



Advances in Understanding Stimulus-Responsive Phase Behavior of Intrinsically Disordered Protein Polymers

Kiersten M. Ruff¹, Stefan Roberts², Ashutosh Chilkoti² and Rohit V. Pappu¹

1 - Center for Biological Systems Engineering and Department of Biomedical Engineering, Washington University in St. Louis, St. Louis, MO 63130, USA

2 - Department of Biomedical Engineering, Duke University, Durham, NC 27708, USA

Correspondence to Ashutosh Chilkoti and Rohit V. Pappu: chilkoti@duke.edu; pappu@wustl.edu

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Abstract

Proteins and synthetic polymers can undergo phase transitions in response to changes to intensive solution parameters such as temperature, proton chemical potentials (pH), and hydrostatic pressure. For proteins and protein-based polymers, the information required for stimulus-responsive phase transitions is encoded in their amino acid sequence. Here, we review some of the key physical principles that govern the phase transitions of archetypal intrinsically disordered protein polymers (IDPPs). These are disordered proteins with repetitive amino acid sequences. Advances in recombinant technologies have enabled the design and synthesis of protein sequences of a variety of sequence complexities and lengths. We summarize insights that have been gleaned from the design and characterization of IDPPs that undergo thermo-responsive phase transitions and build on these insights to present a general framework for IDPPs with pH and pressure responsive phase behavior. In doing so, we connect the stimulus-responsive phase behavior of IDPPs with repetitive sequences to the coil-to-globule transitions that these sequences undergo at the single-chain level in response to changes in stimuli. The proposed framework and ongoing studies of stimulus-responsive phase behavior of designed IDPPs have direct implications in bioengineering, where designing sequences with bespoke material properties broadens the spectrum of applications, and in biology and medicine for understanding the sequence-specific driving forces for the formation of protein-based membraneless organelles as well as biological matrices that act as scaffolds for cells and mediators of cell-to-cell communication.

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Introduction

Polymer solutions undergo phase transitions in response to changes in specific stimuli [1]. Signatures of phase transitions include sharp changes in physical properties such as polymer density, translational and rotational symmetry, miscibility, viscosity, and elasticity [2]. For a two-component polymer plus solvent system, the stimuli can be changes to colligative properties achieved by adding a ternary component or changes to intensive solution parameters such as temperature (T) [3–6], proton chemical potential (pH) [7–9], and hydrostatic pressure (P) [10, 11]. Phase transitions that arise in response to changes in colligative properties involve the coupling among the

polymer, the solvent, and the ternary component. In contrast, responses to changes in intensive solution parameters reflect the contributions from conformational and solvent entropy, and the interplay among chain–chain, chain–solvent, and solvent–solvent interactions that derive from the nature of the solvent and the chemistry of the repeating units as well as side-groups of the polymer. Here, we focus on the physical principles that govern the phase behavior of polymers that respond to changes in intensive solution parameters.

A first-order transition that leads to the emergence of a polymer-deficient phase that is in equilibrium with a polymer-rich phase is known as *phase separation* [12]. This phase transition is governed by the free energy of

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mixing, written in terms of a conserved order parameter, which is the total concentration or volume fraction (ϕ) of the polymer in solution. Phase separation is accompanied by a sharp change in density whereby the polymer-deficient phase is dilute and the polymer-rich phase is dense [12]. Phase separation can drive additional continuous transitions that involve changes to non-conserved order parameters such as translational or rotational symmetry, polymer conformation, and the extent of physical crosslinking among polymers in the dense phase [13–15].

The physical concepts underlying stimulus-responsive phase separation that might be accompanied by additional continuous transitions such as conformational changes and physical crosslinking are of direct relevance to understanding the biogenesis of protein and RNA-based membraneless organelles. These systems, which are also referred to as *biomolecular condensates*, include bodies such as nucleoli, nuclear speckles, cytoplasmic bodies, P granules, and stress granules [16–22]. Other condensates are less well defined as compartments within the cell, and yet, they are important for regulating cellular functions including actin assembly and T-cell signaling [23–25]. Proteins can also form hydrogels such as FG-rich nucleoporins that make up the nuclear pore complex to control the transport of molecules between the nucleus and the cytoplasm [26]. While it is unclear if gelation of FG-rich nucleoporins requires phase separation *in vivo*, many of these proteins have been shown to undergo phase separation and gelation *in vitro* [27–29].

Multivalency, which refers to the presence of multiple folded domains, multiple associative motifs also known as short linear motifs (SLiMs) [30], or some combination of the two, is a defining feature of proteins that drive phase separation [19, 23, 31]. Surface patches/hot spots on domains and/or SLiMs contribute to stereospecific intra- and intermolecular interactions. Non-stoichiometric interactions among collections of multivalent molecules can drive continuous changes such as networking transitions that are also known as sol–gel transitions through physical crosslinks [14, 15, 32]. Depending on the range, hierarchy, and types of interactions involved, multivalent proteins can also undergo phase separation [23]. If phase separation occurs at concentrations that are lower than the gel point, then phase separation can lead to gelation because the concentration within the dense phase can be higher than the gel point. Alternatively, gelation will occur without phase separation if the gel point is lower than the threshold concentration for phase separation.

Intrinsically disordered regions (IDRs) can serve as scaffolds for associative motifs, as enablers of phase separation, as modulators of phase behavior, and as generators of multivalency [31, 33, 34]. Multivalency originating from the presence of IDRs is likely a key determinant of the liquid-like, dynamic

properties of intracellular condensates formed through phase separation [35]. While it is possible for fully folded domains to undergo phase separation, many intracellular condensates include proteins with disordered regions [30]. *In vitro* studies of these disordered domains have revealed their ability to phase separate under physiologically relevant conditions [17, 18, 36]. While some intrinsically disordered proteins (IDPs) do require additional binding events to trigger phase separation [24], many can drive phase separation and gelation without the need for folded domains [17, 18, 33]. Our focus here is on intrinsically disordered protein polymers (IDPPs), which are repetitive sequences defined by a multivalency of associative SLiMs within IDRs.

The key question with regard to sequence-encoded phase transitions of IDPPs is as follows: Why do certain sequences respond spontaneously to specific types of stimuli in specific concentration regimes, producing condensates with distinctive material properties? The ability to design and recombinantly synthesize protein sequences of arbitrary lengths with precise repetitive patterns that engender the requisite valency of associative motifs has opened the door to uncovering the principles that govern stimulus-responsive phase behavior of IDPPs [37–39]. The zeroth-order system of interest is a binary mixture comprising of a finite concentration of a specific IDPP and solvent. All the information required for stimulus-responsive phase behavior of IDPPs should be encoded in the primary sequence. The stimulus alters the balance of chain–chain and chain–solvent interactions, and this response is governed by the sequence and the nature of the stimulus.

Nature uses IDPPs in a variety of contexts, especially as scaffolds for extracellular matrices, plant cell walls, and other fibrous composites [40, 41]. These proteins encompass long repetitive stretches of amino acid motifs, and many of these protein polymers undergo stimulus-responsive phase behavior. Well-known examples of IDPPs that undergo stimulus-responsive phase transitions are polymers made of IDRs derived from elastomeric proteins that include proteins such as resilins, elastins, proteins from spider silks, fibrillin, titin, and gluten [42, 43]. Elastin-like polypeptides (ELPs) were arguably the first IDPPs to be studied as biomaterials, decades before the prevalence and importance of intracellular protein disorder was formally established [44–46]. Sequences of designed polymers that are based on elastomeric proteins serve as ideal systems to understand the physical principles that govern stimulus-responsive phase behavior of proteins with IDRs [47]. Techniques used to study elastomeric proteins through IDPP design can also be applied to understand phase separation of biomolecular condensates [48]. Here, we review the concepts that underlie phase transitions of IDPPs in response to changes in intensive solution

parameters and summarize findings that have emerged from recent studies.

Thermoresponsive phase transitions

IDPPs can undergo four types of phase transitions in response to changes in solution temperature. Schematics of coexistence curves for each of the scenarios are shown in Fig. 1a–d.

Systems that show UCST behavior

In the ϕ – T plane, where ϕ denotes the volume fraction of the IDPP in question, for fixed temperatures below T_{uc} (see Fig. 1a), an increase in ϕ will lead to a metastable or unstable supersaturated mixed phase, leading to phase separation and the formation of two coexisting phases namely, a dilute phase of volume fraction ϕ_L and a dense phase of volume fraction ϕ_H . The driving forces for phase separation weaken with increased temperature and above a critical temperature T_{uc} the IDPP plus solvent system forms a single well-mixed phase. The volume fraction of the system at T_{uc} is denoted as ϕ_c and T_{uc} is the *upper critical solution temperature* or UCST.

The classical Flory–Huggins theory [49, 50] has served as a powerful predictive framework for understanding the phase behavior of systems showing UCST behavior, even for systems that undergo so-called complex coacervation [51]. Flory–Huggins theory is based on mean field considerations. It does not explicitly account for polymer conformations, their fluctuations, and the impact of these fluctuations on changes to ϕ [49, 50, 52, 53]. Other omissions include the impact of the effects sequence patterning on intra- as well as intermolecular interactions. Lin *et al.* [54–56] have introduced a generalization that accounts for sequence correla-

tions among charged residues by adapting the modified random phase approximation for polyelectrolytes first introduced by Ermoshkin and Olvera de la Cruz [57]. It is worth noting, however, that despite the numerous omissions [52], the Flory–Huggins theory serves as a zeroth order “practical device” that can be co-opted to generate predictive phase diagrams of long IDPPs, especially for temperatures that are well below T_{uc} .

In Flory–Huggins theory, entropy favors mixing, whereas the enthalpic contributions can lead to demixing. To zeroth order, the enthalpic contribution captures the balance of chain–chain, chain–solvent, and solvent–solvent interactions in terms of a single parameter denoted as χ , although later refinements showed that χ captures both the enthalpic and entropic aspects of mixing/demixing (see below). If χ is positive, then enthalpy favors demixing and if χ is negative then miscibility is driven in part through favorable chain–solvent interactions. In the original Flory–Huggins theory, the enthalpies assumed to be independent of temperature. Accordingly, since χ is inversely proportional to T it χ approaches zero, becoming less positive, as T increases, and χ is negative in the one-phase regime above T_{uc} .

Figure 1a shows the coexistence curve for a system with UCST phase behavior. At a given solution temperature, $T < T_{uc}$, the width of the two-phase regime is $w_T = (\phi_H - \phi_L)/\phi_c$. For systems that show UCST behavior, ϕ_L will increase, whereas ϕ_H will decrease with increasing temperature. Accordingly, $w_T \rightarrow 0$ as $T \rightarrow T_{uc}$ because ϕ_L approaches ϕ_c from the left and ϕ_H approaches ϕ_c from the right. Mean field theories cease to be applicable as $w_T \rightarrow 0$. It is also well known that in contrast to the expectations from an unmodified Flory–Huggins theory, which ignores changes to volume upon mixing, there in fact are discernible changes to the volume per residue upon mixing. These changes and packing interactions of solvent around the chain

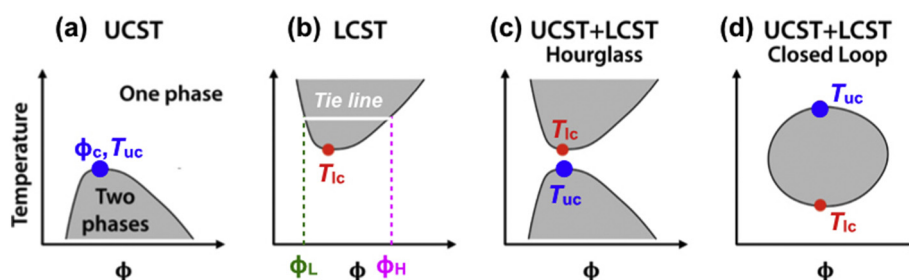


Fig. 1. Four types of thermoresponsive phase transitions for IDPPs. (a) The coexistence curve (binodal) for systems that show *upper critical solution temperature* (UCST) behavior. (b) The binodal for systems that show *lower critical solution temperature* (LCST) behavior. (c and d) Binodals for systems that show UCST and LCST behavior. Gray shaded areas denote the two-phase regimes, whereas white areas denote the one-phase regimes. T_{uc} and T_{lc} denote the upper critical and lower critical solution temperatures, respectively, where a system separates into two phases below T_{uc} and above T_{lc} . ϕ denotes the volume fraction of the IDPP, ϕ_c denotes the volume fraction of the IDPP at the critical temperature, and ϕ_L and ϕ_H denote the volume fractions of the coexisting dilute and dense phases, respectively, at a given temperature, T .

or of chain around chain necessitate recasting χ to ensure that the temperature-independent packing effects are separated from the temperature-dependent weakening of chain–chain as well as chain–solvent interactions and the gain of solvent–solvent interactions. Accordingly, χ can be empirically recast as $\chi \approx A + B/T$, where A is the temperature-independent entropic part and B/T is the temperature-dependent enthalpic part. This empirical formulation was first introduced by Flory [58] and is described in pedagogical detail by Rubinstein and Colby [13].

Systems that show LCST behavior

ELPs and variants of these sequences show a very different phase behavior in response to temperature. These systems form miscible phases at temperatures below the *lower critical solution temperature* or LCST [38, 59]. For temperatures above T_{lc} , the miscible phase becomes saturated as ϕ increases. Above a saturation volume fraction, the system separates to form two coexisting phases, a dilute phase and a dense phase as shown in Fig. 1b.

The physical principles governing LCST behavior have been made clear through detailed studies of synthetic polymer systems such as poly-*N*-isopropylacrylamide (PNIPAM). This system comprises a polymethylene backbone and a propionamide side-group (mimic of the sidechain of glutamine) attached to each of the backbone carbon atoms. For long chain lengths, PNIPAM forms well dispersed solutions below 33 °C and phase separates above 33 °C [60–62]. In systems like PNIPAM, the miscible phase is stabilized by the presence of the secondary amides in the side groups. Solvation of amides requires that the solvent be organized around the hydrophobic moieties that include the backbone carbon chain and the isopropyl group in the sidechain. The entropic cost for organizing solvent molecules around individual chains increases with increasing temperature. Accordingly, above the critical temperature and for volume fractions that are greater than a threshold value, the chain separates from the solvent leveraging a combination of the gain in solvent entropy through the release of solvent molecules and the gain of favorable inter-chain interactions, such as hydrogen-bonding interactions among secondary amides in the PNIPAM system.

Adaptation of the Flory–Huggins theory to describe LCST behavior requires a generalization of the model that uses temperature-dependent interaction strengths, such that the balance of chain–chain, chain–solvent, and solvent–solvent interactions disfavors chain–solvent interactions as T increases. In the mean-field formalism, this can be captured by making χ negative below T_{lc} , zero at T_{lc} , and positive above T_{lc} . This approach is useful because it provides a route to develop a phenom-

enological model that leverages experimental data regarding the temperature dependence of solubilities for repeating units and/or second-virial coefficients as a way to parameterize χ and reproduce LCST behavior. Simon *et al.* [63] pursued a variant of this approach in their recent study of ELPs. Of course, it should be noted that changing the sign of χ is a purely *ad hoc* approach. A more satisfying way to model LCST behavior in the context of a mean-field model is to recognize that the hydrophobic effect, the solvation of charged moieties, and the solvation of proline-rich regions have marked temperature dependencies. These solvation-mediated changes to χ can be captured by weakening/strengthening the solvent-mediated attractive interactions among chain moieties as temperature decreases/increases. Several approaches have been proposed to generalize the Flory–Huggins model for capturing LCST behavior [64–70]. Motivated by the LCST behavior for elastins, Lin *et al.* [71] have described an adaptation of mean-field approaches prescribed in the polymer physics literature that could prove to be useful for fitting to extant data on IDPPs that undergo LCST behavior.

One might reasonably argue that reparameterizing the Flory–Huggins model through temperature-dependent interactions glosses over the cooperative processes at the molecular level that give rise to LCST behavior. This is a valid criticism. A thorough molecular-level understanding of systems undergoing LCST behavior requires detailed molecular simulations using explicit representations of solvent molecules. Alternatively one can also model differences in sequence-specific LCST behavior using atomistic simulations based on implicit solvation models that have the ability to reproduce the temperature dependence of solvation processes [72]. These simulations can provide the necessary inputs for machine-learning aided coarse-graining that helps in modeling phase behavior [73].

The driving forces for LCST behavior of elastin and its derivative IDPPs have been the topics of much discussion. The high prevalence of Gly and Pro residues and evidence for marginally stable turn structures within the polymer have confounded discussions regarding the contributions to the elasticity of elastin and its overall phase behavior [44, 74–79]. After nearly five decades of debate that ultimately required the use of molecular simulations, researchers have concluded that elastomeric IDPPs undergoing phase behavior do not collapse into an entropically disfavored structure rich in beta turns [77, 80–82]. Rather, similar to PNIPAM, unfavorable solvent entropy drives their collapse into amorphous coacervates [42, 82].

Systems with UCST and LCST behavior

Systems with block-copolymeric architectures can have sequence regions with UCST behavior that are

interspersed by sequence regions with LCST behavior. Such systems can give rise to two types of coexistence curves [83]. If the critical temperature T_{uc} for the region with UCST behavior is lower than T_{lc} , the critical temperature for the regions with LCST behavior, then we obtain two distinct coexistence curves with an overall hourglass shape for the phase diagram (see Fig. 1c). If $T_{lc} < T_{uc}$, then the phase diagram will have a closed loop profile as shown in Fig. 1d. Overall, it becomes clear that even for a simple two-component system comprising the IDPP and solvent, the phase diagrams for phase separation can show considerable complexity depending on the numbers (valence) and the types (LCST versus UCST) of sequence blocks that are incorporated into the IDPP [38, 84].

UCST and LCST behavior in IDPPs

Elastomeric proteins such as tropoelastin and titin contain regions that have repetitive motifs that are rich in Pro and Gly [85, 86]. There is emerging consensus that the Gly and Pro rich regions are intrinsically disordered [87]. A threshold composition of Pro and Gly residues seems to be important in order to favor functional elastomeric properties over amyloid formation [87]. Gly residues engender flexibility, whereas Pro residues interrupt the propagation of stable secondary structures. Together, the enrichment of Gly and Pro residues promote disorder within elastomeric proteins thus disfavoring ordered assemblies. However, this compositional bias alone does not account for the rich phase behavior of elastomeric protein polymers. Resilins and tropoelastins are similar in having high Pro and Gly contents. However, resilins show UCST and LCST phase behavior, whereas tropoelastins show LCST phase behavior [38, 86]. These results suggest that additional sequence features modulate the precise control of IDPP phase behavior.

Quiroz and Chilkoti [38] analyzed the compositions of various elastomeric proteins to uncover sequence features that distinguished tropoelastins from resilins. Resilins are enriched in charged and polar residues, with approximately equal numbers of negatively and positively charged residues and a clear preference for Arg over Lys (Fig. 2c). In contrast, in positions that are not occupied by Gly or Pro, there is an enrichment of nonpolar hydrophobic residues in tropoelastins (Fig. 2a). In addition, there is a depletion of negative charges, and a preference for Lys over Arg in tropoelastins.

To test whether the observed sequence features within resilins and tropoelastins could encode UCST versus LCST phase behavior, Quiroz *et al.* designed repetitive sequences with P-X_n-G motifs. Here, *n* varied from zero to four amino acids and X was chosen to mimic the sequence features of either

tropoelastins or resilins. Designed sequences with resilin-like motifs showed UCST behavior, whereas those with tropoelastin-like motifs showed LCST behavior. Taken together, these results suggest that a set of sequence features might be required for UCST behavior, within a background of Pro and Gly residues: (1) the overall fraction of charged residues should be greater than 0.2; (2) the net charge per residue should lie between −0.05 and 0.05; (3) the fraction of positively charged residues that are Arg should be greater than 0.85; and (4) the overall fraction of aromatic residues should be greater than 0.1. The last criterion was added based on the observation that this inclusion was necessary for designed IDPPs to show UCST behavior at physiologically relevant temperatures.

How robust are composition-based heuristics for predicting IDPP phase behavior and do they apply to IDPs that undergo thermoresponsive phase behavior? As a test case, Quiroz *et al.* examined the sequence composition of the low-complexity domains (LCDs) of three RNA-binding proteins known to exhibit UCST behavior (hnRNPA1, hnRNPA2, and TIA-1) [17, 89]. The LCDs showed similar compositional biases to that of resilins, namely, an enrichment in Arg, Asn, Ser, and Tyr, and a similar hydropathy balance (Fig. 2d). However, the arginine enrichment is considerably lower than that of resilins. This provides a rationale for why the LCDs in question phase separate at higher concentrations than those of resilin-like systems. In support of this finding, Wang *et al.* [90] recently showed that the phase behavior of FET family proteins requires both the Tyr-rich LCD and the Arg-rich RNA binding domains. Another system that shows UCST behavior is the Ddx4 protein [18]. Ddx4 is a DEAD-box helicase and a primary constituent of nuage bodies. Full-length Ddx4 and the N-terminal IDR of Ddx4 (residues 1–236) phase separate in cells and *in vitro*. The IDR of DDX4 shows UCST behavior. The IDR of Ddx4 has compositional characteristics that are similar to the IDPPs that Quiroz *et al.* designed to have UCST behavior (Fig. 2d). Mutation of nine or more Phe residues to Ala or 24 Arg residues to Lys decreased the drive to phase separate [36, 91]. Phase separation occurs below 0 °C in the absence of salt when 14 Phe residues are mutated to Ala. Phase separation was not observable under any of the conditions tested when 24 of the Arg residues were mutated to Lys [36]. These results implicate a role for cation–pi and pi–pi interactions as contributors to the driving forces for UCST phase behavior, although the work of Wang *et al.* [90] suggests a more nuanced explanation for the interplay among networks of aromatic and cationic residues. In summary, UCST behavior is the result of both entropic and enthalpic contributions from hydrophobic interactions, electrostatic interactions, cation–pi interactions, and pi–pi stacking. Although it is progressively becoming easier to recognize UCST domains based on their amino acid sequence features,

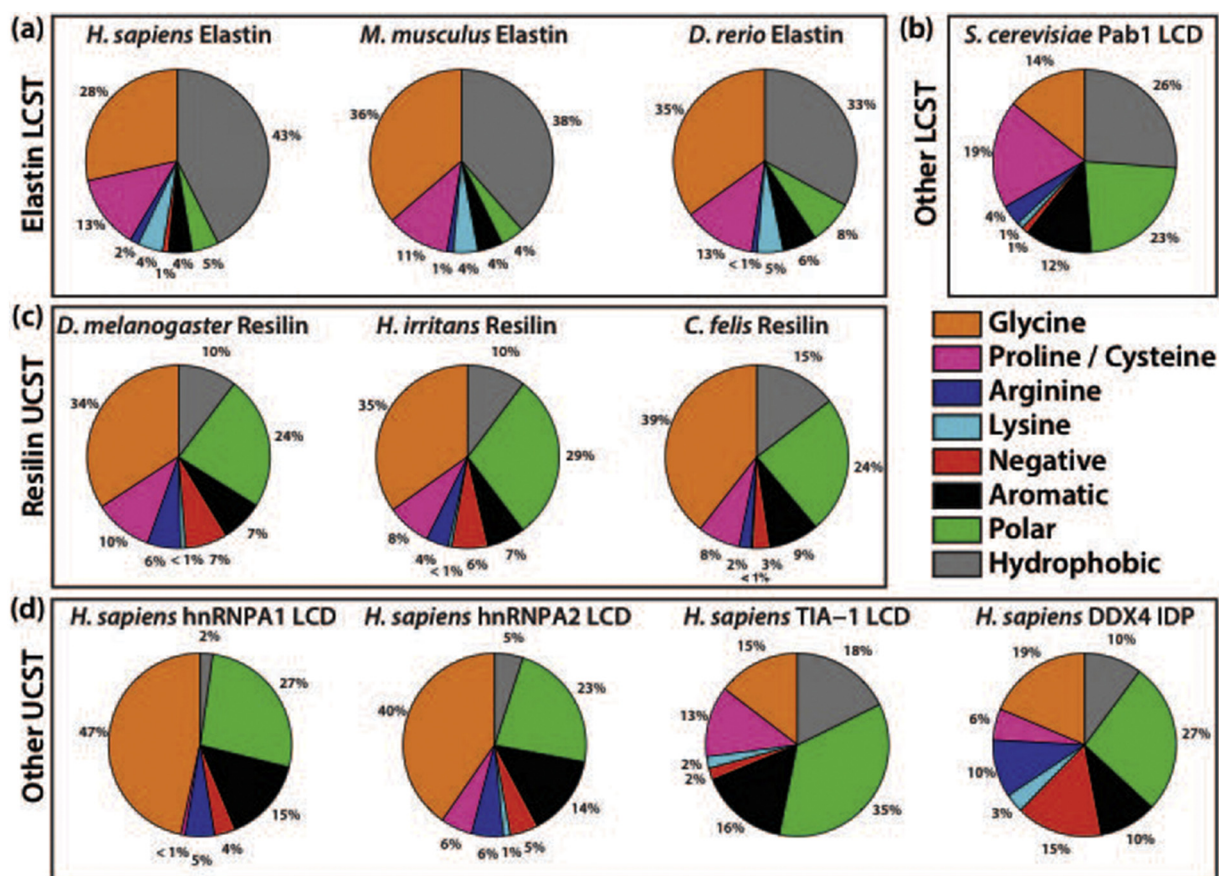


Fig. 2. Fraction of different types of residues within IDPPs and IDPs that undergo LCST (a and b) or UCST (c and d) behavior. (a) Elastin sequences that show LCST behavior examined by Quiroz and Chilkoti (P15502, P45320, gi 121583675) [38]. (b) Low-complexity, proline-rich domain of Pab1 (P04147) [88]. (c) Resilin sequences that show UCST behavior examined by Quiroz *et al.* (Q9V7U0, G4Y9J3, G4Y9J1) [80]. (d) Low-complexity domains (LCDs)/IDRs that show UCST behavior (P09651: 186–320, P22626: 181–341, P21483: 280–375, Q9NQI0: 1–236) [18, 38]. IDPPs and IDPs that show LCST behavior are generally depleted in negative charge residues (red), enriched in hydrophobic residues (gray), and prefer Lys (cyan) to Arg (blue). IDPPs and IDPs that show LCST behavior are generally enriched in polar residues (green), have approximately equal numbers of positively charged (cyan and blue) and negatively charged (red) residues, and prefer Arg (blue) over Lys (cyan).

the *de novo* design of UCST polymers, especially those with specific transition temperatures, remains an intriguing and open challenge.

LCST behavior, on the other hand, is thought to be driven mainly by the gain in solvent entropy and a concomitant chain collapse. Oddly enough, LCST behavior has proven to be easier to design and manipulate. Although Gly aids in chain flexibility, a combination of Pro residues and a suitable number of hydrophobic groups are minimal necessities to encode LCST behavior. Pro residues play a crucial role in determining LCST behavior. High polymers of poly-L-Pro have a measurable LCST at ~70 °C [92–94]. Pro is an imino acid as opposed to an amino acid. N-substitution that leads to its tertiary amide character causes a loss of a hydrogen bond donor. In addition, the cyclization alters the *cis* to *trans* ratio of the peptide bond. Although Pro cannot

participate as a hydrogen bond donor, the backbone carbonyl oxygen remains a potent hydrogen bond acceptor. The carbonyl oxygen moieties are most likely satisfied by hydrogen bonding to water. Accordingly, at room temperature, the solubility of L-Pro is the highest when compared to all other amino acids [95]. The combination of chain stiffness and the unsatisfied acceptor should lead to organization of water molecules along the backbone. As temperature increases, so does the entropic cost for hydrating the backbone. The release of bound water molecules and the increased *cis* content within the chain engenders the formation of dense liquid crystalline phases [92]. The entropic cost for organizing water around hydrophobic groups will decrease with decreasing temperature and this is further offset by the gain in solvent enthalpy.

The transition temperature of sequences that show LCST behavior can be modulated either by changing

the hydrophobicity of the amino acids or their charge [8, 47, 80, 84, 96]. In IDPPs such as ELPs, one can tune the LCST by assigning one of the residues in a repeat motif as a “guest” residue and altering its identity. Increasing the hydrophobicity of this guest residue will lower the LCST assuming all other polymer conditions remain identical. As demonstrated by Quiroz and Chilkoti [38], the average hydrophobicity of a repeat sequence is, however, not a direct predictor of the LCST. Sequence patterning plays an important role and this is reminiscent of findings connecting the patterning of charged and uncharged residues to the conformational features [97–101] and phase behavior of IDPs [22, 31, 37, 54–56, 71, 102]. Intra-chain interactions, particularly hydrogen bonding between Gly and Pro, must contribute others factors which determine the LCST.

Charged residues also provide significant tunability of the LCST behavior, although their presence is not required for realizing LCST behavior. The free energy of solvation of charged groups such as the carboxyl moieties of Asp/Glu, the guanido moiety of Arg, or the tertiary amine in Lys is on the order of -10^2 kcal/mol at room temperature. However, increasing the temperature increases the entropic penalty for organizing water molecules around the ions, and this is reflected in decreased magnitudes of the free energies of solvation as temperature increases [72]. The drop in favorable solvation free energies is rather substantial, thus implicating charges as important determinants of LCST behavior. Although LCST behavior is intimately tied to the details of temperature dependent hydration, it is possible to capture many of the phenomenological trends with accurate atomistic simulation paradigms that use mean-field descriptions for solvation effects, as is the case with the ABSINTH implicit solvation model and forcefield paradigm [72, 103]. This opens the door to systematic designs of sequences with bespoke LCST behavior. A particularly useful feature is the connection between single-chain collapse transition and phase behavior as discussed next.

Connecting collapse transitions of individual IDPPs to phase behavior

The interplay between intra-chain and chain-solvent interactions also influences the collapse transitions that individual polymers undergo in dilute solutions in response to changes in temperature [13, 104]. Unlike phase separation, which is a first-order transition, the collapse transition is a second-order transition, defined by a tricritical point also known as the theta point or theta temperature (T_θ). For systems that show UCST behavior, above T_θ the polymer in dilute solutions adopts conformations that belong to the same universality class as self-

avoiding walks and below T_θ , the chain collapses to form dense globules. Collapse leads to a decrease in radius of gyration (R_g), thus increasing the density of chain units around one other. Thermal blobs define the key length scale for collapse transitions. A thermal blob is the largest segment within the polymer chain that maintains Gaussian behavior irrespective of the temperature [13, 105]. Accordingly, the transitions that a chain undergoes can be described in terms of the interplay between blob-solvent and inter-blob interactions. Above T_θ , blob-solvent interactions are favored, and the inter-blob interactions are repulsive leading to chain expansion above its dimensions at T_θ . Below T_θ , the inter-blob interactions are attractive and the blob-solvent interface is characterized by an interfacial tension that becomes increasingly positive as temperature decreases.

For generic flexible polymers that lack a rich chemical diversity of interactions, there can be a direct connection between the collapse transitions of individual chains in dilute solutions and phase separation that is realized at finite concentrations [106]. The connections between collapse transitions and phase separation are summarized in Fig. 3 for the four categories of temperature-driven transitions. In the collapsed state, blob-blob interactions are to be maximized, while the blob-solvent interface is to be minimized. As ϕ increases, there is an increased likelihood of intermolecular interactions. Clusters of polymers form through intermolecular interactions to minimize chain-solvent interactions and maximize intra- as well as inter-chain interactions. Within clusters of chain molecules, inter-blob interactions can be realized within an individual chain and across multiple chains. When clusters cross a threshold size, phase separation results if the collective interactions among blobs stabilize dense polymer phases characterized by networks of intermolecular interactions. The lowering of blob-solvent interfacial tension in dense polymer assemblies can also engender chain expansion in the context of the dense phase. The two coexisting phases at temperatures well below T_θ would therefore correspond to a dilute phase of dispersed globules that minimize the chain-solvent interface and a dense phase characterized by collective interactions among blobs from networks of molecules that could give rise to chain expansion in the context of the dense phase.

For systems that show UCST behavior, the chain will form collapsed states in dilute solutions and undergo phase separation at finite concentrations for temperatures below T_θ . For finite-sized chains, T_θ will be higher than T_{uc} and the latter will converge upon T_θ as the chain becomes infinitely long. The opposite scenario applies for systems that show LCST behavior, whereby collapse occurs at temperatures above T_θ . Similarly, for finite-sized chains, T_θ will be lower than T_{lc} and the latter will converge

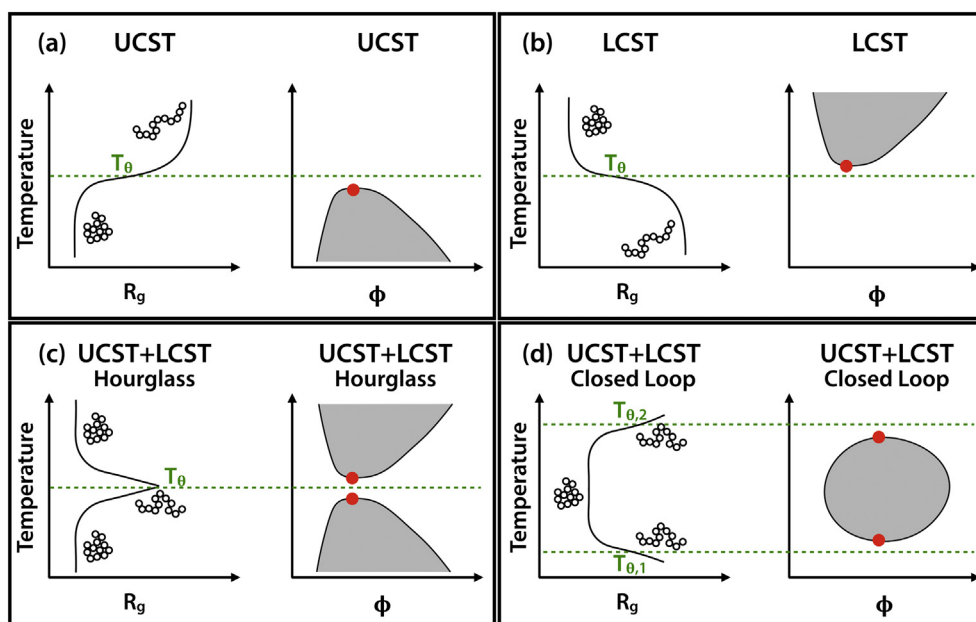


Fig. 3. Connection between the single-chain collapse transition and phase behavior for systems that show UCST behavior (a), LCST behavior (b), or both UCST and LCST behavior (c and d). Inserts in the R_g versus T plots denote representative conformations of the IDPP. T_θ denotes the theta temperature, that is, the temperature at which the IDPP behaves like a Gaussian chain. For UCST systems, the single-chain will collapse below T_θ , whereas the single-chain will expand below T_θ for LCST systems. For the ϕ versus T phase diagrams, the red circles denote the critical temperatures, the gray areas denote the two-phase regimes, and the white areas denote the one-phase regimes. For finite-length polymers, T_θ will be above the critical temperature for UCST systems and below the critical temperature for LCST systems.

upon T_θ as the chain becomes infinitely long. It is worth noting, however, that the requirement for Pro residues in IDPPs that show LCST behavior will engender local stiffness into the chain. As a result, the collapse transition will be considerably more gradual than would be the case for generic flexible polypeptides. The scenario depicted in Fig. 3b will likely require IDPPs that are several hundreds of residues long. The driving forces for collapse and phase separation will become more pronounced with increasing chain length and this is true for systems that exhibit UCST and LCST behavior.

Systems with UCST and LCST behavior will show different types of collapse transitions. There will be a reentrant collapse transition with a single T_θ for chains with hourglass-shaped coexistence curves. The upper and lower critical solution temperatures T_{uc} and T_{lc} will lie below and above T_θ , respectively (Fig. 3). For systems characterized by closed-loop phase diagrams, there will be two theta temperatures that respectively lie below T_{lc} and above T_{uc} for finite-sized polymers.

Leveraging the connections between collapse transitions and phase behavior

Considerable advances have been made toward quantifying the dimensions of individual IDPs and

IDPPs as a function of temperature, pH, and salt concentration. From an experimental standpoint, the dimensions of IDPPs can be measured at the single-molecule level using single-molecule Förster resonance energy transfer, two-focus fluorescence correlation spectroscopy, and single-molecule force spectroscopy [107–109]. If confounding effects of aggregation that arise at high concentrations can be circumvented, then methods like small angle X-ray scattering and pulse field gradient NMR spectroscopy become useful ensemble-based methods to deploy for measuring the change in dimensions of ensembles of non-aggregating molecules as a function of the desired stimulus [110, 111]. For long IDPPs, laser light scattering provides a way to access information about the radius of gyration (R_g) and the hydrodynamic radius (R_h) as has been shown for synthetic polymers in aqueous solutions [112].

Quantitative studies of phase separation that are designed to uncover the driving forces for and the dynamics of the process are difficult because most spectroscopic methods are ill-equipped to enable signal deconvolution given the presence of two coexisting phases or the onset of a phase transition. Microscopy has an important role to play in this regard as does the three-way synergy among experiment, computation, and theory. Experiments can be deployed to quantify the collapse transition as a function of the relevant intensive solution

parameters. From data that quantify the variation of R_g or R_h (preferably both) as a function of T , one can extract the values of the two- and three-body interaction coefficients from knowledge of the extent of compaction, the thermal stability of the collapsed states, and the steepness of the globule to coil transitions. An independent test of the accuracy of the inferred values for the two-body interaction coefficients can be made using laser light scattering or measurements of osmotic coefficients that yield independent estimates of second and possibly even third virial coefficients as a function of temperature. The measured parameters can be deployed in a suitable theoretical framework such as the Flory-Huggins theory or adaptations thereof to calculate phase diagrams for the IDPP in question [49, 50]. This methodological pipeline will be particularly useful for comparative assessments of sequence-specific variations in phase behavior.

The connections between single-chain collapse transitions and phase separation open the door to using molecular simulations as a route to design sequences with bespoke phase behavior or obtain comparative assessments of phase transitions for a family of sequences. This is made possible by advances in simulations based on explicit representations of solvent that are deployable for small numbers of sequences [113, 114]. If one seeks throughput without loss of predictive accuracy, then implicit solvation models such as ABSINTH retooled to capture the temperature dependence of reference solvation free energies becomes a strategy that one can deploy for reasonably high-throughput assessments of collapse transitions across large numbers of sequences [72, 103, 115]. The simulations yield information regarding the density of collapsed states, the diluteness of expanded states, comparative steepness of different collapse transitions, the theta temperatures, and an automatable strategy for identifying blobs [116]. From this information one can extract two- and three-body interaction coefficients and calculate full phase diagrams using suitable theories. Accuracies of the calculated temperature dependent, sequence-specific collapse transitions can be assessed through comparisons to measurements. Machine learning methods provide a way to optimize forcefield parameters to improve the accuracy of the calculations [117].

In effect, the advances in experiments and computations that have enabled improved characterizations of the collapse transitions of IDPPs can be leveraged to obtain high-throughput comparative assessments of sequence-encoded stimulus-responsive phase behavior. The methods will be applicable if there is a demonstrable coupling between collapse and phase separation. Recent studies have shown that the collapse transition can also be decoupled from phase separation for sequences such as the IDR from the protein LAF-1 [53]. This unique feature of heteropolymers requires

a new way of thinking of sequence-encoded phase behavior. However, for highly repetitive IDPPs, the proposed connections between single-chain collapse transitions and phase behavior will hold. Quantitative studies of collapse transitions then become usable in a predictive manner with the aid of suitable theories.

Polyelectrolytic and polyampholytic IDPs can undergo pH-responsive phase behavior

One of the defining hallmarks of many IDRs is the enrichment of charged residues (Asp, Glu, Arg, and Lys). Sequences that are enriched in charges can be cationic or anionic polyelectrolytes, symmetric polyampholytes, or asymmetric polyampholytes. These designations are made clear in terms of compositional parameters such as the fraction of Lys/Arg (f_+) residues, the fraction of Asp/Glu residues (f_-), the fraction of charged residues where $FCR = (f_+ + f_-)$, and the net charge per residue where $NCPR = (f_+ - f_-)$ [98]. These compositional parameters are calculated based on the charge states of amino acid sidechains at neutral pH. Of particular interest are sequences where the FCR is greater than 0.25. These are sequences where the charge content exerts significant influence over the solubility profiles, conformational preferences and overall phase behavior. For sequences where $FCR > 0.25$, anionic polyelectrolytes are enriched in Asp/Glu and deficient in Lys/Arg, whereas cationic polyelectrolytes are enriched in Lys/Arg and deficient in Asp/Glu. The NCPR is close to zero for symmetric polyampholytes, whereas asymmetric polyampholytes have high fractions of positive and negative charge, with a preference for one over the other. Note that the designations listed above only consider the impact of four residues on FCR and NCPR. The presence of ionizable groups such as His will also contribute to changes in FCR and NCPR depending on the charge state. Histidine is of particular interest in biomedical applications as its pK_a is physiologically relevant, creating a polyelectrolyte in mildly acidic conditions. The use of His to induce pH responsiveness has, for example, been modeled previously in elastin-based IDPPs [8] for better intra-tumoral drug distribution. Post-translational modifications such as Ser/Thr/Tyr phosphorylation, Lys acetylation, Arg citrullination, or Asn/Gln deamidation can also contribute to changes in FCR and NCPR.

IDPs/IDPPs that are enriched in charged residues or have charge groups added/structurally regulated via post-translational modifications can undergo pH-responsive changes to conformation and phase behavior. Figure 4 summarizes the types of collapse transitions and phase diagrams we expect to observe as changes to pH cause changes to the NCPR and FCR of polyelectrolytes and polyampholytes. The

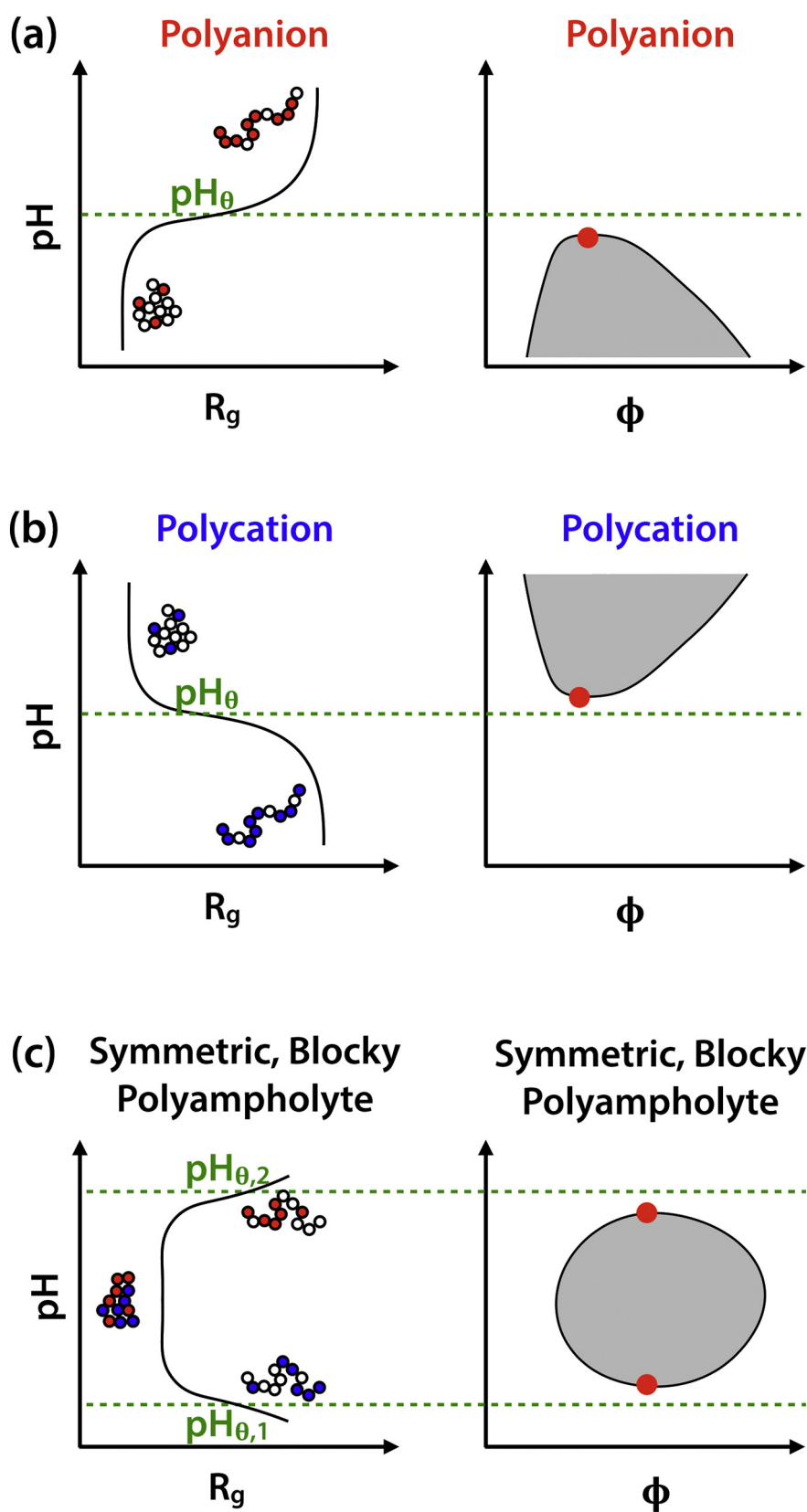


Fig. 4 (legend on next page)

likelihood of charge neutralization will increase as the pH approaches the intrinsic pK_a values of the acidic groups. Protonation of acidic groups will reduce the FCR of anionic polyelectrolytes. Neutralization of acidic groups weakens the preference for chain solvation and drives collapse through chain–chain interactions. Accordingly, anionic polyelectrolytes will undergo a collapse transition below a threshold pH designated as pH_θ . The pH-dependent modulation of the interplay between chain–solvent and chain–chain interactions will drive phase separation as the volume fraction increases. The opposite scenario will obtain for cationic polyelectrolytes. The differences in collapse transitions and phase behaviors that we expect for anionic *versus* cationic polyelectrolytes are summarized in Fig. 4a and b. Phase separation is defined by a critical pH value and anionic polyelectrolytes should have an *upper critical solution pH*, whereas cationic polyelectrolytes should have a *lower critical solution pH*.

Symmetric polyampholytes are characterized by high FCR values (> 0.3) and low NCPR values (≈ 0). In such systems, the collapse transitions are governed by an additional consideration namely the mixing *versus* segregation of oppositely charged residues within the linear sequence [97, 118]. Block copolymeric sequences that segregate oppositely charged residues into distinct blocks might be thought of as polymers of anionic and cationic polyelectrolytes. Such systems are likely to have closed loop phase behavior and show two distinct transition regimes for single-chain behavior characterized by two distinct pH values viz., $pH_{\theta,1}$ and $pH_{\theta,2}$ (Fig. 4c). Changes to the mixing *versus* segregation of oppositely charged residues within the linear sequence have a profound effect on the collapse transitions and the predicted phase behavior of symmetric polyampholytes [55, 97]. Lin and Chan [55] have uncovered a striking inverse correlation between the critical temperature and the extent of collapse. Inasmuch as the latter is determined by the linear segregation/mixing of oppositely charged residues, there is a positive correlation between the critical temperature and the extent of linear segregation of oppositely charged residues. A similar effect of the linear clustering of charged residues has been shown to impact the driving forces for complex coacervation of polyelectrolytes *in vitro* and in cells [37, 119]. What remains unclear is the effect of pH on collapse transitions and phase

behavior of symmetric polyampholytes with different levels of sequence-intrinsic charge segregation or mixing. This lacuna comes from our deficiencies in understanding how sequence context influences the charge states of ionizable groups at different pH values. Preliminary unpublished work suggests that it is reasonable to expect a combinatorial diversity of charge states for symmetric polyampholytes as a function of pH and that this diversity depends on the intrinsic patterning of oppositely charged residues. How this diversity will impact collapse transitions and phase behavior remains an open question, but the possibilities are tantalizingly rich.

Making a case for pressure-dependent phase behavior of IDPPs

The driving forces for stimulus-responsive phase behavior come from the interplay among chain–chain, chain–solvent, and solvent–solvent interactions. This simplified mean-field picture becomes considerably more esoteric when one accounts for the contributions from conformational fluctuations that in turn dictate fluctuations in volume fractions. The amplitudes of these fluctuations, which diverge near the critical point, are also governed by the interplay between chain–chain interactions on the one hand and solvation effects on the other. The centrality of solvation effects and the realization that changes to chain/residue volumes accompany mixing/demixing [13] implies that hydrostatic pressure might also be a suitable stimulus for phase transitions. This proposal comes from observations made in the synthetic polymer literature as well as the protein literature [10, 11]. Protein condensation has been studied for decades in the context of protein crystallization [120]. Numerous studies have shown that proteins in supersaturated solutions undergo liquid–liquid demixing to form dense metastable precursors that act as crucibles for lowering the nucleation barrier for protein crystallization [121, 122]. The stabilities of these dense liquid phases can be tuned by a combination of changes to pressure and temperature because of the unique phase behavior of liquid water on the P – T plane.

Evidence for pressure-dependent changes to proteins comes from the protein folding literature, where it has been established that increases in pressure can lead to protein denaturation [123]. This happens when

Fig. 4. pH-responsive single-chain and phase behavior for anionic, polyelectrolytic IDPs (a), cationic, polyelectrolytic IDPs (b), and symmetric, blocky polyampholytic IDPs (c). Inserts in the R_g *versus* pH plots denote representative conformations of the IDP. White circles denote neutral residues; red, negative residues; and blue, positive residues. For anionic polyelectrolytes, the single-chain will collapse below pH_θ , whereas the single-chain will collapse above pH_θ for cationic polyelectrolytes as a result of charge neutralization. For symmetric, blocky polyampholytes, the single-chain will expand below $pH_{\theta,1}$ as the acidic residues get neutralized and above $pH_{\theta,2}$ as the basic residues get neutralized. For the ϕ *versus* pH phase diagrams, the red circles denote the pH values at the critical points, the gray areas denote the two-phase regimes, and the white areas denote the one-phase regimes.

the partial molar volume of unfolded state is lower than the partial molar volume of the folded state. By reasoning through correspondence with protein-folding, we propose that IDPPs can also be designed to show pressure-dependent phase behavior. A suitable thermodynamic framework would have the following ingredients: We denote the partial molar volumes of the IDPP in the dense and dilute phases as $\bar{V}_{\text{IDPP}}^{(2)}$ and $\bar{V}_{\text{IDPP}}^{(1)}$, respectively. The difference in the partial molar volumes across the two phases is written as: $\Delta\bar{V} = \bar{V}_{\text{IDPP}}^{(2)} - \bar{V}_{\text{IDPP}}^{(1)}$. Pressure and volume are conjugate variables. Accordingly, an increase in pressure causes a decrease in volume, and therefore, an increase in pressure will favor the phase with lower partial molar volume. Therefore, if $\Delta\bar{V} < 0$ at atmospheric pressure, then increasing pressure at constant temperature will lead to increased stability of the dense phase. Conversely, the dense phase will be destabilized with increasing pressure if $\Delta\bar{V} > 0$ at ambient pressure. Comparative studies of the sequence-specific pressure responsive phase transitions of designed IDPPs will help uncover the sequence-encoded responses that result from the interplay among chain-chain, chains-solvent, and solvent-solvent interactions.

The volume of the dense phase will depend on the partial molar volumes and hence the concentrations of the IDPP and solvent in the dense phase. In addition, global dimensions of the IDPP should also contribute to the partial molar volume of protein in the dense phase. Knowledge of the pressure dependence of the global dimensions in the dilute phase combined with the pressure dependence of the stability of the dense phase will provide a route to infer the global dimensions of IDPPs within dense phases—a topic of considerable interest to the phase separation community. In addition, investigating the pressure dependence of IDPP phase behavior should help unmask the phase behavior of solvent, the properties of solvents in the dense *versus* dilute phases, the densities within the dense phases, and the linkage between solvent and IDPP phase equilibria. Even if the pressures required are beyond the physiological realms for mammalian systems, the studies designed to uncover the phase behavior of IDPPs on the P - T plane have the desirable consequence of affording insights into hydration-mediated driving forces for phase separation. Such studies might also have a bearing on the role of phase separation in the evolution and adaptation of piezophilic organisms and for tuning IDPP phase behavior in the context of biotechnological applications.

The tools developed to study pressure dependence of protein conformational equilibria in dilute solution seem versatile enough to be adapted, with suitable modifications, to study the pressure dependence of IDPP phase behavior. Along these lines, Cinar *et al.* [124] investigated the pressure and temperature dependence of the phase behavior of

α -elastin. They observed a novel reentrant pressure-induced phase separation for α -elastin. Importantly, Cinar *et al.* provide a molecular-level theory invoking hydrophobic interactions and void volumes to explain the observed phase behavior of α -elastin.

Cells leverage stimulus-responsive phase behavior in response to stress

The preceding narrative has focused on stimulus-responsive phase behavior of IDPPs from a purely conceptual standpoint. At first glance, these concepts would appear to be of interest only in the *in vitro* setting and/or from an engineering perspective. This inference derives from the realization that temperature, pH, and hydrostatic pressure do not vary significantly for mammalian systems. However, such an assertion glosses over the degree to which unicellular organisms explore the three-parameter space of T , pH, and P . In addition, T , pH, and P phase diagrams are binary with concentration as the second axis. Thus, small changes in protein concentrations, induced through protein expression, can induce a T -, pH-, or P -responsive phase transition. Concentration-dependent phase transitions have been readily observed in the formation of membraneless organelles, but they are rarely connected to other intensive solution parameters [18, 21, 48, 125]. Direct changes in intensive parameters also occur in the context of biological adaptation, evolution, and stress response. Yeast is proving to be a model system to understand the extent of biological exploration of the T , pH, and P space [102, 126, 127].

It has been established that the cytosolic pH drops when yeast respond to stresses such as osmotic or heat shocks [127]. This lowering of the pH drives intracellular stress responses, which are manifest in the form of phase separation driven by the yeast prion protein Sup35 [128]. Recent work suggests that the normal function of Sup35 might be the formation of pH-sensitive biomolecular condensates in order to promote yeast cell survival under stress conditions [102]. Sup35 contains an N-terminal prion domain (N), a charged middle-domain (M), and a C-terminal GTPase domain (C) [129, 130]. The M domain contains a cluster of negative charges, predominantly Glu residues, at its C-terminus. When this charge cluster is disrupted, Sup35 can still phase separate but loses its pH sensitivity. In addition, although the sequence of the M-domain is not conserved across fungi, the charge patterning of the M-domain is conserved. These results suggest that clustering of Glu residues within the M-domain allows Sup35 to form condensates in response to a drop in pH [102]. Lowering of the pH by less than one pH unit enables a neutralization of Glu residues within an acidic tract of the M-domain. This neutralization, caused by apparent pK_a shifts of Glu residues, perhaps due to the clustering of acidic residues within the M-domain,

enables phase separation. Details of how this transition occurs are being investigated.

In addition, upon heat shock, cells form protein and RNA condensates called heat shock granules. A constituent component of heat shock granules is the poly(A) binding protein, Pab1 [131]. Under normal growth conditions, Pab1 binds A-rich tracts in the 5' untranslated region of mRNA, including heat shock chaperone mRNAs. However, after heat shock, Pab1 releases mRNA and phase separates [88]. This suggests that heat-induced phase separation of Pab1 allows for efficient translation of chaperones in response to stress. In addition, these chaperones in turn dissolve Pab1 assemblies, restoring the cell back to its normal state. In *Saccharomyces cerevisiae*, Pab1 is composed of various domains including four RNA recognition motifs and a disordered proline-rich domain (P-domain). The P-domain is not necessary for Pab1 phase separation but its presence and amino acid composition tune the temperature at which phase separation occurs [88]. The P-domain is poorly conserved in length and sequence across organisms. However, the P-domains show a preference for certain aliphatic residues compared to other disordered proteins and the yeast proteome. In addition, aliphatic residues appear to be somewhat interchangeable across species. Mutational studies aimed at characterizing the biophysical properties of the P-domain suggest that the wild-type P-domain samples disordered collapsed conformations and that the collapse is driven by hydrophobic interactions. The degree of collapse of monomeric P-domain correlates with the temperature at which Pab1 phase separates *in vitro*. Mutations of Met and Val residues to more hydrophobic residues (Ile, Leu) lead to aggregation at lower temperatures *in vitro* but do not affect fitness of yeast after heat shock *in vivo*. However, mutation of Met and Val residues to a less hydrophobic residue (Ala), increasing the polar amino acid content, or deleting the P-domain leads to reduced fitness after heat shock. These results suggest that the functional LCST behavior of Pab1 under physiological stress conditions is dependent on the hydrophobicity of the P-domain. The importance of hydrophobic, nonpolar residues for physiologically sensitive LCST behavior and fitness is consistent with the sequence properties observed for LCST behavior of elastomeric like polymers (Fig. 2a and b) [38].

Conclusions and broader implications

We have provided a conceptual overview of phase transitions that IDPPs undergo in response to changes in intensive solution parameters. In doing so, we have connected the stimulus-responsive phase behavior to collapse transitions of IDPPs. The concepts and proposals presented here are directly relevant for understanding the driving forces for and

mechanisms of sequence-encoded phase transitions. Through a high-throughput effort that combines design, synthesis, characterization, and modeling we expect to be able to uncover the relationships among sequence-encoded information and stimulus-responsive collapse transitions at the single-chain level and overall phase behavior. This should pave the way for the design of *de novo* condensates that confer novel responses in model organisms and the development of novel biomaterials with bespoke material properties.

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Abbreviations used:

IDPPs, intrinsically disordered protein polymers; SLiMs, short linear motifs; IDRs, intrinsically disordered regions; IDPs, intrinsically disordered proteins; ELPs, elastin-like polypeptides; UCST, upper critical solution temperature; LCST, lower critical solution temperature; PNIPAM, poly-*N*-isopropylacrylamide; LCDs, low-complexity domains.

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