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# Molecular determinants of protein-based coacervates

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**Abstract**

Protein–polyelectrolyte coacervates have gained interest for their potential to stabilize proteins or function as adhesives and their biological implications in the formation of membraneless organelles. To effectively design these materials or predict their biological formation, knowledge of the macromolecular properties that dictate phase separation is required. This review highlights recent advances in the understanding of molecular determinants of protein–polyelectrolyte phase behavior. Properties that promote the phase separation of protein–polyelectrolyte pairs are covered from the perspective of synthetic systems and simplified biological condensates. Prominent factors that determine coacervate formation and material properties include nonspecific intermolecular interactions, as well as specific biological interactions and structures. Here, we summarize the essential roles of electrostatics, including charge magnitude and distribution, (bio) polymer chemistry and structure, and post-translational modifications to protein phase separation in both a synthetic and cellular context.

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## Introduction

The first scientific report of complex coacervation described the liquid–liquid phase separation of biomacromolecules — the anionic protein gelatin and the cationic polysaccharide gum arabic [1]. In the intervening century, our understanding of protein–polyelectrolyte coacervation has improved through studies of varying combinations of synthetic polymers [2,3], polypeptides [4–8], and proteins [9–13]. In addition to experimental characterization of coacervates, theoretical descriptions, initiated by Overbeek and Voorn [14] in 1957, have elucidated underlying thermodynamic principles governing complex coacervation. While initial experimental and theoretical efforts highlighted the primary role of electrostatic interactions in driving phase separation, more recent efforts have demonstrated that hydrophobic interactions and hydrogen bonding also play important roles [15,16]. As our understanding of the molecular determinants of protein complex coacervation has advanced, so has the ability to design these materials for a range of applications. For example, these advances have made it possible to use protein complex coacervates in a variety of applications including food science [17–19] and protein drug delivery [20,21].

Moreover, in the last decade protein complex coacervation has also been found to be one of the underlying driving forces for membraneless subcellular compartment formation [22,23]. These phase separated condensates form via weak, multivalent interactions between biomacromolecules. A subset of these organelles is formed through associative phase separation of multiple biopolymers, such as proteins and nucleic acids. These biomolecular condensates share many properties with complex coacervates composed of synthetic polymers. For example, many protein domains that promote phase separation *in vivo* are intrinsically disordered and have low sequence complexity. The intrinsically disordered regions (IDRs) behave similarly to synthetic polymers than globular proteins. Several studies have probed the molecular interactions between IDRs and other biomacromolecules to understand the driving forces of intracellular phase separation. These studies have evaluated phase separation in simplified *in vitro* systems that mimic the condensate environment and have validated these *in vitro* findings in living cells.

Recent efforts have revealed the role of molecular features, both electrostatic and nonelectrostatic, that contribute to the formation and stability of protein–polyelectrolyte coacervates. Complex coacervation is defined by, and primarily driven by, electrostatic interactions between oppositely charged polyelectrolytes [24]. Additional molecular interactions such as hydrogen bonding, π-π interactions, specific biological interactions, and hydrophobic effects can contribute to,

further enhance, or otherwise alter phase behavior. Here, we provide an overview of our current understanding of protein and (bio)polymer properties that inform molecular design parameters for protein coacervation. We begin by summarizing recent *in vitro* studies of proteins and synthetic polyelectrolytes followed by discussion of cellular coacervates formed from proteins and charged biopolymers. These investigations have established molecular-level features that influence coacervate formation and material properties.

## Synthetic protein-based coacervates

Protein-based coacervates, unlike polymer coacervates, require at least one protein as a polyelectrolyte partner. Consequently, a key protein design parameter for protein coacervates is the protein net charge. This can be calculated using the pKa of the amino acid monomers that make up the protein. Protein modeling software, such as APBS and PROPKA, further refines this calculation by accounting for the protein structure and solvent-exposed surface of the protein [11,25,26]. Experimentally, zeta potential measurements and isoelectric focusing can be used as indicators of protein net charge [25,27]. However, the molecular specificity of proteins also enables biological interactions to play a significant role in coacervate formation. In addition to potential applications of protein-based coacervates, proteins provide a powerful scaffold for studying complex coacervate design criteria as they allow for control of both the monomer sequence and the three-dimensional arrangement. We review how protein charge and charge patchiness, as well as specific biological interactions, contribute to phase separation with polyelectrolytes. While protein properties play a key role in protein−polyelectrolyte coacervates, the polymer component has an equal influence on the formation and properties of these coacervates. As the chemical functionality found in synthetic polymers exceeds that of the 20 amino acid monomers that make up proteins, additional intermolecular interactions of the polymer can contribute to phase separation. This chemical diversity coupled with the opportunity for sequence definition makes synthetic macromolecules a valuable tool kit for studying coacervates. Here, we present key features of polyelectrolytes in determining complexation and phase separation with proteins. We additionally cover recent findings using synthetic polypeptides as these biomacromolecules bridge the gap between synthetic polymers and biological proteins and provide important insights into the molecular determinants of phase separation.

### Protein properties that influence phase separation

#### *Electrostatic interactions and charge patterning*

Given the importance of long-range electrostatic interactions to coacervate formation, one of the most studied protein design characteristics is that of surface charge and surface charge distribution. As a primary example, genetically engineered cationic green fluorescent proteins (GFPs) with varying net charge were used to establish molecular properties of both proteins and polyanions that govern globular protein phase separation [26]. As expected, this study established a positive correlation between increasing protein charge and phase separation. While phase separation was largely driven by protein net charge, the critical salt concentration was also dependent on the chemical structure of the corresponding polyanion. This study also demonstrated that the nature of the second phase (liquid, viscoelastic gel, or solid precipitate) depends not only on the protein charge but also on the polyanion properties and the mixing ratios of proteins and polymers.

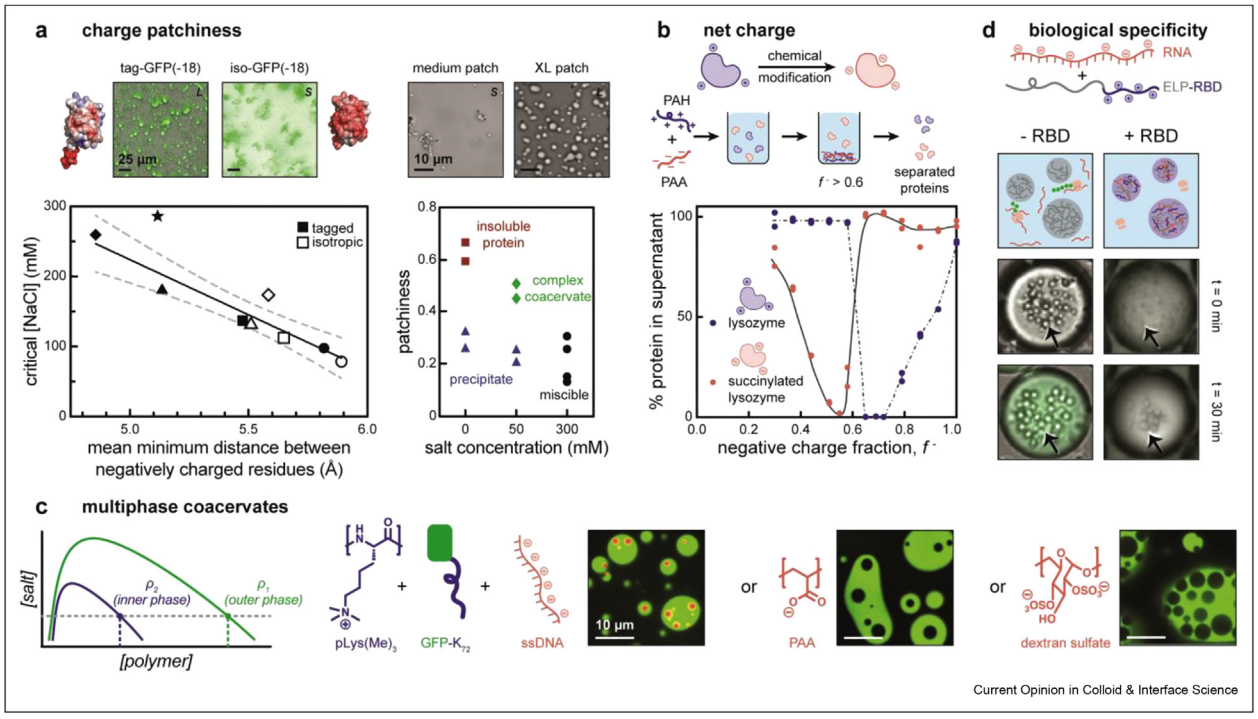
Protein supercharging and charge 'patchiness' were also determined to be the critical design parameters in heteroprotein coacervates. In one example, symmetrical protein oligomers were engineered using two oppositely supercharged variants of fluorescent proteins, a cationic cerulean protein with an expected charge of +31 (ceru (+31)) and an anionic GFP variant with an expected charge of −17 [28]. At low salt concentrations, the two protein phases separated, but at intermediate salt concentrations (100−300 mM NaCl), a well-defined oligomer formed. The resulting oligomer structure was predicted to consist of two stacked octamer rings. Within each ring, the model proteins were ordered by alternating charge. The two rings were oriented in a way that both hydrogen bonds and electrostatic interactions between the two rings contributed to the oligomer formation. Similarly to studies of mixed protein/polymer coacervates, the investigation of the phase separation of anionic β-lactoglobulin (BLG) with cationic rapeseed napin (NAP) and lysozyme demonstrated a key role for charge anisotropy in heteroprotein coacervation [27]. NAP and lysozyme were determined to have the same net positive charge, while NAP had a relatively patchy charge distribution. It was determined that under slightly basic conditions, BLG and lysozyme underwent solid−liquid phase separation, forming micron sized complexes. Under slightly acidic conditions, liquid−liquid phase separation was observed. This behavior was attributed to the weaker electrostatic interactions at lower pH. BLG and NAP never underwent phase separation. It was determined that the patchy nature of the charges on NAP facilitated weaker electrostatic interactions between the two proteins. Mixing BLG and NAP resulted in nanometer size structures that remained soluble.

In addition to the overall surface charge, an area of great interest is the effect of protein charge 'patchiness' on phase separation. The role of charge distribution on protein coacervation has been studied in two complementary ways using genetically engineered proteins. In the first approach, GFP was supercharged using a

polyanionic tag. It was determined that the disordered supercharged domain facilitates liquid–liquid phase separation with a strong polycation (Figure 1a) [25]. In addition, it was observed that a tagged protein of equivalent charge had a higher critical salt concentration than an isotropically charged protein. In the second approach, conducted by Kim et al. [29], a panel of proteins with the same net charge but varying degrees of charge clustering was used to study the effect of charge patchiness on phase separation (Figure 1a). This study quantified a patchiness parameter, which represents the charge correlation between neighboring sites on the protein surface, that was well correlated with the likelihood of phase separation, as well as the nature of phase separation. Both approaches found that increased 'patchiness' promotes phase separation and plays an important role in determining if the condensed phase is liquid or solid-like.

While proteins can directly phase separate with an oppositely charged polyelectrolyte, they can also partition into coacervates. These three macromolecule systems, comprising one protein and two polymers, demonstrate a similar dependence on the net protein charge and charge patchiness as the two component systems described above. For example, differentially charged lysozyme variants partitioned into coacervates formed from poly (acrylic acid) (PAA) and poly (allylamine hydrochloride) (PAH) (Figure 1b) [30]. Protein partitioning was determined by the combination of the mixing ratio of PAA and PAH (negative charge fraction, $f^-$) and the protein net charge. When either synthetic polyelectrolyte was in slight excess, the lysozyme with opposite charge was efficiently incorporated in the coacervate, while the lysozyme with the same charge was excluded. In addition to the net charge, patches of like-charge residues also influence protein partitioning into coacervates. McTigue and Perry [31] demonstrated

**Figure 1**




**Protein design characteristics that influence phase separation. (a)** (left) Phase behavior of GFP(-18) mutants with localized and isotropic charge distributions with qP4VP. Adapted from the study by Kapelner and Obermeyer [25] with permission from the Royal Society of Chemistry. (right) Phase behavior of GFP(-4) mutants with varying size charge patches with qP4VP. Adapted from the study by Kim et al. [29] with permission. Copyright (2020) American Chemical Society. **(b)** Partitioning behavior of lysozyme (blue) and succinylated lysozyme (red) with PAH and PAA as a function of the negative charge fraction, *f*-. Adapted from the study by van Lente et al. [30] with permission from the American Chemical Society. Further permissions related to the material excerpted should be directed to the ACS. **(c)** Formation of multiphase coacervates is possible if the densities of the two condensates are significantly different. Confocal microscopy of multiphase complex coacervate droplets formed by mixing (L to R) ssDNA/PLys $(Me)_3$ core coacervates with ssDNA/GFP-$K_{72}$ outer coacervates, PAA/PLys $(Me)_3$ core coacervates with PAA/GFP-$K_{72}$ outer coacervates, dextran sulfate/PLys $(Me)_3$ core coacervates with dextran sulfate/GFP-$K_{72}$ outer coacervates (AlexaFluor-647 labeled ssDNA [red channel], GFP [green channel]). Adapted from the study by Lu and Spruijt [32] with permission from American Chemical Society. Further permissions related to the material excerpted should be directed to the ACS (https://pubs.acs.org/doi/full/10.1021/jacs.9b11468). **(d)** Phase separation of the ELP ± RBD (50 μM) with *in vitro* transcription and translation components and sfGFP plasmid (20 ng $\mu L^{-1}$). Black arrow indicates liquid coacervates. Adapted from the study by Simon et al. [33] with permission from Elsevier. PAH, poly(allylamine hydrochloride); PAA, poly(acrylic acid); ssDNA, single stranded DNA; RBD, RNA-binding domain; ELP, elastin-like polypeptide; PLys $(Me)_3$, polycation trimeythylated poly-L-lysine.

that the presence of charge patches resulted in significantly higher uptake of proteins into coacervates formed from poly-L-lysine and poly-D,L-glutamate. The charge patches on hen egg white lysozyme were calculated using a radial distribution function between the charged residues on the protein. The charge patches on hen egg white lysozyme improved encapsulation efficiency over bovine serum albumin and hemoglobin as these other globular proteins had more evenly distributed charge. It was hypothesized that the charge patch enabled a strong binary electrostatic interaction between the polypeptides and the protein, while bovine serum albumin and hemoglobin relied on more indiscriminate electrostatic interactions.

In addition to fully miscible coacervates, these three component systems have the ability to form multiphase complex coacervates. One example of multiphase coacervate droplets, consisting of three coexisting immiscible layers, was implemented by Lu and Spruijt [32]. Three components from a panel of commercially available polymers including synthetic polyelectrolytes, polysaccharides, proteins, oligopeptides, and nucleotides were mixed and observed by fluorescence microscopy. The results from this study indicate that multiphase droplet formation will occur if the phases have sufficiently different densities, which can be determined easily by comparing the critical salt concentrations for each individual coacervate (Figure 1c). In a series of experiments with a cationic GFP (GFP-$K_{72}$) and the polycation trimeythylated poly-L-lysine , multiphase coacervates were formed with many different polyanions: single stranded DNA, PAA, and dextran sulfate (Figure 1c). In all three examples, the inner droplet consists of polycation trimeythylated poly-L-lysine and the polyanion, while the outer droplet consists of GFP-$K_{72}$ and the polyanion. Although the polyanion is present in both condensed phases, it is found at a higher concentration in the denser, core coacervate. These multiphase complex coacervates were also experimentally described by Mountain and Keating [34]. Similarly, using a panel of synthetic polymers and polypeptides, it was found that a critical determinant of the formation of multiphase coacervates was that all potential pairs underwent liquid−liquid phase separation at the given condition, particularly at the given ionic strength. In this work, however, a triple coacervate system was developed using six polyelectrolytes: PAH, protamine, an arginine-rich peptide (2xRRASL), PAA, poly (uridylic acid), and poly-L-glutamate (Glu100). By controlling the order of addition, the likelihood of solid−liquid phase separation was reduced and a three layered coacervate with a PAA/PAH core, a predominantly protamine/Glu100 middle phase, and a predominantly 2xRRASL/poly(uridylic acid) outer phase was created.

### *Biological interactions*

While most work has focused on the influence of protein−polyelectrolyte electrostatic interactions, one recent study investigated the impact of the biological interactions between an RNA-binding domain (RBD) and RNA on the temperature-dependent phase separation of an engineered intrinsically disordered polypeptide (Figure 1d) [33]. Although the RNA binding motif is a native biological interaction domain, it still relies on ionic interactions between arginine (R) and RNA. Both the lower critical solution temperature (LCST) behavior of the engineered polypeptide and the RBD domain were necessary for associative phase separation. In this system, engineered proteins with an RBD and elastin-like polypeptide domain were able to sequester mRNA into a liquid droplet, akin to an RNP granule. In addition to exhibiting temperature-dependent phase separation, the biomimetic RNP granules were also functional. When protein translation machinery was added to the system, only the polypeptides with an RBD successfully sequestered mRNA and prevented protein translation.

## Polymer properties that influence phase separation

### *Charge patterning*

The ability to precisely control monomer sequence and polymer chain length makes polypeptides an important class of polymers that allow for the study of the relationship between polyelectrolyte monomer sequence and phase separation. A study by Chang et al. [6] capitalized on these properties to establish an entropic relationship between charge patchiness and phase separation. The role of monomer sequence was studied using two polypeptides: an anionic polyglutamate homopolymer (N = 50) and a panel of cationic copolymers comprising lysine and glycine (N = 50). The polycation charge patches ranged in size from 1 to 8 lysine residues. It was determined, both experimentally and by simulation, that there is a sequence effect from charge patterning that can be attributed to the differences in entropic confinement of condensed counterions on larger charge patches. Expansion of this work evaluated the phase behavior of nonsymmetric, charged patterning using a panel of 16 cationic polypeptides [35] (Figure 2a). From the experimental and theoretical work, they were able to develop a theoretical framework using a transfer matrix approach that accounts for the local electrostatic charge along the polyelectrolyte. From this analysis, it is suggested that both the charge blockiness of the polymer chain and the position of the charge block within the polymer influence phase behavior.

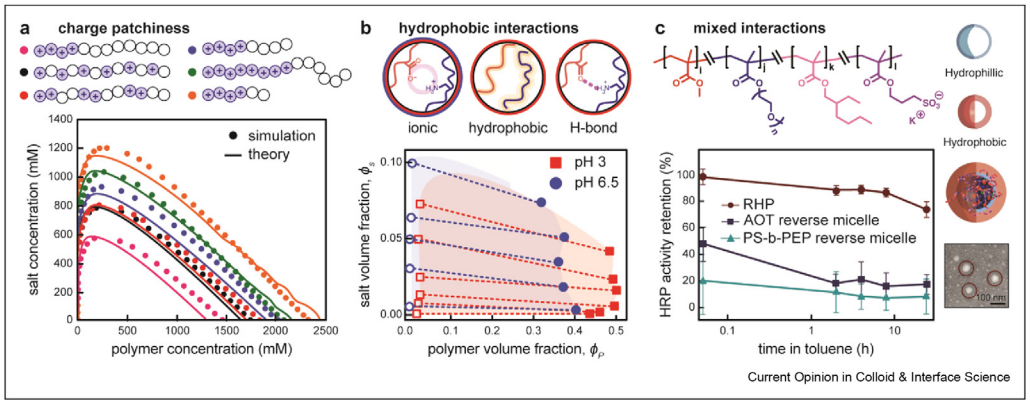
### *Hydrophobic interactions*

Recent advances in complex coacervation use synthetic polymers and polypeptides as a scaffold to better understand the phenomena. Using well-defined synthetic

polyelectrolytes enables the careful examination of polymer hydrophobicity on coacervation. A study by Li et al. [36] looked at a system containing PAA and PAH over acidic, neutral, and basic conditions (Figure 2b). Under neutral and basic pH, PAA and PAH formed precipitates at no salt and spherical droplets as the salt concentration increased, which also resulted in decreased polymer concentration in the condensed phase. Under acidic conditions, PAA and PAH formed solid precipitates where the polymer concentration initially increased with increasing salt concentration (Figure 2b graphs). This was attributed to the fact that, at acidic pH, the PAA chains were partially ionized, so both pure PAA precipitates and PAA–PAH polyelectrolyte complexes were present. The accumulation of pure PAA precipitates was attributed to backbone hydrophobicity and hydrogen bonding, and PAA–PAH complexes were thought to be the result of electrostatic interactions. A recent study from the same group confirmed the role of hydrophobic interactions in the high salt stability of complex coacervates in water. The phase behavior of PAA and PAH was compared with that of polypeptides, poly-L-lysine and poly-D,L-glutamate, with the same charged groups, a primary amine and carboxylic acid. In addition to these two extrema, the synthetic polymers were also mixed with the oppositely charged synthetic polypeptides. It was observed that the more hydrophobic polymer mixtures had a significantly higher critical salt concentration than the hydrophilic polypeptides, while the hybrid mixtures PAA–poly-L-lysine and PAH–poly-D,L-glutamate demonstrated intermediate critical salt concentrations [38]. This was attributed to poor backbone–solvent interactions in the relatively hydrophobic synthetic polymers, where short-range hydrophobic interactions stabilize the coacervates.

Synthetic polymers have also been used to simulate the phase behavior of intrinsically disordered proteins. While unable to control monomer sequence, random heteropolymers developed by de Panganiban et al. [37] had a precisely controlled monomer distribution. The study used four methacrylate-based monomers and reversible deactivation radical polymerization to synthesize random heteropolymers with a statistical distribution of monomers of varied charge and hydrophobicity to match the surface properties of the proteins studied (Figure 2c). In the presence of the optimized random homopolymer (RHP), horseradish peroxidase retained 80% of its native enzymatic activity after being exposed to toluene for 24 h (Figure 2c). Another study by Nguyen et al. [39] used shape-based coarse grain modeling and random copolymers as a proxy for intrinsically disordered proteins (IDPs) to understand how proteins are incorporated into membraneless organelles or could be stabilized in unfavorable environments. The results from these simulations indicate that random copolymers comprising a solvophilic and solvophobic monomer will selectively adsorb to the protein, suggesting that the protein self-selects specific sequences to minimize solvent exposure.

**Figure 2**

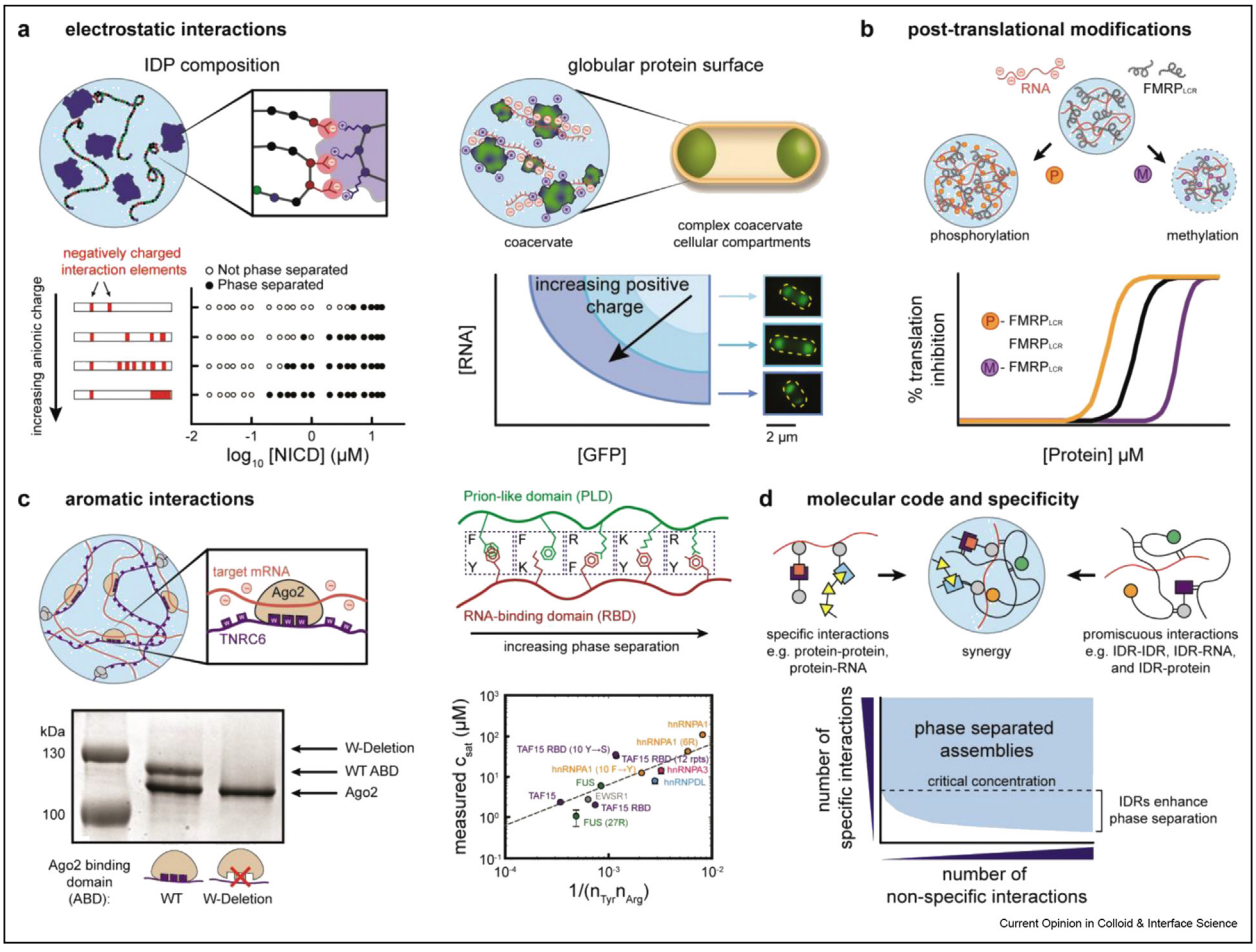



**Polymer design characteristics that influence phase separation. (a)** Phase diagram of coacervation predicted from simulation and transfer matrix theory for poly (glutamate) and poly (lysine-*co*-glycine) with the charge patterning indicated in the schematic. Adapted from the study by Lytle et al [35] with permission from the American Chemical Society. Further permissions related to the material excerpted should be directed to the ACS (https://pubs.acs.org/doi/10.1021/acscentsci.9b00087). **(b)** Phase diagram of PAA and PAH complexes prepared under acidic and neutral pH ($C_{P,0}$ = 5% w/v). The phase boundary under acidic conditions deviates from the pH neutral boundary because of hydrophobic interactions and hydrogen bonding. Adapted with permission from the study by Li et al. [36]. Copyright (2020) American Chemical Society. **(c)** Random heteropolymer (RHP) was designed with statistical distribution of hydrophobic and hydrophilic monomers to match the heterogeneous interactions on the protein surface. Representative coassembly of a protein with an RHP in organic media to form a patchy particle with maximum protein–RHP interaction. HRP activity retention in toluene encapsulated in: RHP, reverse micelles based on sodium bis(2-ethylhexyl) sulfosuccinate (AOT) or polystyrene-block-poly (ethylene oxide) (PS-b-PEO) polymeric surfactant. Representative transmission electron microscopy (TEM) micrograph of RHP/HRP particles dried from a toluene solution. Adapted from the study by Panganiban et al [37] with permission from AAAS. PAH, poly(allylamine hydrochloride); PAA, poly(acrylic acid); HRP, horseradish peroxidase.

## Intracellular protein-based coacervates

Over the past decade, protein coacervation with other biomacromolecules has been shown to be an essential mechanism for cellular organization. After the landmark discovery that liquid–liquid phase separation can form dynamic, intracellular organelles [40], more recent studies have begun to probe how molecular features impact intracellular phase separation. This has been accomplished by decreasing system complexity *in vitro* and via molecular simulations of course-grained protein phase behavior. Here, we highlight molecular features of proteins that impact associative coacervation with biopolymers and vice versa. Important protein molecular features include charged and aromatic content, post-translational modification, and amino acid composition. Recent findings demonstrate that changing the density of charged, aromatic, and hydrophobic residues on

**Figure 3**




**Protein molecular features that impact their coacervation with biopolymers. (a)** (left) Electrostatic interactions between basic and acidic residues drive the phase separation of the IDP, NICD, with globular proteins. Increased charge density can enhance coacervation. Reprinted with permission from the study by Pak et al. [41]. Copyright (2016) Elsevier. (right) Increasing the net surface charge on engineered GFPs promotes phase separation in a charge-dependent manner. *In vitro* coacervation of supercationic GFP with RNA predicts the formation of intracellular condensates in *E. coli*. Adapted with permission from the study by Yeong et al [43]. **(b)** Phosphorylation of $FMRP_{LCR}$ promotes phase separation and translation inhibition, whereas methylation of $FMRP_{LCR}$ does the opposite. Adapted with permission from the study by Tsang et al. [44]. Copyright (2019) National Academy of Sciences. **(c)** (left) Aromatic residues play important roles in protein–biopolymer coacervation between Ago2 and TNRC6B. The removal of Trp residues abolishes TNRC6B–Ago2 binding as observed in pull-down assays with the band corresponding to the Trp deletion mutant TNRC6B absent in the SDS–PAGE gel. Reproduced with permission from the study by Sheu-Gruttadauria and MacRae [45]. Copyright (2018) Elsevier. (right) Cation–π interactions drive phase separation depending on the interaction strengths determined by residue identity. Tyr–Arg interactions are the strongest promoters of phase separation. Correspondingly, higher numbers of Tyr and Arg residues in human proteins with disordered regions are correlated with lower saturation concentrations. Adapted with permission from the study by Wang et al. [13]. Copyright (2018) Elsevier. **(d)** Specific interactions dictated by residue identity and nonspecific ('promiscuous') interactions can work synergistically to enhance phase separation at regimes below the critical concentration. Adapted with permission from the study by Protter et al. [46]. Copyright (2018) Elsevier. NICD, nephrin intracellular domain; FMRP, Fragile X mental retardation protein; Ago2, Argonaute2.

proteins mediate phase separation with nucleic acids. For the corresponding biopolymers, we focus on nucleic acids and how their composition, molecular interactions, and structure influence the formation and material properties of the condensate.

## Molecular features of proteins impact their coacervation with biopolymers

### *Electrostatic interactions*

Tuning the strength of associative interactions influences biomolecular phase separation; these associative interactions can be specific biological interactions or nonspecific intermolecular interactions. While several studies have characterized modular, multivalent interactions between specific binding partners (e.g. SUMO/SIM, SH3/PRM), there is emerging support for the importance of nonspecific intermolecular interactions, such as electrostatic interactions, for intracellular protein phase separation [12]. Recent studies have explored amino acid composition and charge patterning of intrinsically disordered peptides, as well as globular protein surface charge.

Studies investigating the importance of nonspecific molecular interactions highlight the roles of charged residues in driving phase separation [41–43]. Recent work on liquid-like nuclear bodies formed from the nephrin intracellular domain (NICD) demonstrated that aromatic residues and high anionic charge density facilitate intracellular complex coacervation [41]. The NICD contains blocks of anionic charge interspersed with aromatic residues that enable phase separation with cationic biomacromolecules in the cell through nonspecific associative interactions (Figure 3a, left). The authors varied the local charge density of wild-type NICD by rearranging charged and polar residues to produce clusters of aspartic or glutamic acid residues. Phase diagrams testing various NICD concentrations indicated that while charge patterning enhances phase separation, local charge density is not necessary for phase separation; instead, total charge magnitudes (and therefore, amino acid composition) are essential drivers. Similar trends have been observed in the bacterium, *C. crescentus*, where the IDR in the C-terminal domain of RNase E contains charge patches that enable electrostatic interactions with intracellular RNA and are required for BR-body assembly [42].

In addition to examining the charge sequence of native IDPs, globular proteins have been engineered to probe how charge at the protein surface impacts phase separation. Using a panel of engineered GFPs, Yeong et al. [43] found that increased surface charge mediated phase separation with RNA under physiological conditions with higher surface charge broadening the two-phase region (Figure 3a, right). Increasing the protein surface charge was sufficient for condensate formation in bacteria, and the extent of intracellular phase separation was similarly dependent on the magnitude of cationic charge.

### *Post-translational modifications*

Beyond protein sequence and surface charge, intracellular phase separation can also be tuned by cellular signals. The resulting modifications can influence phase separation by altering intermolecular interactions (e.g. electrostatics, hydrophobicity, etc.) on the protein's surface or by mediating specific biological interactions. As a primary example, phosphorylation or methylation of Fragile X mental retardation protein (FMRP) in neurons regulates the formation of neuronal granules and inhibition of translation [44]. The C-terminal domain of FMRP contains a low complexity domain ($FMRP_{LCR}$) that is necessary and sufficient for droplet formation with RNA *in vitro*. Phosphorylation of $FMRP_{LCR}$ promotes phase separation and lowers the concentration required for translational inhibition (Fig. 3b). In contrast, arginine methylation at RGG motifs on $FMRP_{LCR}$ limits phase separation and raises the inhibition concentration. Notably, translational repression exhibited a switch-like behavior that correlated with droplet formation. The post-translational modifications were proposed to fine-tune intracellular phase separation, and therefore, the critical concentration threshold for forming condensates may also provide a mechanism for tunable and switch-like translation inhibition.

### *Aromatic interactions*

Molecular interactions between aromatic and cationic residues have been shown to promote intracellular phase separation. As an example, multivalent interactions between a scaffold protein and a cluster of tryptophan (W)-binding sites on its interaction partner are sufficient to drive the condensation of an RNA processing complex *in vitro* and in mammalian cells [45] (Figure 3c, left). The miRNA-induced silencing complex (miRISC) aids in RNA processing by identifying and targeting mRNAs for repression. Sheu-Gruttadauria and MacRae demonstrated that three closely located W-binding pockets in Argonaute2 interact with an unstructured glycine- and tryptophan-rich IDR found in the argonaute binding domain of the scaffold protein, TNRC6B, to enable the formation of miRISC droplets [45]. Tryptophans on the argonaute binding domain interact with cationic residues in two of the three W-binding pockets, suggesting that cation–$\pi$ interactions play a role in miRISC condensation. Furthermore, Argonaute2 sequestered in droplets remained functionally active, and droplets could colocalize target RNAs and miRISC components that facilitate RNA decay.

Similarly, other native IDPs rely on different aromatic interactions for phase separation. Phase separation of two fused in sarcoma (FUS) domains relies on associative interactions between aromatic and cationic

residues, Tyr and Arg [13]. In their investigation of sequence determinants of phase separation in the FUS family proteins, Wang et al. [13] observed that the saturation concentration of a protein was dependent on the number of Tyr and Arg residues and were able to predict the saturation concentrations of mutated proteins based on amino acid composition (Figure 3c, right). Interactions between tyrosine residues on a low complexity prion-like domain (PLD) and arginine residues on a RBD aided in phase separation. *In vitro* assays demonstrated that the RBD does not phase separate alone and the PLD self-assembles at concentrations two orders of magnitude higher than the full length FUS protein (above 120 μM); however, when these two domains were mixed at an equal molar ratio, they phase separated at a significantly lower concentration (15 μM). Moreover, while most polymer complex coacervates undergo phase separation at charge neutrality, phase separation of the PLD/RBD mixture occurred far from charge neutral conditions (the expected charge ratio of PLD to RBD at the tested buffer condition, pH 7.4, is −4.3:17.8 as calculated by the Henderson–Hasselbach equation). This further emphasizes the importance of aromatic interactions in enhancing phase separation.

Interestingly, phase separation cannot be explained purely by generic cation−π interactions as not all residue combinations drive phase separation to the same extent. This lead to the investigation of how molecular grammar influences properties of the condensate. Different combinations of interactions between Arg, Lys, Tyr, and Phe were tested both *in vitro* and *in vivo* by generating a Tyr → Phe PLD variant and a Arg → Lys RBD variant [13]. In both cases, Tyr–Arg interactions were preferred for phase separation (the order of saturation concentrations from lowest to highest: Tyr–Arg > Tyr–Lys = Phe–Arg > Phe–Lys). This provided evidence that the propensity for phase separation is residue specific as certain chemical moieties confer stronger intermolecular interactions. In the case of R–Y interactions, the delocalization of electrons in arginine's guanidinium group may promote stronger interactions with aromatic residues than the primary amine of lysine. Furthermore, proteome-wide analysis revealed an inverse correlation between saturation concentration and the number of R and Y residues. In contrast, they observed a positive correlation between partition coefficients and the number of R and Y residues, indicating that partitioning of clients can also depend on the number of these residues.

### *Molecular code and specificity*

In addition to saturation concentration, specific residue identity can also influence coacervate behavior. *In vitro* experiments in which all Gly residues in the FUS PLD were replaced with Ala (Gly → Ala) did not change the saturation concentration but slowed the rate of droplet fusion, demonstrating glycine's role as a spacer that offers polypeptide flexibility [47,48]. Decreasing the fraction of hydrophilic amino acids, Ser → Ala and Gln → Gly mutations, also did not sufficiently change the saturation concentration but showed that glutamine and serine promote droplet hardening.

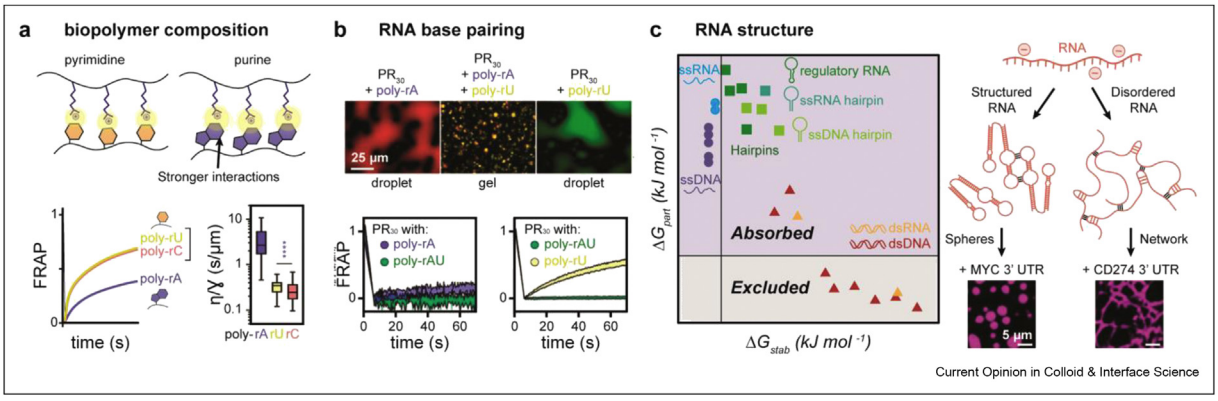
Both specific multivalent interactions and nonspecific, 'promiscuous' interactions can affect the propensity for biomacromolecular phase separation. While most recent studies have focused on how molecular code and sequence determine the propensity for phase separation, promiscuous interactions can also tune the coacervate phase by working synergistically with specific interactions (Figure 3d). One study proposes that the selectivity of RNP granules is attributed to specific protein–protein and protein–RNA interactions that drive its formation [46]. In contrast, the promiscuous interactions of IDRs are not necessary or sufficient to recruit proteins to RNP granules. Moreover, IDRs may even preclude phase separation with desired proteins as IDRs can have more favorable homotypic interactions or nonspecific interactions with competing proteins. However, when locally concentrated, nonspecific interactions between IDRs and proteins or RNA further enhance the assembly of RNP granule formed through specific interactions.

## Molecular features of biopolymers impact their coacervation with proteins

### *Nucleotide composition*

Nucleic acid composition is an important factor for determining the properties of protein–nucleic acid coacervates. *In vitro* experiments investigating phase separation of intrinsically disordered PR (proline–arginine) peptides with different biopolymers — including microtubules, RNA, heparan, and polyphosphate — revealed that the material properties and morphology of the condensate depend on the chemistry of the constituent monomers [8]. In addition, experiments testing phase separation of PR with homopolymeric RNA highlighted the importance of purine/pyrimidine content (Figure 4a). Biolayer interferometry indicated that PR–poly-rA interactions were significantly stronger than PR–poly-rC or PR–poly-rU interactions. Moreover, fluorescence recovery after photobleaching was used to investigate PR mobility in the coacervate phase. A lower fractional recovery was observed for PR–poly-rA, indicating PR is less mobile in the coacervate phase due to stronger molecular interactions (Figure 4a). Boeynaems et al. [8] also tested PK (proline–lysine) peptides to see if cationic residues were sufficient to explain the observed coacervate properties. At neutral pH, where arginine and lysine are both protonated, mixtures of PKand RNA homopolymers were less viscous than their PR counterparts regardless of RNA sequence, suggesting that PK engages in weaker

**Figure 4**




**Biopolymer molecular features that impact their coacervation with proteins. (a)** Nucleic acid composition affects the material properties of the coacervate. Purine–polypeptide interactions are significantly stronger than those of their pyrimidine counterparts and result in reduced polypeptide mobility and higher coacervate viscosity. Adapted with pending permission from the study by Boeynaems et al [8]. Copyright (2019) Aaron Gitler. **(b)** Microscopy images depict that PR (proline–arginine) polypeptide forms droplets with poly-rA or poly-rU. A mixture of all three components in an aqueous environment results in a gel due to poly-rA/poly-rU base-pairing. FRAP dynamics depict mobility of homopolymeric nucleic acids in PR-single oligonucleotide systems and no recovery in coacervates with all three components. Reprinted with pending permission from the study by Boeynaems et al [8]. Copyright (2019) Aaron Gitler Lab. **(c)** (left) Plot depicting the partition free energy ($\Delta G_{part}$) as a function of predicted stability of the nucleic acid structures ($\Delta G_{stab}$) reveals that nucleic acids with a sufficiently low stabilization (i.e., single stranded oligonucleotides) partitioned into coacervates even if they formed secondary structures. In contrast, nucleic acid duplexes were primarily excluded. Adapted with permission from the study by Nott et al [52]. Copyright (2016) Springer Nature. (right) RNAs with distinct secondary structure form spherical condensates, whereas disordered RNAs form networks. Microscopy images depict each of these coacervate morphologies formed by mGFP-FUS-TIS with the 3′UTRs of two different RNAs. Adapted with permission from the study by Ma et al. [53]. Copyright (2020) Christine Mayr Lab.

interactions with RNA than PR. This finding is further supported by recent studies of poly-lysine and poly-arginine coacervation with uridine oligomers of various lengths [49]. These *in vitro* results with simplified IDP sequences are consistent with the mutational studies of FUS [13]. The stronger PR–RNA interactions were attributed to higher order ($\pi$–$\pi$) interactions between arginine's guanidinium group and aromatic nucleotide bases. Finally, it was observed that RNA concentration in the coacervate phase can affect the dynamics of the encapsulated protein by tuning the density of interaction sites between the associated protein and RNA [8]. Partitioning of RNA into droplets formed from PR in the presence of a molecular crowder (polyethylene glycol or PEG) revealed a negative correlation between total RNA concentration and PR mobility in the coacervate phase.

### *RNA base pairing interactions*

While the purine and pyrimidine content of RNA can influence protein mobility within coacervates, interactions with other RNA molecules can also stabilize and influence the material properties of coacervates [8,50,51]. One study examining RNA base-pairing found that RNA–RNA interactions produced rigid coacervate networks instead of spherical droplets in a four-component system consisting of a PR peptide, poly-rA, poly-rU, and a solvent (Figure 4b) [8]. Simulations using a course-grained model predicted kinetically trapped, percolated networks when base-pairing interactions between complementary homopolymeric RNAs were present. Upon heating, the percolated networks rearranged into spherical droplets, which is the morphology observed for ternary systems consisting of the cationic peptide, individual homopolymeric RNA, and solvent. In addition, *in vitro* studies demonstrate that interactions between specific functionally related mRNAs can promote the assembly of distinct, immiscible condensates [50]. Taken together, these findings show that RNA interactions (RNA–peptide versus RNA–RNA) can affect the assembly, material properties, and dynamics of the condensate.

### *RNA structure*

Structural features of naturally existing biopolymers can influence their propensity to phase separate with proteins as well as partition into preformed coacervates [50,52,54,55]. Nott et al. [52] demonstrated that single stranded nucleic acids (i.e., DNA and RNA) preferentially partition into coacervates formed from the intrinsically disordered N-terminus of Ddx4 ($Ddx4^{N1}$) (Figure 4c, left). Moreover, shorter oligonucleotides in general had larger partition coefficients. In contrast, double stranded nucleic acids longer than 20 nucleotides were excluded from the condensate. dsDNA could partition into $Ddx4^{N1}$ coacervates in the presence of cationic GFP; however, the DNA duplex melted upon entry. This restructuring was likely due to steric

hindrance and competition with $Ddx4^{N1}$ for cation−π interactions with the exposed nucleotide bases of single stranded nucleic acids.

In addition to hybridization, nucleic acid secondary structures have been reported to influence partitioning and coacervate morphology. Additional studies reported that mRNA secondary structure can determine whether it is recruited or excluded from coacervates [50]. One hypothesis is that stem loops control whether the constituent sequences are hidden from or hybridize with other RNA strands. Changes in secondary structure may ultimately affect intermolecular interactions between RNAs that allow for RNA recruitment to preassembled droplets. Moreover, a recent study suggests that RNA can also contain intrinsically disordered regions, which may influence coacervate morphology (Figure 4c, right) [53]. Studies of TIS granules reported that large unstructured RNAs could adopt many conformations and were more likely to form networks through multivalent RNA−RNA interactions. In contrast, RNAs that had a high propensity for base-pairing tended to form spherical condensates.

## Conclusion and outlook

The complex coacervation of proteins has potential for protein stabilization, compartmentalization, and delivery. However, to design these protein-based materials, enumeration of the molecular features that mediate protein−polyelectrolyte coacervation is necessary. Reports from the last decade have also highlighted the role of protein phase separation *in vivo*, further necessitating an improved understanding of the molecular determinants of the associative phase separation of proteins and polyelectrolytes. Several approaches have been implemented to investigate the molecular parameters that influence the coacervation of synthetic and biological macromolecules. Studies using a bottom-up approach to systematically increase complexity *in vitro* have provided an improved understanding of parameters for the rational design of protein−polyelectrolyte coacervates. Complementary studies that decrease biological complexity to simpler, controlled *in vitro* experiments have similarly established contributions of both nonspecific intermolecular interactions and specific molecular code to intracellular phase behavior. These combined recent investigations have identified critical molecular parameters of proteins and biopolymers that govern phase separation in synthetic or cellular systems. Protein molecular features that influence phase behavior include electrostatics, charge patterning, aromaticity, post-translational modifications, and molecular specificity (and/or lack thereof). In addition, molecular features of biopolymers can also influence phase separation, including charge blockiness, hydrophobicity, monomer identity, nucleic acid secondary structure, and base-pairing interactions. This review highlights how these factors can be used to regulate aspects of protein phase behavior such as coacervate formation and client partitioning.

Despite significant insights into the effects of molecular interactions on the resulting formation and material properties of protein−polyelectrolyte coacervates, much remains unexplored. To better control protein phase separation, the relative contributions of these molecular interactions on determining coacervate properties need to be investigated. For example, how does protein structure and sequence impact the viscosity, mesh size, or dielectric of the resulting condensed phase? A fundamental understanding of the role of intracellular coacervates in various cellular functions has been established over the last decade. But how do interactions at the residue level affect coacervate biological function? Our nascent understanding of the molecular features that govern coacervate function is broadly unknown and could have huge implications in the design of these materials and in disease pathogenesis. Moreover, many studies have extensively examined single-phase coacervates, but recent findings report that the complex cellular environment can assemble multiphase coacervates. Multiphase coacervates have been reconstituted using synthetic biopolymers *in vitro*; however, understanding how to design the layered assembly of multiphase coacervates is still in its infancy. A deeper understanding of these parameters will confer better control of condensate formation, material properties, and function. This improved knowledge is critical as the liquid-like behavior, selectivity, and dynamics of protein coacervates make them a promising platform for disease mitigation, protein purification, and artificial organelle construction.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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