracellular phase behavior to affect the flow of information and chemicals represent a tremendous opportunity for collaborative efforts between fundamental and applied scientists to develop biomimetic 'smart' materials.

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Conflict of interest statement

Nothing declared.

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