The Effects of Protein Charge Patterning on Complex Coacervation

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The complex coacervation of proteins with other macromolecules has applications in protein encapsulation and delivery and for determining the function of cellular coacervates. Theoretical or empirical predictions for protein coacervates would enable the design of these coacervates with tunable and predictable structure-function relationships; unfortunately, no such theories exist. To help establish predictive models, the impact of protein-specific parameters on complex coacervation were probed in this study. The complex coacervation of sequence-specific, polypeptide-tagged, GFP variants and a strong synthetic polyelectrolyte was used to evaluate the effects of protein charge patterning on phase behavior. Phase portraits for the protein coacervates demonstrated that charge patterning dictates the protein's binodal phase boundary. Protein concentrations over 100 mg mL^{-1} were achieved in the coacervate phase, with concentrations dependent on the polypeptide sequence. In addition to shifting the binodal phase boundary, polypeptide charge patterning provided entropic advantages over isotropically patterned proteins. Together, these results show that modest changes of only a few amino acids alter the coacervation thermodynamics and can be used to tune the phase behavior of polypeptides or proteins of interest.

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The effects of protein charge patterning on complex coacervation

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Abstract

The complex coacervation of proteins with other macromolecules has applications in protein encapsulation and delivery and for determining the function of cellular coacervates. Theoretical or empirical predictions for protein coacervates would enable the design of these coacervates with tunable and predictable structure-function relationships; unfortunately, no such theories exist. To help establish predictive models, the impact of protein-specific parameters on complex coacervation were probed in this study. The complex coacervation of sequence-specific, polypeptide-tagged, GFP variants and a strong synthetic polyelectrolyte was used to evaluate the effects of protein charge patterning on phase behavior. Phase portraits for the protein coacervates demonstrated that charge patterning dictates the protein's binodal phase boundary. Protein concentrations over 100 mg mL⁻¹ were achieved in the coacervate phase, with concentrations dependent on the polypeptide sequence. In addition to shifting the binodal phase boundary, polypeptide charge patterning provided entropic advantages over isotropically patterned proteins. Together, these results show that modest changes of only a few amino acids alter the coacervation thermodynamics and can be used to tune the phase behavior of polypeptides or proteins of interest.

Introduction.

Membraneless compartments in eukaryotic cells, such as the nucleolus, Cajal bodies, and stress granules, have been observed for centuries.^{1–4} However, in the last decade, understanding of the underlying molecular interactions that govern the formation and behavior of these phase separated microcompartments has dramatically improved.^{2,5–8} It has been speculated that many membraneless organelles comprised of proteins and nucleic acids phase separate into two coexisting immiscible phases via complex coacervation, a liquid-liquid phase separation phenomenon driven by electrostatic interactions between oppositely-charged polyelectrolytes.^{9–17} These dense liquid compartments are dynamic, retain material exchange with their environment, and exhibit selectivity in the macromolecules that they encapsulate.^{18–20} Biologically, the phase transitions of proteins (and other biopolymers) are essential for intracellular organization, and have demonstrated functions such as regulating RNA and protein homeostasis, responding to heat shock, and enhancing cellular signaling processes.^{21–24} In addition to the role in normal cell physiology, biomolecular condensates are implicated in disease progression, such as the formation of protein aggregates observed in neurodegenerative diseases.^{24–28}

Phase transitions of macromolecules are also ubiquitous in nonliving systems. Particularly, in polymer sciences, understanding and controlling phase transitions has enabled considerable progress in membrane technologies, porous glasses, composites, and thermoelectrics.^{29–33} As the fundamental understanding of the phase behavior of synthetic polymers has progressed, efforts have turned to sequence-defined biopolymers such as proteins.^{34–36} Similarly to synthetic macromolecules, understanding the phase behavior of proteins has potential to enable applications in the biotechnology domain, namely in protein isolation and protein delivery systems.^{20,37–39} Complex coacervates can encapsulate high concentrations of

protein³⁷ ($\sim 200 \text{ mg mL}^{-1}$) with increased stability to organic solvents,⁴⁰ proteases,⁴¹ ionic strength,^{41,42} and time.⁴³ In addition to protein stabilization, complex coacervates have also been shown to increase the activity of encapsulated enzymes.^{42,44} Developing the proper design rules to tune protein complex coacervation would enable novel opportunities in biomedicine and biocatalysis, while also potentially providing molecular-level insights into the relationship between protein sequence and membraneless organelle formation.

A predictive framework for the associative phase separation of globular protein biopolymers would enable the rational design of this behavior. For example, existing theories for simple coacervation of synthetic polymers or intrinsically disordered proteins (IDPs), such as elastin-like polypeptides (ELPs), have facilitated the rational design and tunable control of the phase behavior of these macromolecules.^{17,45–51} In polymer physics, describing coarse-grained effective interactions with a χ parameter is often a sufficient descriptor of the structure-function relationship of a polymer in a particular solvent.^{46,47,52} Similarly, design rules have been established for ELPs that have enabled a structure-function relationship between canonical biopolymer sequences and their respective lower critical solution temperatures (LCST).^{48,49} Unfortunately, analogous structure-function relationships for globular protein phase separation via complex coacervation remain unresolved. Complex coacervation of linear polyelectrolytes has been accurately and theoretically described using adaptations of the Voorn-Overbeek liquid lattice model of polymer solutions.^{16,17,53–55} This original model developed a quantitative theoretical treatment for the entropy of mixing and electrostatic attraction by combining the Debye-Huckel equations for electrical interactions and the Flory-Huggins equations for entropy.^{15–17} Adaptations by de Pablo,^{55,56} Ganesan,^{57,58} Wang,^{59–61} and Olvera de la Cruz^{62,63} have accounted for polymer chain connectivity, excluded volume interactions, and charge

fluctuations to develop a comprehensive and quantitative description of the phase behavior of simple, flexible polyelectrolytes and colloids. However, globular proteins represent a more complicated subset of polyelectrolytes due to their ampholytic nature and relatively fixed secondary structure. In order to develop robust theoretical predictions, additional experimental data relating protein-specific properties, such as charge patterning and conformational heterogeneity, to phase behavior are needed. Manipulating the charge and charge patchiness of a protein via protein engineering or chemical modifications is a strategy that can be used to promote coacervation.^{64–66} Additionally, several studies have shown that proteins with intrinsically disordered regions (IDRs) are more likely to phase separate.^{27,67,68} Critically, it has been observed that the sequence of intrinsically disordered or synthetic polypeptides impacts the phase behavior.^{12,51,69–72} Recent work from Perry and Sing has demonstrated the sequence-dependent complex coacervation of linear polypeptides, where the repeat length, or blockiness, of charged and neutral amino acids governed the thermodynamic driving force for phase separation.^{69,70,73,74} Here, a similar strategy was employed for globular proteins with polypeptide tags consisting of charged and neutral residues. The polypeptide tag charge blockiness was varied in an attempt to tune the binodal phase boundary of GFP variants and a strong polyelectrolyte as a function of salt concentration (Figure 1A).

With these considerations, a library of protein mutants was created to determine the effects of protein charge density and charge patterning on complex coacervation (Figure 1B). This was done by genetically fusing charged tags onto the C-terminus of superfolder GFP (sfGFP). The tag polypeptide sequences consisted of ionizable aspartate (D) and glutamate (E) and neutral glycine (G) and serine (S) residues. The polypeptides charge density, patterning, and lengths were varied in order to assess the effect of charge patchiness on complex

coacervation when coupled with an inherently patchy globular protein domain. The tags exist as a source of structural disorder that was expected to promote phase separation as previously demonstrated with other IDRs. To narrow the large parameter space, the complex coacervation of these sfGFP variants was investigated with a single strong synthetic polyelectrolyte (quaternized poly(4-vinyl *N*-methyl pyridinium iodide)). The balance between the globular protein (sfGFP) and ionic polypeptide tags with varying sequence enables evaluation of the relative importance of phase separation design criteria such as electrostatic interactions, entropy gains from the release of condensed counterions, and conformational heterogeneity. Here, we demonstrate that both charge and charge patterning dictate the protein's binodal phase boundary as a function of salt (NaCl) concentration. Additionally, protein concentrations over 100 mg mL⁻¹ were achieved in the dense coacervate phase. This high protein concentration was maintained at elevated ionic strengths in a sequence-dependent manner, with blockier sequences promoting higher protein concentrations in the coacervate phase. Differences in the thermodynamics of complex coacervation were also investigated by isothermal titration calorimetry (ITC) and revealed that coacervation was driven by sequence specific entropic gains as has been seen in polypeptide-based complex coacervates.

Figure 1. (A) Qualitative illustration of a typical phase portrait for complex coacervate forming (bio)macromolecules. (B) Schematic of the sequence defined polypeptide materials used in this study. (C) Representative example of the complex coacervation of an anionic GFP variant (τ 6-2) with qP4VP. Fluorescence microscopy image of the condensed phase with no added salt and protein concentration in the coacervate and dilute phases at low salt conditions.

Results.

A library consisting of sfGFP and 7 polypeptide tagged variants were designed and biosynthesized to analyze the effects of polypeptide charge patterning on the complex coacervation of globular proteins. The polypeptide tags were designed to have a net charge of -3 or -6 and were fused to the C-terminus of sfGFP. The naming convention was defined according to the polypeptide sequence periodicity, τ , and numbers x-y, where x corresponds to the periodicity, and y corresponds to the number of repeat units. Ultimately, this resulted in eight protein variants with three possible theoretical net charges at pH 7.4: -7 (▲ sfGFP), -9 (■ τ 1-2, τ 2-1, τ 6-1), and -12 (● τ 1-2, τ 2-2, τ 6-2, τ 12-1). Additionally, an isotropically charged variant, GFP(-12), was used as a control for protein net charge in the binodal composition assays and thermodynamic analysis.

GFP variants were expressed in *E. coli* and purified via Ni-NTA affinity chromatography. Purified samples were characterized via MALDI-TOF and gel electrophoresis (Figure S1 and S2). While the central goal of appending polypeptide tags onto the globular protein domain was to tune the protein's phase behavior, for this approach to be practical, it was essential that the charged tags did not affect protein biosynthesis. Cell growth kinetics and protein expression for each mutant were quantified by monitoring optical density at 600 nm (OD₆₀₀) and total cell fluorescence, respectively (Figure S3 and S4). Cell growth and protein expression were not affected by the addition of charged polypeptide tags on the C-terminus of sfGFP, indicating that this method of protein supercharging does not impose additional stress on the cell or affect protein yields.

Preliminary phase separation assays were performed to determine the relative mixing ratios of protein and quaternized poly(4-vinyl *N*-methyl pyridinium iodide) (qP4VP) that maximize phase separation and incorporation of GFP in the coacervate phase. The results of these preliminary coacervation assays are summarized in Table S1. The turbidity at 600 nm was used as a marker for phase separation of each mutant when mixed with the strong polycation qP4VP. All of the GFP mutants phase separated at low ionic strength with a maximum turbidity of approximately 90%. Turbidity values were plotted as a function of negative charge fraction (Figure S5A). For simple polyelectrolyte systems, the expected maximum in turbidity would occur at a charge fraction of 0.5.^{75–77} For this protein-polyelectrolyte system, turbidity maximums were observed at $f^- \sim 0.2$, indicating that an excess of polymer is required relative to the theoretically optimal charge neutral mixing ratios. This shift is thought to be specific to protein systems as ionizable amino acids may undergo induced charging in the presence of cationic polymer.^{65,78,79} While turbidity was used as a marker of phase separation, optical microscopy

was used to distinguish the morphological nature of the resulting phases; the phase separated protein and polymer mixtures formed dense, liquid-like coacervate phases (Figure S6iv). At the negative charge fraction corresponding to maximum turbidity, all of the tagged GFP variants were efficiently incorporated in the coacervate phase at conditions of low ionic strength (Figure S5B). In other words, charge distribution had minimal impact on the phase separation at low ionic strength.

Ionic strength is an important factor governing complex coacervation.^{15,80,81} To evaluate the salt dependence of coacervation for each mutant, protein and polymer were mixed at the ratio corresponding to maximum turbidity, 5 M NaCl was titrated into the mixture, and the turbidity was measured as a function of salt concentration (Figure 2A and S5C). All tagged proteins phase separated at moderate (near physiological) ionic strength (-9 ~ 100 mM, -12 ~ 150 mM). This represents a 25 – 75 mM increase in salt stability compared to sfGFP with the inclusion of only 3-6 charged amino acids. Detailed experimental results for each GFP variant can be found in Figure S6.

In this case, turbidity decreases with the addition of salt due to a gradual change in the free energy of the system until phase separation is no longer favorable. The increased concentration of free counterions in solution via the addition of salt reduces the release of bound counterions, resulting in smaller entropic gains.^{70,75,77} Proteins with increased net negative charge were expected to be more resistant to salt screening due to stronger electrostatic interactions with the polymer and an increased initial number of bound counterions. Likewise, the sequence defined polypeptide tags were hypothesized to demonstrate varying salt stability due to charge anisotropy or charge patchiness.^{65,70,73} This behavior was expected as patchier polypeptides have more confined condensed ions, and thus lower entropy, such that when they

are released, the entropy gains are larger.⁷⁰ In order to assess these design criteria and the relation to protein concentration in the coacervate phase, the binodal phase boundary of proteins was approximated by measuring the protein concentration in both the coacervate and dilute phases at discrete salt concentrations.

A thorough analysis of protein incorporation in the coacervate was performed to determine the interplay between protein net charge and polypeptide charge patterning. This enabled the construction of phase portraits that accounted for the volume of the coacervate as well as the ionic strength of the initial solution (Figure 2B). This analysis was performed at the macromolecule ratio corresponding to maximum protein concentration in the coacervate with no added salt. Coacervate volumes were determined via overhead fluorescence images where the coacervate droplet was easily identified, thresholded, and analyzed (Figure S7). Tagged GFP variants remained fluorescent through the phase separation process, indicating that the model proteins retained their secondary structure and activity upon concentration in the dense coacervate phase. Consistent with other findings, the preservation of fluorescence suggests that complex coacervation may be capable of incorporating proteins while maintaining their activity, an essential feature for enzyme nanoreactors or therapeutic delivery vehicles.^{82–84} Due to the small volume of the condensed phase, it was not possible to measure both the protein and the salt concentration in each phase. It was therefore assumed that the salt concentration in the two phases were equal. For this reason, tie lines were omitted between each pair of points. While tie lines for other polyelectrolyte complex coacervates have demonstrated slightly negative slopes, a decidedly uneven distribution of salt ions between the two phases would decrease the entropy of mixing and therefore be unlikely.^{55,85} Above the ionic strengths displayed for each protein, phase separation did not occur.

Figure 2. Salt titrations into turbid protein-polymer mixtures (A) and binodal phase portraits (B) for tagged protein library with qP4VP. (Top) Salt titrations and phase portraits for sfGFP and lesser charged mutants (charge = -9). (Bottom) Salt titrations and phase portraits for sfGFP and higher charged mutants (charge = -12). (A) Titration with salt (5 M NaCl, 1 μ L increments) to investigate GFP and qP4VP complex dissolution for charged protein mutants compared to sfGFP. (B) Phase portraits for sfGFP and tagged mutants with qP4VP at discrete NaCl concentrations (0*, 25, 50, 75, 100, 125, 150, 200 mM). Protein concentrations in the dilute phase (left) and coacervate phase (right) were determined using fluorescence measurements, using a calibration curve of overhead UV Images to determine the volume of the bulk coacervate phase (Figure S7). Dotted lines approximating the binodal phase boundary are simply a visual aid and do not represent theoretical modeling or analysis. The initial protein concentration of 1 mg mL⁻¹ has been represented using a dotted line. *Protein concentrations at 0 mM NaCl have been omitted due to the formation of less-hydrated films, where the volume of the coacervate could not be determined.

Consistently across the library of proteins, the concentration of protein in the coacervate phase decreased as the thermodynamic driving force for dissolution increased (i.e. increasing [NaCl]). sfGFP, -9 charged mutants, and -12 charged mutants phase separated up until salt concentrations of 75 mM, 100 mM, and 150 mM, respectively – a result consistent with the salt titration data (Figure 2A). For -9 charged mutants (Figure 2B, top), the concentrations of protein in the coacervate phase were comparable for each protein. At the highest salt concentration, more blocky polypeptide tags achieved slightly higher protein concentrations in the coacervate phase, but overall, the tag sequence had minimal effect on coacervation. In this regime, the

sfGFP globular domain remains the dominant element as the net charge of the globular domain (-6) is great than that of the polypeptide tag (-3). However, the effect of charge patterning on complex coacervation was expected to be more pronounced for the -12 charged group as the net charge of the polypeptide tag was equal to the net charge of the globular protein domain. At the maximum salt concentration with liquid-liquid phase separation (150 mM), τ 6-2 and τ 1-2 had protein concentrations an order of magnitude higher than the less blocky τ 2-2 and the sterically blocked τ 12-1. Interestingly, all of the tagged mutants demonstrated an increase in their binodal phase boundary compared to GFP(-12), a sfGFP variant with isotropically distributed charge, reinforcing the notion that charge distribution is an essential design parameter in protein phase separation. To ensure that the dilute and coacervate phases were equilibrated when establishing these phase portraits, the experimental protocol was repeated for protein τ 6-2 using a variety of equilibration methods (Figure S8). Thermal processing and longer equilibration times did not impact the determined binodal phase boundary significantly, suggesting that the analyzed phase behavior was at or near equilibrium. This phase behavior can be used to tune protein partitioning and stability in complex coacervates. For example, for enzymes with functional active sites, charged tags can potentially be used as a mechanism for protein supercharging without compromising protein structure and activity.

The underlying thermodynamic driving forces for complex coacervation were investigated for a subset of the sfGFP variants by isothermal titration calorimetry (ITC). This technique determines the amount of power that must be supplied to maintain constant temperature as macromolecules interact. As the titration proceeds, the amount of power needed to increase or decrease temperature is indicative of both the sign and magnitude of any interactions taking place, and thus can be used with an appropriate model to calculate thermodynamic properties

and affinity constants for the interaction.^{70,77,86} Analysis of ITC data was performed using a Two-Step Binding Model previously described by both Priftis et al. and Chang et al, which is separated into two regimes: ion pairing and coacervation, shown in Figure 3A in blue and red, respectively.^{70,77} The sum of both contributions is the total energy and was used to fit the data (black). The thermodynamic criteria for complex coacervation is not unique and is common to any spontaneous process. However, the driving force for coacervate formation is unique in that it is system-dependent. While polyelectrolyte-polyelectrolyte coacervation tends to be entropically driven, colloid-polyelectrolyte coacervation can be driven by both entropy gains in the form of counterion release or by electrostatic interactions.^{15,75} Predicting a protein's phase behavior thermodynamics would guide the rational design of a charged polypeptide tag given the particular globular protein and polymer, while also providing guidance for simulations to approximate the phase space of genetically modified globular proteins.

Figure 3. (A) Example (variant: τ 2-2) of fitting ITC data using a two-step binding model with parameter space Θ . Parameter values and individual fits are described in Figure S9. (B) Calculated thermodynamic quantities for each GFP variant during the process of electrostatic attraction and phase separation with qP4VP. Free energy (ΔG), entropy ($T\Delta S$), and enthalpy (ΔH) are plotted from left to right. Error bars represent the standard error relative to the mean ($N = 3$).

ITC was performed for a subset of variants in the library to determine if the observed shift in the binodal curves and salt stability correlated with the system thermodynamics (Figure 3B). The complex coacervation thermodynamics of sfGFP was compared with three of the -12 mutants: τ 2-2, τ 6-2, and the isotropic control GFP(-12) (summarized in Figure S9). Enthalpically, increasing charge and/or charge anisotropy only produced minor unfavorable changes. As expected in PE-PE systems, entropy was the primary thermodynamic driving force for phase separation and this driving force increased with increasing charge. Anisotropically supercharged variants (i.e. tagged) demonstrated thermodynamic advantages over their isotropic counterparts and these differences were consistent with the trends observed in the

titrations and phase portraits shown in Figure 2. Specifically, as the protein net charge increased so did the entropic driving force. When comparing the relative entropic gains compared to sfGFP, τ 6-2 demonstrated a 3-fold increase (2.4 kJ/mol) in its entropic driving force relative to the isotropic GFP(-12) control (0.8 kJ/mol). This shift in thermodynamic driving force is critical to the variant's phase behavior and once again demonstrates that complex coacervation is a multifaceted phenomenon that depends as much, if not more, on charge distribution and not only the protein's net charge. This observation is consistent with trends observed in linear polypeptides, where condensed counterions are confined along with backbone of more charge blocky polypeptides.⁷⁰ Here, the increase in thermodynamic driving force was attained using this relationship between charge distribution and confinement effects; together, these modest changes to the protein sequence with a short 12 amino acid polypeptide tag result in a thermodynamic difference that can be used in either linear polypeptides or globular proteins to tune phase behavior.

Conclusions.

The design rules for the complex coacervation of globular proteins were investigated using a library of anionic sfGFP variants tagged with polypeptide sequences consisting of both ionizable and neutral amino acids in different arrangements. Protein supercharging via charged tags required only modest changes to the protein sequence and this method of supercharging did not affect protein yield. Using qP4VP as the cationic counterpart, the phase behavior of this system was analyzed and optimized. All of the variants underwent complex coacervation with modest, if any, differences in turbidity and encapsulation efficiency at low ionic strength. When interactions were screened with salt, the net charge and charge distribution of sfGFP mutants synergistically affected the formation and salt stability of complex coacervates. These phase

portraits are the first of their kind for engineered protein coacervates, and this thorough evaluation of encapsulation efficiencies indicated that charge distribution has a pronounced effect on globular protein phase behavior, especially at elevated ionic strengths. Isothermal titration calorimetry indicated that the binodal shift in critical salt concentration was due to increased entropic gains upon phase separation. Charge blocky tags like τ 6-2 doubled the critical salt concentration and increased the entropic driving force substantially (2.4 kJ/mol) when compared to GFP without a tag. The lack of structure-function relationships for the complex coacervation of globular proteins has prevented the theoretical prediction of such thermodynamic data. Moving forward, we hope to help in the collective effort to bridge this gap to better understand, design, and optimize protein phase behavior for a wide-range of applications.

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Supporting Information. (PDF) The supporting information is available free of charge at [ACS website].

- General materials and methods, experimental procedures, experimental data, models, and codes (PDF)

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Supporting Information

The effects of protein charge patterning on complex coacervation

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1. Materials and Methods

All enzymes for molecular biology and competent cells [NiCo21 (DE3), BL21 (DE3), NEB-5 α] were purchased from New England Biolabs (Ipswich, MA). All primers were purchased from Integrated DNA Technologies (Coralville, IA). All chemicals and media components were purchased from Fisher Scientific (Pittsburgh, PA) or Sigma Aldrich (St. Louis, MO) and were used as received. Poly(4-vinyl-*N*-methylpyridinium iodide), qP4VP ($M_n = 28,000$, $\bar{D} = 1.2$), was purchased from Polymer Source (Montreal, QC). qP4VP was dissolved in 10 mM Tris to a concentration of 1 mg mL⁻¹. The cationic polymer solution was adjusted to a pH of 7.4. Both protein and polymer solutions were stored at 4 °C until use. Unless otherwise noted, all experiments were conducted using 1 mg mL⁻¹ working solutions of protein and polymer in 10 mM Tris (pH 7.4).

2. Cloning

The sfGFP plasmid was a gift from the Banta lab (Columbia University). Anionic GFP mutants were cloned using sfGFP (N-terminal, 6xHis) as a template. Forward and reverse primers were designed specifically for each amino acid tag sequence using NEBuilder (<https://nebuilder.neb.com>). The tag sequences were appended to the 3' end (protein C-terminus) of the sfGFP gene using restriction enzyme cloning or HiFi assembly. For both methods, the insert was amplified by PCR to include the mutations at the 3' end of the gene. Briefly, template DNA, dNTPs (final conc. 200 μ M), primers (final conc. 0.5 μ M), Phusion DNA polymerase, and HF Buffer were combined as per the PCR protocol for Phusion High-Fidelity DNA polymerase with a total sample volume of 50 μ L. PCR reactions were denatured (98 °C), annealed, and extended (72 °C). PCR was performed for a total of 35 cycles at annealing temperatures of 52 °C for restriction enzyme cloning or as recommended by NEBuilder for HiFi assembly. PCR products were purified using a Qiagen PCR purification kit. For restriction enzyme mediated cloning prior to PCR purification, the PCR reaction was treated with Dpn1 to digest the template DNA and NcoI and XhoI to digest the PCR amplified inserts. Insert DNA and vector DNA were purified via the Qiagen PCR purification kit and agarose gel electrophoresis (followed by Qiagen Gel Purification kit), respectively. Otherwise, HiFi assembly was performed following the HiFi DNA Assembly Reaction protocol using a 2:1 molar ratio of insert DNA to vector and incubating for 15 minutes at 50 °C. Ligated DNA was transformed into NEB-5 α cells and the sequence of the resulting plasmid was confirmed by Sanger sequencing (Genewiz).

Amino Acid Sequences of sfGFP mutants:

Tag sequence is in bold, **anionic residues in the tag** in red, thrombin cleavage site is underlined

sfGFP (-7)

MGHHHHHHGASKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLTCLKFI
CTTGKLPVPWPTLVTTLTYGVCFSRYPDHMKQHDFFKSAMPEGYVQERTISFKDDG
TYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNFSHNVIYITADKQKNGIKAN
FKIRHNVEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMLVLE
FVTAAGITHGMDELYK

T 1-1 (-9)

MGHHHHHHHGGASKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLTCLKFI
CTTGKLPVPWPPTLVTTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTISFKDDG
TYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNFNSHNVIYITADKQKNGIKAN
FKIRHNVEDGGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALS KDPNEKRDHMLLE
FVTAAGITHGMDELYKLVPRGS**DEE**

T 2-1 (-9)

MGHHHHHHHGGASKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLTCLKFI
CTTGKLPVPWPPTLVTTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTISFKDDG
TYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNFNSHNVIYITADKQKNGIKAN
FKIRHNVEDGGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALS KDPNEKRDHMLLE
FVTAAGITHGMDELYKLVPRGS**DGDGES**

T 6-1 (-9)

MGHHHHHHHGGASKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLTCLKFI
CTTGKLPVPWPPTLVTTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTISFKDDG
TYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNFNSHNVIYITADKQKNGIKAN
FKIRHNVEDGGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALS KDPNEKRDHMLLE
FVTAAGITHGMDELYKLVPRGS**DDEGGS**

T 1-2 (-12)

MGHHHHHHHGGASKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLTCLKFI
CTTGKLPVPWPPTLVTTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTISFKDDG
TYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNFNSHNVIYITADKQKNGIKAN
FKIRHNVEDGGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALS KDPNEKRDHMLLE
FVTAAGITHGMDELYKLVPRGS**DEEEDD**

T 2-2 (-12)

MGHHHHHHHGGASKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLTCLKFI
CTTGKLPVPWPPTLVTTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTISFKDDG
TYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNFNSHNVIYITADKQKNGIKAN
FKIRHNVEDGGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALS KDPNEKRDHMLLE
FVTAAGITHGMDELYKLVPRGS**DGDGESDGDGES**

T 6-2 (-12)

MGHHHHHHHGGASKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLTCLKFI
CTTGKLPVPWPPTLVTTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTISFKDDG
TYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNFNSHNVIYITADKQKNGIKAN
FKIRHNVEDGGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALS KDPNEKRDHMLLE
FVTAAGITHGMDELYKLVPRGS**DDEGGSDEGGS**

T 12-1 (-12)

MGHHHHHHHGGASKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLTCLKFI
CTTGKLPVPWPPTLVTTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTISFKDDG
TYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNFNSHNVIYITADKQKNGIKAN

FKIRHNVEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALS KDPNEKRDH MVLL
 FVTAAGITHGMDELYKLVPRGS**DDEDEGGSGGS**

Summary of protein mutants:

Displayed electrostatic maps are approximate structures meant to illustrate differences between the charge distribution of protein mutants. Electrostatic maps were generated using the optimized sfGFP structure (PDB). Minimized polypeptide tag sequence structures were determined using PEP-FOLD3 (<http://mobyle.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py#forms>). Tag structures were imported into Pymol and manually appended to the C-terminus of sfGFP. PQR files were generated at pH 7.4 (http://nbc-222.ucsd.edu/pdb2pqr_2.1.1/) and the solvent-accessible surface was visualized using the APBS plugin in Pymol.

GFP(-12) – Isotropic Control, **Mutations from sfGFP** in bold/underlined¹

MGHHHHHHGASKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATE**E**GKLT LKFI
 CTTGKL PVPWPTLVTTLT YGVQCFS RYPDHMKQH DFFKSAMPEGYVQERTISFKDDG
 TYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNFN SHNVYITADKQ**E**NGIKAN
 FKIRHNVEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALS KDPNE**D**RDH MVLL
 FVTAAGITHGMDELYK

3. Protein Expression, Purification, and Preparation

Protein Expression. Anionic GFP mutants were expressed in NiCo21 (DE3) or BL21 (DE3) cells in 1 L cultures of LB media supplemented with 100 µg mL⁻¹ ampicillin. Cells were grown at 37 °C, with shaking at 250 rpm to an OD₆₀₀ between 0.6 and 0.8. Subsequently, protein expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cultures were grown for an additional 16-20 h after induction at 37 °C with shaking at 250 rpm.

Protein Purification. Cells were harvested by centrifugation (4000 rpm for 20 min), resuspended in 15-20 mL of cell lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0), and stored at -20 °C. Immediately prior to cell lysis by sonication, 200 µL of DMSO solubilized Protease Inhibitor Cocktail (Sigma Aldrich, P8849) was added to resuspended cells. Cells were lysed in a -20 °C aluminum bead bath using probe tip sonication for 10 min (2 s pulse on, 4 s pulse off). Desired soluble components were separated from cell debris by centrifugation (10,000 rpm for 30 min). GFP was isolated from other soluble components via Ni-NTA affinity chromatography. Wash buffer consisted of 50 mM NaH₂PO₄, 300 mM NaCl, and 35 mM imidazole (pH 8.0), and elution buffer consisted of 50 mM NaH₂PO₄, 300 mM NaCl, and 250 mM imidazole (pH 8.0). Relative volumes of wash and elution buffers were similar to the manufacturer's instructions with slight modifications made dependent on the mutant. Fractions of the flow through, wash, and

elution were collected and analyzed by SDS-PAGE. Pure fractions were combined and concentrated by centrifugal ultrafiltration with a molecular weight cutoff (MWCO) of 10 kDa. Buffer was exchanged into 10 mM Tris (pH 7.4) with 1 mM EDTA by dialysis using regenerated cellulose dialysis membranes (MWCO of 3.5 kDa) at 4 °C in the dark. At least seven buffer changes were performed over a minimum of 21 h (3 h per exchange).

Sample Preparation. GFP mutants were characterized by MALDI-TOF mass spectrometry to confirm the mass and purity of each protein. Samples were prepared using a 10 mg mL⁻¹ sinapinic acid matrix (7:3 acetonitrile to H₂O with 0.1% trifluoroacetic acid) and calibrated with Protein Standard II (Bruker). Matrix (60%) and protein sample (40%) were premixed and 1 µL was spotted for analysis.

The concentration of GFP was determined by measuring absorbance at 488 nm ($\epsilon = 83,300 \text{ M}^{-1} \text{ cm}^{-1}$)² using a Cary 60 UV-Vis spectrophotometer. GFP working solutions were prepared at 1 mg mL⁻¹ in 10 mM Tris (pH 7.4).

4. Cell Growth Kinetics and Protein Expression

Cell growth kinetics and protein expression for different protein mutants were quantified in a Tecan Infinite M200 Pro plate reader. Glycerol stocks were streaked on a LB agar plate containing ampicillin; a single colony was selected and grown in a 5 mL culture (LB media supplemented with 100 µg mL⁻¹ ampicillin) overnight to an OD₆₀₀ of approximately 2. This culture was back-diluted to an OD of 0.60 and 1 mM IPTG was added to quantify cell growth kinetics (Figure S3) and protein expression (Figure S4). All dilutions were done by using the OD₆₀₀ of the overnight culture to calculate the respective volumes of overnight culture and LB media needed for a final volume of 1 mL. Each dilution was split into 6 wells (100 µL each) in flat, clear bottom, black polystyrene 96-well plates (Corning). Absorbance at 600 nm was used to measure optical density. The measured absorbance was corrected to that of a 1 cm pathlength using a measured path correction.³ Fluorescence of GFP mutants ($\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 530 \text{ nm}$, Gain = 50) was used to measure relative protein concentration.⁴ The assay was run at 37 °C for 15 h with orbital shaking, with measurements taken every 20 min. To verify that the direct back-dilution to mid-log phase (OD = 0.6) did not impact the induced protein expression, a similar experiment was performed with a different back-dilution. An overnight culture was grown to an OD₆₀₀ of approximately 2. The overnight culture was back-diluted to 1 mL samples (n = 3) with an OD of 0.10. Replicates were then grown to an OD of 0.60 and IPTG (1 mM final conc.) was added to induce protein expression. Each method resulted in nearly identical protein yields and cell growth kinetics.

5. Complex Coacervation Assays

Results from these assays are summarized in Table S1.

Turbidimetric Titration. Protein and polymer solutions were mixed at protein mass fractions ranging from 0 to 1 in increments of 0.04 with a total sample volume of 50 µL. Each mass fraction corresponded to a specific negative charge fraction, calculated using the expected polymer and protein charge. The expected protein charge was calculated using the Henderson-Hasselbalch equation and pK_a values of the isolated amino acids.⁵

Samples at each mass fraction were prepared in triplicate in tissue culture-treated polystyrene 96-well half-area plates (Corning). Absorbance ($\lambda = 600$ nm) was used to evaluate phase separation of the protein/polycation mixtures and was measured using a Tecan Infinite M200 Pro plate reader after 10 s of orbital shaking. The turbidity (τ) was calculated from the absorbance (A) using the formula $\tau = 100 - 10^{(2-A)}$. Turbidity values are plotted as a function of negative charge fraction $f^- = M^-/(M^- + M^+)$, where M^- and M^+ correspond to the charge per mass of the negative (protein) and positive (polymer) species, respectively (Figure S5A).

Encapsulation Efficiency. From the turbidity data, five specific mass fractions were selected for each GFP mutant to analyze the partitioning of GFP in the dense coacervate phase (Table S1 and Figure S5B). At these ratios, protein and polymer were mixed in triplicate in 1.5 mL tubes at a total sample volume of 100 μ L. After spontaneous phase separation and 10 min incubation, the samples were microcentrifuged at 13,000 rpm for 25 min to physically separate the dense and dilute phases. The dilute phase was removed via pipette and diluted 2-fold and 10-fold with 10 mM Tris (pH 7.4) in flat, clear bottom, black polystyrene 96-well plates (Corning). The absorbance ($\lambda = 488$ nm) and fluorescence ($\lambda_{\text{ex}} = 488$ nm, $\lambda_{\text{em}} = 530$ nm, Gain = 47, 55) of the dilute phase was measured using a Tecan Infinite M200 Pro plate reader. The concentration of GFP (mg mL⁻¹) in the dilute phase was calculated using calibration curves previously determined from serial dilutions of GFP from 0.25 μ g mL⁻¹ to 0.4 mg mL⁻¹.

Salt (NaCl) Titration. The ionic strength dependence of GFP/polycation phase separation was determined by titrating NaCl into protein/polymer mixtures (Figure S5C). Macromolecule composition was optimally selected from turbidity and encapsulation data at the ratio with the highest protein encapsulation. Microscopy images were considered in this optimization process on the basis of condensate morphology, where selected mixing ratios appeared to phase separate into two distinct liquid states. Mixtures were prepared in 4 mL poly(methyl methacrylate) cuvettes at a total sample volume of 1 mL. The sample was stirred at 850 rpm and the percent transmittance (%T) of the phase separated mixture was measured using a Cary 60 UV-Vis spectrophotometer. 5 M NaCl (in 10 mM Tris, pH 7.4) was added to the mixture in 1 μ L increments. Transmittance measurements were taken 5, 10, and 15 s after each incremental addition of salt to allow for sample equilibration. NaCl was added following this procedure until %T stabilized and remained unchanged following 4-5 subsequent additions of salt. Turbidity (τ) was calculated from percent transmittance (%T) using the formula $\tau = 100 - \%T$.

Phase Portraits. Protein and polymer solutions were prepared at 2 mg mL⁻¹. A salt solution of 1 M NaCl in 10 mM Tris (pH 7.4) was prepared and used to investigate the effect of ionic strength on the partitioning of macromolecules in the dilute and coacervate phases. Protein and polymer were mixed at the maximum encapsulation ratio, as described above, reported in Table S1, at various salt concentrations with a total sample volume of 50 μ L. Samples were prepared in triplicate in 96-well round-bottom plates (Nunc) and incubated for approximately 10 min. Phases were separated by centrifugation at 3,000 rpm for 30 min. The supernatant was extracted and diluted 10-fold (1 M NaCl, 10 mM Tris, pH 7.4). The remaining coacervate pellet was imaged using a Gel-Doc XR+ (Bio-Rad), thresholded using ImageJ, and computationally analyzed to determine the

volume using a calibration curve of known volumes (Figure S7, see Matlab script). The coacervate pellet was dissolved and diluted in 350 μL of 1 M NaCl, 10 mM Tris (pH 7.4). The absorbance ($\lambda = 488 \text{ nm}$) and fluorescence ($\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 530 \text{ nm}$, Gain = 47, 55) of the coacervate and dilute phases were measured in flat, clear bottom, black polystyrene 96-well plates (Corning) using a Tecan Infinite M200 Pro plate reader. To determine the binodal phase boundary, the concentration of each anionic GFP mutant (mg mL^{-1}) at a range of ionic strengths (25 mM to 200 mM) was calculated for both the dilute and coacervate phase using calibration curves (Figure 2B).

Equilibration Experiments. Phase portraits were also investigated for multiple equilibration conditions. Figure S8 represents this equilibration data for variant τ 6-2. In each of these cases, the equilibration step was performed and immediately followed by the standard binodal composition assay described above. These included longer equilibration times prior to centrifugation (24 h, 1 week) and thermal equilibration ($20^\circ\text{C} \rightarrow 50^\circ\text{C} \rightarrow 20^\circ\text{C}$) in a thermal cycler. This temperature ramping was well below the thermal denaturation temperature of sfGFP.^{6,7}

Microscopy. Microscopy images were collected using an EVOS FL Auto 2 inverted fluorescence microscope (Invitrogen). From the turbidity data, five specific mass fractions were selected for each GFP mutant for analysis via microscopy. GFP mutants and polycation were mixed together in black 384-well plates with optically-clear bottoms (Nunc) at a total sample volume of 30 μL . Samples were incubated at room temperature for 3 h. Images were taken using a 20X, long working distance objective (NA 0.4) in both GFP fluorescence ($\lambda_{\text{ex}} = 470 \text{ nm}$, $\lambda_{\text{em}} = 525 \text{ nm}$) and phase-contrast channels.

6. Isothermal Titration Calorimetry.

Data Acquisition. Isothermal Titration Calorimetry was performed in a Malvern MicroCal Auto-iTC200 in triplicate for a subset of protein mutants to compare coacervation thermodynamics. This subset included the proteins: sfGFP, τ 2-2, τ 6-2, and isotropic GFP(-12). This was done with 1 μL injections of polymer into a 200 μL cell containing protein at a temperature of 25°C , waiting 300 s between injections. Additionally, multiple blanks were performed using 10 mM Tris buffer as the titrant and each respective protein in the cell. This blank was subtracted from collected data as a minor correction accounting for heat of mixing. Similarly, titrations of polymer into buffer were performed to account for heat of dilution. Both of these contributions to measured enthalpy changes were relatively minor. qP4VP was used at a concentration of 4.2 mg mL^{-1} (0.150 mM) and proteins were used at a concentration of 1 mg mL^{-1} ($\sim 0.034 \text{ mM}$). Data were exported in a CSV file, corrected, and parameterized in MATLAB. Rigorous cleaning with detergent and salt-in-salt titrations with PBS were performed after experimental runs to ensure dissolution of the coacervate and to prevent cross contamination between trials.

ITC Data Analysis. Data analysis was performed via the Two Step Model described by Priftis et al.^{8,9} This model includes 6 parameters in the parameter space: ion-pairing stoichiometry (n_{IP}), coacervate stoichiometry (n_{coac}), characteristic enthalpies (ΔH_{IP} , ΔH_{coac}), affinity constant (K_A), and a constant proportional to the width of a gaussian curve approximating the range where coacervation occurs (α). Parameter estimates are

provided with the corresponding standard error measurements. The MATLAB code used to fit the data is also included in SI Section 16.

7. Fig. S1 - MALDI-TOF of Protein Mutants

Figure S1. MALDI-TOF MS characterization of purified proteins. Expected molar mass refers to the adjusted theoretical mass, accounting for methionine cleavage and chromophore formation. Observed molar mass refers to the corresponding methionine-adjusted peak (bold, underlined peak). The higher mass peak corresponds to the protein with methionine.

8. Fig. S2 - Electrophoresis Analysis of Engineered Proteins

Figure S2. SDS-PAGE analysis of sfGFP and tagged GFP mutants. For each case, bands corresponding to the ladder and the GFP mutant were cropped from the same gel. 4-12% Bis-Tris Bolt gels (ThermoFisher) were run at 200 V for 30 minutes.

9. Fig. S3 - *E. Coli* Cell Growth Kinetics

Figure S3. Cell growth kinetics of *E. coli* expressing each member of the protein library were analyzed using whole-cell measurements. Overnight cultures of *E. coli* cells expressing each protein mutant were grown to an optical density between 1 and 2 and back-diluted to $OD_{600} = 0.60$. IPTG was added and back-dilutions were plated with 6 replicates and the absorbance at 600 nm was recorded over 15 h. Absorbance was converted to OD_{600} using a pathlength correction for the 100 μ L sample volume. Error bars represent the standard deviation for absorbance measurements in each sample group at that time, scaled according to the pathlength correction.

10. Fig. S4 - Whole Cell Fluorescence Measurements in *E. Coli*

Figure S4. Whole-cell fluorescence measurements of *E. coli* cells (induced with IPTG) expressing each protein mutant. Fluorescence measurements of the induced sample group are normalized to the optical density of the induced sample group. Overnight cultures of *E. coli* cells expressing each protein mutant were grown to an optical density between 1 and 2 and back-diluted to $OD_{600} = 0.60$. Back-dilutions were plated with 6 replicates and OD (600 nm) and GFP fluorescence ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 530$ nm, Gain = 50) were recorded over 15 h. Error bars represent the error propagation of absorbance and fluorescence measurements in each sample group, computed via the standard method of adding in quadrature for measurement errors assumed to be governed by independent Gaussian distributions (sqrt. of the sum of squared fractional uncertainty)

11. Table S1 - Summary of Protein and Phase Separation Parameters

Table S1. Summary of complex coacervation behavior of GFP mutants used in this study. Five charge fractions were selected from turbidimetric assays for protein encapsulation studies. **Bold fractions** indicate the relative amount of protein/polymer used in subsequent analysis (salt titrations, phase portraits). These fractions are highlighted in the microscopy images of Figure S6 with a black border. GFP(-12), an isotropically charged sfGFP variant, was used as a control in this study. Complex coacervation parameters for this protein were previously reported by Kapelner.¹

	Expected Charge	Expected Molecular Weight (kDa)	Negative Charge Fraction (f-)	Protein Mass Fraction	Turbidity	Fraction Encapsulated
WT sfGFP	-7	27.64	0.092	0.64	49.81	0.790
			0.128	0.72	78.99	0.921
			0.153	0.76	82.65	0.971
			0.230	0.84	80.14	0.992
			0.396	0.92	67.65	0.590
τ 1-1	-9	28.62	0.114	0.64	88.09	0.935
			0.133	0.68	90.98	0.986
			0.144*	0.70	90.47*	0.989
			0.224	0.80	77.67	0.986
			0.275	0.84	84.87	0.874
τ 2-1	-9	28.82	0.097	0.60	74.93	0.868
			0.132	0.68	86.84	0.968
			0.156	0.72	87.09	0.999
			0.223	0.80	85.76	0.977
			0.345	0.88	79.70	0.685
τ 6-1	-9	28.82	0.113	0.64	73.41	0.907
			0.132	0.68	84.72	0.962
			0.156	0.72	90.76	0.989
			0.223	0.80	75.62	0.901
			0.274	0.84	79.23	0.804
τ 1-2	-12	28.99	0.147	0.64	88.03	0.974
			0.171	0.68	91.39	0.996
			0.184*	0.70	89.71*	0.998
			0.279	0.80	89.83	0.966
			0.337	0.84	81.96	0.898
τ 2-2	-12	29.37	0.145	0.64	76.29	0.952
			0.169	0.68	87.89	0.994
			0.197	0.72	90.79	0.998
			0.276	0.80	92.87	0.970
			0.334	0.84	82.05	0.872
τ 6-2	-12	29.37	0.145	0.64	91.19	0.993
			0.169	0.68	90.29	0.998
			0.197	0.72	87.26	0.998
			0.276	0.80	93.62	0.923
			0.334	0.84	83.79	0.809
τ 12-1	-12	29.37	0.125	0.60	65.10	0.887
			0.169	0.68	89.60	0.989
			0.232	0.76	67.92	0.980
			0.334	0.84	81.06	0.853
			0.412	0.88	48.23	0.654
GFP(-12)	-12	27.62	0.207[†]	0.72	87.41 [†]	0.862 [†]

* denotes charge fractions that were not investigated in turbidimetric assays. In each case, the turbidity value listed is an average of the adjacent points (0.68 and 0.72).

[†] values and parameters reported by Kapelner.¹

12. Fig. S5 - Summary of Complex Coacervation Assays with sfGFP Mutants and qP4VP

Figure S5. Summary of phase separation assays performed with sfGFP and tagged mutants with qP4VP. All assays were performed at a total macromolecule concentration of 1 mg mL⁻¹ in 10 mM Tris, pH 7.4. (A) Turbidity measurements for different ratios of protein and polymer are shown as a function of negative charge fraction. (B) Encapsulation efficiency of tagged GFP mutants compared to sfGFP for 5 macromolecule ratios selected by initial turbidity studies (Table S1). The fraction of protein encapsulated was quantified by measuring the protein concentration in the dilute phase after centrifugation where, *fraction encapsulated* = $1 - [protein]_{dilute\ phase} / [protein]_{initial}$. (C) GFP mutant and qP4VP complex dissolution as a function of NaCl concentration was evaluated via titration studies. The macromolecule ratios analyzed were selected on the basis of encapsulation efficiency and microscopy data and were subsequently the ratios investigated in phase portraits, as indicated in Table S1. Transmittance (%T) was measured after 1 μ L additions of 5 M NaCl.

13. Fig. S6 - Detailed Experimental Results for sfGFP and Tagged Protein Mutants

Figure S6. Detailed experimental results for sfGFP and tagged protein mutants: (A) sfGFP, (B) τ 1-1, (C) τ 2-1, (D) τ 6-1, (E) τ 1-2, (F) τ 2-2, (G) τ 6-2, (H) τ 12-1. All experiments were conducted with GFP and qP4VP at a total macromolecule concentration of 1 mg mL⁻¹ in 10 mM Tris, pH 7.4. (i) Individual turbidimetric assays for each anionic protein with cationic polymer, qP4VP across a range of negative charge fractions. (ii) Encapsulation efficiency for each protein mutant was approximated for 5 macromolecule ratios (See Table S1). These ratios were selected on the basis of turbidity results. (iii) Salt titrations with NaCl to investigate complex dissolution for the GFP and qP4VP system. NaCl (5 M) was added in 1 μ L increments while stirring. Transmittance (%T) was measured in a UV-Vis spectrophotometer to approximate the critical salt concentration of protein/polymer complexes. (iv) Microscopy images of protein/polymer complexes after approximately 3 h of incubation. Merged images are displayed of the phase contrast and GFP channels. The same 5 macromolecule ratios were studied and are displayed in increasing negative charge fraction from left to right. The macromolecule ratio investigated in titrations and phase portraits is emphasized with a black border in both turbidity graphs (when ratio was part of turbidimetric assay) and microscopy images. Scale bar = 125 μ m.

14. Fig. S7 - Measuring Coacervate Volumes: Calibration and Analysis

C

D

NaCl Concentration (mM)	Average Droplet Volume (μL)	Standard Deviation
0	-	-
25	0.60	0.05
50	0.76	0.02
75	0.89	0.03
100	1.09	0.02
125	1.15	0.02
150	0.94	0.03
200	-	-

Figure S7. Outline and example of the method used to determine coacervate droplet volume. Protein and polymer mixtures were centrifuged in round bottom plates and overhead images were used to determine volume using a calibration. (A) Calibration curves were generated using mixtures of water, glycerol, and a small amount of Coomassie Blue. This range of glycerol concentrations spans a density range of $1.0 - 1.13 \text{ g mL}^{-1}$, compared to an approximate coacervate density range of $1.06 - 1.15 \text{ g mL}^{-1}$. Small volume droplets were submerged in a droplet of mineral oil to prevent evaporation. Raw images (top) were thresholded (bottom) and analyzed to create a calibration for droplet volume using the number of pixels detected. (B) Calibration curves for each glycerol concentration were generated spanning a volume of $0.5 - 2.0 \mu\text{L}$ (left). Due to varying amounts of salt, protein, and polymer in the coacervate phase a range of coacervate densities was expected. An average trendline (right) was used to compute the coacervate volume (X) from the number of pixels (Y). (C) Example overhead UV images of GFP and qP4VP coacervates (in triplicate) after centrifugation: raw (top), thresholded (middle), and pixels detected for each well (bottom). (D) Calculated average droplet volumes using the trendline in (B) at each NaCl concentration. Volumes at 0 mM were omitted due to spreading and an inability to threshold in many cases.

15. Fig. S8 - Testing for Equilibration in Phase Portraits for GFP mutant τ 6-2

Figure S8. Phase portraits for GFP mutant τ 6-2 at discrete NaCl concentrations (0*, 25, 50, 75, 100, 125, 150, 200 mM) under different incubation conditions. Phase portraits were made for sample groups that underwent longer equilibration times (24 h, 1 wk) compared to the standard incubation time of 10 min. Thermal equilibration was investigated using a thermal cycler, where protein, polymer, and salt were mixed according to protocol, heated to 50 °C (below denaturation temperature^{5,6}) and slowly ramped (0.1 °C/s) back to room temperature. Protein concentrations in the dilute phase (left) and coacervate phase (right) were determined using fluorescence measurements, using a calibration curve of overhead UV Images to determine the volume of the bulk coacervate phase (Figure S7). Dotted lines approximating the binodal phase boundary are simply a visual aid and do not represent theoretical modeling or fitting. The initial protein concentration of 1 mg mL⁻¹ has been indicated using a dotted vertical line. *Protein concentrations at 0 mM NaCl have been omitted due to the formation of less-hydrated films, where the volume of the coacervate could not be determined.

16. Fig. S9 - Summary of ITC Results and Parameters

A

	n_{IP}	n_{coac}	ΔH_{IP}	ΔH_{coac}	K_A	α
WT	0.270 ± 0.004	0.102 ± 0.005	3.99 ± 0.01	0.198 ± 0.040	54.7 ± 0.5	0.068 ± 0.006
τ 2-2	0.271 ± 0.004	0.165 ± 0.013	4.00 ± 0.01	0.682 ± 0.052	76.2 ± 1.3	0.086 ± 0.002
τ 6-2	0.303 ± 0.060	0.174 ± 0.005	4.00 ± 0.01	0.760 ± 0.095	117.5 ± 22.5	0.093 ± 0.005
(-12)	0.329 ± 0.023	0.138 ± 0.011	3.98 ± 0.01	0.446 ± 0.077	67.5 ± 5.0	0.110 ± 0.016

B

Figure S9. Summary and visualization of ITC fits for selected subset of protein mutants (A) Parameter values for ITC fits determined by a parameter fitting script written in MATLAB using the model described previously by Priftis et al.^{7,8} (B) MATLAB generated ITC fits using the Two-Step Binding Model. Error in parameter values represents the standard deviation between trials (N = 3).

17. MATLAB Code.

Coacervate Droplet Volume Analysis

```

I = imread('Droplet_Image_File.tif');
[J, rect] = imcrop(I);
I2 = imcrop(I,rect);

% Get the dimensions of the image. numberOfColorBands should be = 3.
[rows columns numberOfColorBands] = size(I2)

blockSizeR = 145; % Rows in block.
blockSizeC = 145; % Columns in block.

% Figure out the size of each block in rows.
% Most will be blockSizeR but there may be a remainder amount of less than that.
wholeBlockRows = floor(rows / blockSizeR);
blockVectorR = [blockSizeR * ones(1, wholeBlockRows), rem(rows, blockSizeR)];
% Figure out the size of each block in columns.
wholeBlockCols = floor(columns / blockSizeC);
blockVectorC = [blockSizeC * ones(1, wholeBlockCols), rem(columns, blockSizeC)];

% Now display all the blocks.
plotIndex = 1;
numPlotsR = size(ca, 1);
numPlotsC = size(ca, 2);
for r = 1 : numPlotsR;
    for c = 1 : numPlotsC;
        fprintf('plotindex = %d, c=%d, r=%d\n', plotIndex, c, r);
        % Specify the location for display of the image.
        subplot(numPlotsR, numPlotsC, plotIndex);
        rgbBlock = ca{r,c};
        imshow(rgbBlock); % Could call imshow(ca{r,c}) if you wanted to.
        [rowsB columnsB numberOfColorBandsB] = size(rgbBlock);
        caption = sprintf('Block #%d', ...
            plotIndex, numPlotsR*numPlotsC, rowsB, columnsB);
        title(caption);
        drawnow;
        % Increment the subplot to the next location.
        plotIndex = plotIndex + 1;
    end
end

A=nan(numPlotsR,numPlotsC); % A is a matrix of pixels
for n=1:numPlotsR
    for m=1:numPlotsC
        BW= imbinarize(ca{n,m});
        BW1=double(BW);
        [ymax,xmax] = size(ca{n,m});
        White_pix=0;
        Black_pix=0;
        for j=1:(xmax)-1
            for i=1:(ymax)-1
                if BW1(i,j)==1
                    White_pix = White_pix+1;
                else
                    Black_pix=Black_pix+1;
                end
            end
        end
        A(n,m)=Black_pix;
    end
end
end

```

ITC Fitting Analysis

Script

```
% Script to fit ITC data using a method by Sarah Perry's Group featured in Nature Communication 2017
% Sequence and entropy based control...

% Fitting for complexation (ion pairing) and complex coacervation will be
% separately done

% This method of analysis will include the fitting of 6 parameters:
% n_IP, n_coac, DH_IP, DH_coac, K_assoc, and alpha

C_Inj = 0.150; % mM
Vo = 200; % uL
```

Load Data

```
Data = importdata("t221.xlsx");
%InjV = Data(:,2); % uL
Xt = Data(:,3); % mM
Mt = Data(:,4); % mM
Ratio = Data(:,5);

Enthalpy_Exp = Data(:,9); % kJ/mol

% Theta_IP = 0.5.*(1 + (Xt./(n_IP.*Mt)))+(1./(K_assoc.*n_IP.*Mt))-
% sqrt((1+(Xt./(n_IP.*Mt)))+(1./(K_assoc.*n_IP.*Mt))).^2-(4.*Xt./(n_IP.*Mt)));
```

Optimization

```
params0 = [1,1,1,2,5,0.10];
lb = [0,0,0,0,0,0];
ub = [1,1,4,10,200,1];
options = optimoptions('fmincon','Algorithm','interior-point',...
    'MaxFunctionEvaluations',1e5,'MaxIterations',1e5,'TolX', 1e-10);

Z = fmincon(@(params)predictor(params,Data),params0,[],[],[],[],...
    lb,ub,[],options);

n_IP = Z(1);
n_coac = Z(2);
DH_IP = Z(3);
DH_coac = Z(4);
K_assoc = Z(5);
alpha = Z(6);

InjV = Data(:,2); % uL
Xt = Data(:,3); % mM
Mt = Data(:,4); % mM
Ratio = Data(:,5);
Enthalpy_Exp = Data(:,9); % kJ/mol

Theta_IP = 0.5.*(1 + (Xt./(n_IP.*Mt)))+(1./(K_assoc.*n_IP.*Mt))- ...
    sqrt((1+(Xt./(n_IP.*Mt)))+(1./(K_assoc.*n_IP.*Mt))).^2-...
    (4.*Xt./(n_IP.*Mt)));
```

```

Q_IP = (n_IP.*Theta_IP.*Mt.*DH_IP*Vo)./(C_Inj.*InjV);

DQ_IP = Q_IP(2:end) - Q_IP(1:end-1) + InjV(1:end-1).*(Q_IP(2:end) +...
    Q_IP(1:end-1))/(2*Vo);

f = Xt./(Xt+Mt);
f_coac = n_coac/(1+n_coac);
Theta_coac = exp(-(f-f_coac).^2/alpha.^2);

Q_coac = (n_coac.*Theta_coac.*Mt.*DH_coac*Vo)./(C_Inj.*InjV);

DQ_coac = Q_coac(2:end) - Q_coac(1:end-1) + InjV(1:end-1).*(Q_coac(2:end) +...
    Q_coac(1:end-1))/(2*Vo);

DQtot = DQ_coac + DQ_IP;

err = sum((Enthalpy_Exp(2:end) - DQtot).^2);

```

Predictor Function

```
function err = predictor(params,Data)
```

```

C_Inj = 0.150; % mM
Vo = 200; % uL

n_IP = params(1);
n_coac = params(2);
DH_IP = params(3);
DH_coac = params(4);
K_assoc = params(5);
alpha = params(6);

Enthalpy_Exp = Data(:,9); % kJ/mol

```

IP

```

Theta_IP = 0.5.*(1 + (Xt./(n_IP.*Mt)))+(1./(K_assoc.*n_IP.*Mt))- ...
    sqrt((1+(Xt./(n_IP.*Mt)))+(1./(K_assoc.*n_IP.*Mt)).^2-(4.*Xt./(n_IP.*Mt))));

Q_IP = (n_IP.*Theta_IP.*Mt.*DH_IP*Vo)./(C_Inj.*InjV);

DQ_IP = Q_IP(2:end) - Q_IP(1:end-1) + InjV(1:end-1).*(Q_IP(2:end) + Q_IP(1:end-1))/(2*Vo);

```

Coac

```

f = Xt./(Xt+Mt);
f_coac = n_coac/(1+n_coac);
Theta_coac = exp(-(f-f_coac).^2/alpha.^2);

Q_coac = (n_coac.*Theta_coac.*Mt.*DH_coac*Vo)./(C_Inj.*InjV);

DQ_coac = Q_coac(2:end) - Q_coac(1:end-1) + InjV(1:end-1).*(Q_coac(2:end) + Q_coac(1:end-1))/(2*Vo);

DQtot = DQ_coac + DQ_IP;

err = sum((Enthalpy_Exp(2:end) - DQtot).^2);

```

18. References.

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