Metastability of Biological Matter in Liquid Phase Separation

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Abstract

Membraneless organelles (MLOs) form via liquid-liquid phase separation (LLPS). The liquid-like MLOs afford multifold peculiarities including high dynamics, reversibility and responsiveness. The MLOs are typically metastable with a tendency to undergo fast, drastic and reversible formation and dissolution, as well as transition into more stable glassy or gel-like states. Moreover, the alteration of metastability of LLPS is linked with cellular pathology. Here, we review the crucial and ubiquitous 'metastability' of MLOs driven by liquid phase separation, from multifaceted regards including energy state, molecular interactions, molecular structure, materials state, as well as the associations with diseases. This review can help to advance the insight into properties and pathogenesis associated with LLPS of biological matter.

Review

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Keywords

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Abstract

Membraneless organelles (MLOs) form via liquid-liquid phase separation (LLPS). The liquid-like MLOs afford multifold peculiarities including high dynamics, reversibility and responsiveness. The MLOs are typically metastable with a tendency to undergo fast, drastic and reversible formation and dissolution, as well as transition into more stable glassy or gel-like states. Moreover, the alteration of metastability of LLPS is linked with cellular pathology. Here, we review the crucial and ubiquitous 'metastability' of MLOs driven by liquid phase separation, from multifaceted regards including energy state, molecular interactions, molecular structure, materials state, as well as the associations with diseases. This review can help to advance the insight into properties and pathogenesis associated with LLPS of biological matter.

Key points

Liquid-liquid phase separation emerges as a universal tool for the intracellular biomolecule organization, particularly via the formation of membraneless organelles.

The liquid-like membraneless organelles afford unique properties and have aroused wide interests among cell biologists.

Metastability is a general feature of liquid-like membraneless organelles, which has manifested in multiple aspects including energy state, molecular interactions, molecular structures, biological assemblages and its association with diseases.

Understanding of metastability of biological liquid phase separation can not only help to elucidate the formation and properties of membraneless organelles, but also pave the way to the insight into pathogenesis.

Metastable Biological Matter

Cells compartmentalize to organize a myriad of biomolecules and biochemistry hierarchically, conventionally known to form lipid bilayer membrane-bound organelles [1]. Nonetheless,**liquid-liquid phase separation** (LLPS, see **Glossary**) emerges recently as another universal phenomenon for intracellular organization, particularly via the formation of **membraneless organelles** (MLOs) hosting **intrinsically disordered proteins**(IDPs) as scaffolds, which have important roles in cellular physiology and disease [2–4]. The liquid-like MLOs are biological soft matter that afford multifold peculiarities, including high dynamics, reversibility and environmental responsiveness[5]. Notably, there is growing evidence that '**metastability** ' is a universal and pivotal nature of liquid-like MLOs [6]. For one thing, MLOs are capable of undergoing fast, drastic and reversible formation and dissolution events commensurate with surrounding intracellular environment[7]. For another, the liquid state has an inherent tendency to transit into more stable glassy or gel-like states[8], simply over time [9] or expedited by disease-associated mutations [10–13]. Moreover, the alteration of metastable state has been revealed to be closely tied with cellular physiology and pathology [14], whilst the manipulation of LLPS can be promising route towards effective treatment of diseases [15].

In this review paper, we highlight the ubiquitous yet essential metastability of liquid phase separation of biological soft matter. We first depict the **free energy** landscape of biological matter, and underscore the metastable state of MLOs with liquidity. We next discuss about the distinctive metastable molecular interactions and molecular structures implicated in liquid-like MLOs. Through examples, we then highlight the metastable material state of biological assemblages formed through liquid phase separation. Lastly, we describe how metastability provides a pivotal link between physiology and disease.

Metastability of energy state

The liquid condensate state of MLOs is energetically**metastable** , namely, resides in a local minimum of Gibbs free energy (**Figure 1A**). A thermodynamic system will spontaneously minimize the free energy to attain states with higher stability. As such, liquid condensate state can spontaneously transit to more energetically favorable states, namely, other local minima with lower free energy or the global minimum, simply over longer time[16–18] or expedited by disease-associated mutations[10–13,19]. For example, liquid condensates can evolve into reversible hydrogels or irreversible amyloid-like aggregates, which has lower free energy and higher stability. Additionally, LLPS can be an intermediate process to facilitate nucleation and lower the free energy of crystallization and aggregation[18,20,29–32,21–28]. To maintain the liquid condensate state, specific quality control machineries [33] are required to stop them from spontaneous solidification[34–37], including post-translational modification (PTM) [38,39], RNA binding [40], chaperon [11,41–45], hydrotrope [46]and disaggregase [47].

In cell biology, proteins are commonly hydrophilic biomacromolecules dispersed in the state of dilute solution. How can the metastable liquid condensates form from the dispersed solution thermodynamically? Basically, there are two types of interactions in a dispersed protein solution, namely, the homotypic interactions between two biomolecules (e.g., protein or nucleic acid) and two water molecules, as well as the heterotypic interactions between a biomolecule and a water molecule[48]. The phase transition from dispersed state to phase-separated state can occur if the homotypic interactions are favored over heterotypic interactions. The enthalpy and entropy change of this phase transition can be categorized into four quadrants (**Figure 1B**). The formation of liquid condensates is thermodynamically favored within two quadrants (**Figure 1B** , highlighted with green color), namely, when$\Delta H \bullet \Delta S > 0$. When $\Delta H > 0$ and$\Delta S > 0$, ΔG is only negative at high temperature. This means the LLPS is spontaneous when temperature is higher than a threshold value, thus exhibiting a lower critical solution temperature (LCST) phase behavior, which has been found in extracellular IDPs including elastin, elastin-like polypeptide [49] and histidine-rich squid beak proteins [50–52]. When$\Delta H < 0$ and $\Delta S < 0$, ΔG is only negative at low temperature. This means the LLPS is spontaneous when temperature is lower than a threshold value, thus exhibiting an upper critical solution temperature (UCST) phase behavior, which has been found in intracellular IDPs including Ddx4 [53], LAF-1 [54], FUS [55], TDP-43 [56] and hnRNP[17] protein. By contrast, the formation of liquid condensates is thermodynamically disfavored within two quadrants (**Figure 1B** , highlighted with red color), namely, when$\Delta H \bullet \Delta S < 0$. When $\Delta H > 0$ and$\Delta S < 0$, phase separation is always thermodynamically unfavorable, and solution remains in the dispersed state becauseΔG is always positive. When $\Delta H < 0$ and$\Delta S > 0$, phase separation is always thermodynamically favorable. Dispersed solution will form irreversible phase separation spontaneously, either liquid-solid phase separation or LLPS, asΔG is always negative.

Molecular interaction and molecular structure for metastability

In accordance with the metastable free energy state, IDPs, as the scaffold protein of MLOs, often harbor transient and weak molecular interactions [34]. The **low complexity domains**(LCDs), as the domains that mediate the LLPS of IDPs, are largely enriched with charged, polar and aromatic residues whilst commonly devoid of hydrophobic residues [22]. Weak, multivalent and non-specific interactions, including electrostatic, pi–pi, cation–pi and dipole–dipole interactions (between polar amino acids), are prevalent among residues in LCDs (**Figure 2A**)[36]. The long-range electrostatic interactions among charged blocks may facilitate the initiation of LLPS, while short range interactions, including pi–pi, cation–pi and dipole–dipole interactions, may mediate the multivalent contacts among weakly interacting motifs [34]. Among these molecular interactions, cation–pi interactions are considered as the strongest, with the free energy of binding (ΔG_{bind}) around –3.6 kcal/mol [57]. This magnitude is lower than the average ΔG_{bind} per residue implicated in the formation of $A\beta_{17–42}$ amyloids-β protofibrils (–19.3 kcal/mol) [58], thus suggesting molecular interactions driving the formation of MLOs are much weaker than amyloid plaques. Compared with static amyloids [34], LCDs harbor transient interactions among residues with higher dynamics. This can be quantified by fluorescence recovery after photobleaching (FRAP), commonly showing a half time of recovery ($t_{\frac{1}{2}}$) on the order of seconds (normalizing the diameter of bleaching spot to 1 μm)[34]. Efforts have been made to shed light on the possible reason of liquid phase formation from the molecular level, including theory of amyloids-β fibril formation [59], multivalent domain interaction network model [60] and theory of polymer physics [22].

IDPs lack a stable and well-defined 3D molecular structure[61–63]. IDPs are always devoid of stable tertiary structures under physiological conditions, albeit collapsed IDPs could harbor some stable secondary structure elements [64]. The lack of stable structure can be possibly considered as one common and crucial feature for IDPs to form metastable MLOs[65]. The unstable conformation allows the flexibility of IDPs as major scaffold constituents, which may contribute to the physical fluidity of MLOs [65]. The unstable conformation allows formation of the weak and multivalent interactions, which is a common hallmark for the interactions that contribute to LLPS[3]. The IDPs harbor 'stickers-and-spacers' structural features, wherein modules provide attractive interactions are considered as 'stickers', and flexible linkers provide no significant attractive interactions are considered as 'spacers'. The unstable structure with 'stickers-and-spacers' features allows the multivalent presence of PTM sites [66], whilst PTMs can efficiently alter the stability of MLOs [53].

Modular interaction domains connected by disordered linkers can mediate multivalent interactions that drive LLPS. Rosen *et al.* reported the LLPS of multivalent signaling proteins. Neural Wiskott–Aldrich syndrome protein (N-WASP), the actin-regulatory protein, interact with its established biological partners NCK and phosphorylated nephrin1 to form LLPS, wherein NCK contain three SH3 domains that can bind to the six proline-rich motif (PRM) ligands of N-WASP [67]. Similar multivalent system were also reported in T cell receptor signaling pathway [68], nucleophosmin (NPM1) interacting with proteins comprising arginine-rich linear motifs and ribosomal RNA[69] and pair of polySUMO–polySIM interacting multivalent scaffold proteins [70].

Weakly interacting motifs are prevalent in LCDs to mediate LLPS. It has been widely known that tightly self-complementing '**steric zipper** ' structure forms solid-like amyloid-β plaques with hydrophobic interfaces and high stability [71–73]. By contrast, IDPs largely host motifs that can form thermodynamically metastable '**κινκεδ β σηεετς** ' [74–77] molecular structure,*i.e.* , the archetypical [G/S]Y[G/S] motifs of FUS protein. These motifs can form close interactions as quantified by the structural complementarity (S_c) (**Table 1**). However, side chains cannot interdigitate across the β-sheet interface owing to the prevention of kinks. They thus harbor smaller buried solvent-accessible surface area (A_b) and more hydrophilic interfaces, thus exhibiting much lower stability [74] (**Figure 2B**and **Table 1**). This is exemplified by metastable interaction motifs in LCDs of FUS [55,78–82], Tau[83], TDP-43 [84] and hnRNP[17,76,85] proteins. Specifically, short associative peptide motifs within LCDs can form metastable fibrils *in vitro* , whilst exhibiting melting behavior in response to mild heating, which is distinctive from stable amyloid fibrils (**Table 2**).Besides kinked β sheets, other interaction motifs that can mediate multivalent interactions were also reported, including repeated [F/R]G and G[F/R] pair motifs of Ddx4 proteins [53], α-helix-forming ^{321}AMMAAAQAAL^{330}motif of TDP-43 proteins [86], VPGXG (X is a guest residue except proline that can modulate phase behaviour) motifs of elastin-like proteins [87] and GHGLY motif of histidine-rich squid beak proteins [52]. In addition, specific motifs may hinder LLPS, *i.e.* , FGDF can bind to G3BPs to block the formation of stress granules [88].

The metastable molecular structures and phase behavior of IDPs can be drastically altered simply by mutations[17,55,76,80,84,85,89,90] or PTMs[55,82,84] on one single residue. For example, The phosphorylation of FUS protein by kinase at the Ser42 site drastically altered the molecular interactions of LCDs, haltering the formation of metastable fibrils and LLPS formation [55]. This prominent alteration of phase behavior can be attributed to disruption of metastable kinked structure. The Ser42 site is the primary phosphorylation site by DNA-dependent protein kinase (DNA-PK)[91]. The phosphorylation at Ser42 can significantly disrupt the hydrogen bonds between Ser42 and Tyr38, interfere with the interaction of mating sheet and destabilize the RAC1 interacting motif, thereby modulating the ability of FUS to undergo LLPS. Additionally, the mutation of Ser42 to Asp (S42D) can also remarkably depress the LLPS of LCD of FUS protein, decreasing the critical temperature of LLPS by 5 °C, as the mutation S42D is a change that mimics serine phosphorylation[55].

There are two major types of phase behavior for a biological LLPS system of interest, namely, the entropy-driven LCST phase behavior and enthalpy-driven UCST phase behavior [24]. How is the type of phase behavior encoded in motifs of protein sequences? Chilkoti*et al.* synthesized artificial IDP-like polymers harboring several tens of repeats of short peptide motifs [87]. They found that motifs with low-charge content and high hydrophobicity feature tend to engender IDP-like polymer with LCST behaviour, which is reminiscent of

tropoelastins. By comparison, motifs with high-charge content and low hydrophobicity feature tend to engender IDP-like polymer with UCST behaviour, which is reminiscent of the dual UCST and LCST behaviour at extremes of temperature of resilin. Furthermore, Chilkoti *et al.*found that hysteresis behavior can also be encoded and tuned at the motif level by the precise position of an amino acid within a motif, as well as at the macromolecule level by chain length [92].

The unique molecular interaction and molecular structure allow metastable MLOs with unique properties, including liquidity, high dynamics and environmental responsiveness. Learning from nature, the responsible domains and motifs of IDPs have also been exploited as building blocks to design bio-inspired materials[93–95], which have been reviewed elsewhere[96].

Environment-responsive biological matter exhibiting metastability

The MLOs, as biological assemblages, reside in a metastable material state. They can readily form under stress conditions as induced by heat[12,97,98], pH change [98,99], starving[98] or chemicals (e.g. sodium arsenate[12,40]) in living cells. For example, the exposure of HeLa cells to heat stress by heating from 37 °C to 42 °C, or to chemical stress of 1 mM sodium arsenate, can significantly induce liquid-like stress granules in both the nucleus and cytoplasm [12]. Also, the starvation of yeast by glucose depletion or simply drop of cytosolic pH (to 5.7) can trigger the LLPS of polyU-binding protein (Pub1) into stress granules [98].

Liquid-like MLOs can readily reverse to dispersed state in response to a wide range of environmental stimuli including heating[53,100], pH [98], ionic strength[11,53], light [101,102] and enzymatic reaction, including phosphorylation [80] and proteolytic cleavage [54], which may indicate the liquid state of assembly is a metastable state. For example, heating from 10 °C to 20 °C dissolved the liquid droplets formed from N-terminal Argonaute binding domain (ABD) of TNRC6B protein *in vitro* [100]. The phase separation of Pub1 *in vitro* can be dissolved with the increase of ionic strength from 187 mM to 1 mM, as well as the change of pH from 5.7 to 7.5 [98]. The liquid-like droplets formed from the LCD of FUS protein gradually dissolved within two hours*in vitro* , via the phosphorylation at serine and threonine sites by kinase [80]. Hammer *et al.*[54] demonstrated the controllable dissolution and formation of LLPS induced by proteolytic cleavage. They reconstituted proteins from the fusion of two RGG domains of LAF-1 protein, linked by Glu-Asn-Leu-Tyr-Phe-Gln-Gly recognition sequence by tobacco etch virus (TEV) protease. This RGG-x-RGG (x=TEV cleavage site) protein forms LLPS, whilst the dissolution of liquid droplets can be triggered by TEV proteolytic cleavage of linkage of RGG domains, as was demonstrated*in vitro* and in living HEK293 cells. Additionally, they fused RGG-RGG with maltose-binding protein (MBP) domain via linkage of TEV cleavage site, which has been widely used as a solubility-enhancing tag to prevent phase separation of IDPs [79]. This MBP-x-RGG-RGG protein remains dispersed, while the formation of liquid droplets can be triggered by proteolytic cleavage of MBP domain with the treatment of TEV protease, as was demonstrated in water-in-oil protocells *in vitro* .

MLOs can spontaneously transit into more stable material state over time (known as '**maturation** '[16,103–106]), including viscous liquids [28], gels[28] and amyloid-like fibrils[12,42,107] (**Figure 3A**). For example, the liquid droplets from 8 μM FUS protein transit into amyloid-like fibrillar structure after 6-hour incubation *in vitro*[12]. Likewise, the liquid droplets from the LLPS of p-tau protein evolve into viscoelastic liquids, gels and finally amyloid-like aggregates after 1-day incubation *in vitro* , and liquid droplets almost completely converted to amyloid-like aggregates after 10-day incubation [28]. Parker *et al.* fused polypyrimidine tract-binding protein (PTB, an RNA-binding domain) with LCDs of IDPs including PUB1, LSM4, EIF4GII, TIA1 and FUS. These reconstituted proteins form LLPS in complexation with RNAs *in vitro* . Notably, all of these complexed droplets mature into much more stable and solid-like assemblies within 48 hours [9]. This natural and spontaneous liquid-to-solid transition of materials state can be accelerated under disease-associated conditions[11,12], which will be discussed in the next section.

Metastability is associated with diseases

It costs to form metastable LLPS in cells. The LLPS of IDPs derives from a state of **supersaturated** compositional proteins wherein their cellular concentrations are high relative to their solubilities[67,83,108], a phenomenon that can drive the aberrant protein aggregation in **neurodegenerative diseases**[108,109].

The liquid condensed phase is intrinsically metastable and vulnerable to uncontrolled aggregation leading to pathological consequences [9,10,14,37,110]. The metastable liquid MLOs is susceptible to alteration simply over time[9,12], which is commonly expedited under pathological conditions [12,19,111]. The transition from metastable MLOs to stable aggregates can further lead to the gain of biological changes, including cancer, neurodegenerative diseases and aging[4,11,118,119,35,75,112–117]. A wide array of factors can engender the alteration of metastability, such as PTMs, mutations and overexpression of proteins (**Figure 3B**), which are reviewed below.

Loss of metastability can be triggered by aberrant PTMs[120]. George-Hyslop *et al.* [11]showed hypomethylation of arginine can drive the formation of hydrogel structure from FUS protein, which can disrupt ribonucleoprotein (RNP) granule function and damage protein synthesis in neuron terminals, thus suggesting a plausible mechanism for frontotemporal lobar degeneration (FTLD), as well as other neurodegenerative diseases. Conversely, metastability can be preserved by specific PTMs. Fawzi *et al.*[85] revealed that arginine methylation can help to maintain the metastability of hnRNPA2 protein by disrupting interactions between arginine guanidyl group and aromatic residues, thereby pointing out a possible method for the regulation of solid-like assemblies and modify the toxicity. Fawzi *et al.* [39]investigated the impact of phosphorylation using phosphomimetic mutations. All the twelve serine/threonine–glutamine (S/TQ) sites of FUS protein were mutated to glutamate–glutamine (EQ) sites. The disordered nature of LCDs was preserved, whilst allowing the ability to form LLPS *in vitro* . Distinctively, the aggregation-prone characteristics were reduced. With one-day orbital agitation to induce maturation of IDPs, most of the droplets formed from wild-type FUS protein evolved into irregular assemblies, while the droplets formed from phosphomimetic variant 12E remained spherical *in vitro* . They further demonstrated both the aggregation propensity and cytotoxicity in yeast *Saccharomyces cerevisiae* were reduced, suggesting that maintaining metastability of liquid-like MLOs can be an effective way to tackle neurodegeneration caused by aberrant protein aggregation. Ferreon*et al.* [121] found PTM can counteract LLPS-initiated protein aggregation. Tau protein underwent LLPS under physiology-relevant conditions *in vitro* , whilst the liquid-to-solid transition can be induced in the presence of heparin. The 24-hour maturation process was visualized by morphological change into solid-like irregular aggregates and quantified by prominent fluorescence increase of Thioflavin T (ThT) fluorescence assay. Tau protein was also hyperacetylated with p300 histone acetyltransferase. Distinctively, hyperacetylated Tau protein forms much less irregular solid after 24-hour maturation. The aggregation propensity of Tau is largely decreased by acetylation, suggesting a way to ameliorate neurodegenerative diseases associated with toxic Tau aggregation, including Alzheimer's disease.

Disease-associated mutations often strengthen homotypic interactions of IDPs, thereby impairing the liquidity of MLOs, decreasing dynamics and promoting protein aggregation [112]. Neurodegenerative diseases have been widely reported to link with aberrant transition of MLOs. Alberti *et al.* [12] showed the maturation of FUS protein liquid droplets can be drastically accelerated by mutations associated with amyotrophic lateral sclerosis (ALS). After 8-hour maturation *in vitro* , droplets formed by the LLPS of FUS protein was mostly preserved, only demonstrating minute solidification. By contrast, droplets from the G156E mutant of FUS protein maturated into stable fibrous aggregates (a state without metastability) almost completely. How is the loss of metastability linked with disease? Da Cruz *et al.* [122] further clarified that ALS/frontotemporal dementia (FTD)-linked mutant of FUS protein drives disease by a gain of toxicity, rather than a loss of function. Taylor*et al.* [19] investigated the hnRNPA1 mutant, D262V, which is associated with multisystem proteinopathy and ALS[123]. At the initial state, D262V mutant formed LLPS comparable with wild-type hnRNPA1, demonstrating similar propensity of droplet formation and critical melting temperature. Repeated heating-cooling cycling was applied to both D262V mutant and wild-type hnRNPA1. Notably, within minutes of maturation, the reversible droplet formation of D262V mutant was concomitant with the fibrilization and formation of irregular solid-like assemblies, which is stable, amyloid-like and ThT-positive. By contrast, no such liquid-to-solid conversion was observed for wild-type hnRNPA1, thus suggesting the metastability of MLOs was impaired by the D262V mutation, which is associated with neurodegeneration. Fawzi *et al.*[85] studied the P298L and D290V mutants of hnRNPA2 protein with connections to Paget's disease and multisystem proteinopathy (MSP), respectively. Freshly prepared wild-type hnRNPA2, P298L and D290V

mutants all formed similar spherical droplets with liquidity and comparable dynamics, as was quantified by FRAP. Nonetheless, P298L and D290V mutants prominently transited into stable solid-like assemblies after 30-min maturation *in vitro* , in stark contrast to the preservation of liquid droplets of wild-type hnRNPA2 for at least 120 mins, thereby indicating disease-associated mutations impaired metastability of MLOs. Fawzi *et al.* [86]reported A321V, an ALS variant of C-terminal domain (CTD) of TDP-43, formed liquid droplets similar to wild-type TDP-43, as was confirmed by turbidity, spherical shape and FRAP. After one hour incubation *in vitro* , A321V variant changed into irregular assemblies which is distinct from the spherical shape of wild type TDP-43. Similar phenomena have also been found for P362L mutants in the LCD of T cell-restricted intracellular antigen-1 (TIA1) [124], and A4V mutants in superoxide dismutase 1 (SOD1) [45], which are associated with ALS. Taylor *et al.* [118] studied the expansion of a hexanucleotide repeat GGGGCC in C9ORF72, which is the most common cause of ALS and FTD, presumably through the expression of toxic dipeptide repeat proteins. They identified that arginine-containing dipeptide repeat proteins, namely, polyGly-Arg and polyPro-Arg, can interact with LCDs of IDPs to alter the biophysical properties of MLOs. Both polyGly-Arg and polyPro-Arg can impair the liquidity, dynamics and metastability of MLOs *in vitro* and in living HeLa cells, as was substantiated by FRAP and change of spherical shape. Moreover, the polyGly-Arg and polyPro-Arg can promote the assembly of stress granules lacking metastability in HeLa cells, which can inhibit cellular translation and increase the risk of cell death events. Vale *et al.* [125] further showed RNAs harboring CAG and CUG repeats (length 31, 47 and 66 repeats) underwent LLPS event into droplets *in vitro* , albeit rapidly evolving into solid-like RNA hydrogels maintaining sphere-like shape. RNAs harboring 47 CAG repeats can form liquid-like nuclear foci in living U-2OS cells. By contrast, RNAs with 10 or 23 GGGGCC repeats forms irregular solid-like gels *in vitro* , and RNAs with 29 GGGGCC repeats forms irregular solid-like gels in living U-2OS cells. This report demonstrated that expansion of the GGGGCC in the C9ORF72 gene can trigger formation of solid-like RNA gels with impaired dynamics and metastability. As the accumulation of the repeat-containing transcripts into aberrant RNA foci in the nucleus is a common feature in neurodegeneration, this research suggests sequence-specific gelation of RNAs can be one cause of neurodegenerative diseases. Hsp27 is molecular chaperone that is vital in maintaining the metastability and dynamics of liquid droplets of stress granules from FUS protein, whilst mutations of chaperons can directly engender hereditary motor neuron diseases[42]. Liu *et al.* [42] mutated residues (I120, H124, 126–131, I134, F136, R140 and T143) of Hsp27 to alanine to synthesize Hsp27-A, which can largely change the binding surface between Hsp27 and FUS protein. Hsp27 bound to FUS LCD to preserve the liquid phase against amyloid fibril formation, whilst the mutant Hsp27-A had little inhibitory effect on the amyloid-like aggregation of FUS LCD, as has been quantified by the ThT fluorescence assay. This study suggests loss of metastability can lead to diseases through the mutations of chaperone. Besides neurodegenerative diseases, Mittag *et al.* [126] investigated the impact of cancer mutations on LLPS and functioning of MLOs. Tumor suppressor speckle-type POZ protein (SPOP) formed liquid-like MLOs with death-domain-associated protein (DAXX) in living HeLa cells, and CUL3 ubiquitin ligase activity is found in the MLOs formed. By contrast, cancer mutants (W131G and F133V) of SPOP failed to co-localize and form liquid-like MLOs with DAXX in living HeLa cells, resulting into reduced protein ubiquitination, thus suggesting cancer mutations are associated with loss of function of MLOs.

The overexpression of IDPs can also trigger the alteration of metastable MLOs. Carra *et al.* [127] revealed overexpression of small heat shock proteins (HSPBs) engendered aberrant MLOs in nucleus, which can mislocalize nuclear intermediate filament protein lamin-A/C (LMNA) and chromatin, resulting in the abnormal distribution of LMNA and chromatin and thereby damaging the integrity and function of nucleus.

Concluding remarks

Membraneless organelles are peculiar liquid-like compartments in cells. Membraneless organelles are unique because of their multifold metastability, including free energy, molecular interaction, molecular structure, and materials state. What's more, the alteration of metastability has been tightly linked to diseases. We envision that further elucidation of metastability in liquid phase separation, especially in the formation, maintenance and modulation of liquid-like MLOs, can contribute to the insight into cell physiology and mechanisms of pathogenesis (see **Outstanding Questions**).

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Conflict of interest disclosure

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Data availability statement

Data sharing not applicable – no new data generated, or the article describes entirely theoretical research.

Glossary

Free energy: In a thermodynamic system, the portion of energy available to perform work at constant temperature. Specifically, the Gibbs free energy is used for constant temperature and pressure, which is true for solution-phase chemistry including biochemistry. Systems tend to transit into a state where the free energy is lower, which is more thermodynamically stable.

Intrinsically disordered proteins (IDPs) : The most common scaffold proteins of MLOs with a strong tendency to form LLPS, which is often concomitant with nucleic acids.

Κινκεδ β σηεετς: The β-sheet with kinks that prevent side chains from tight interdigitating across the interface. Compared with the steric zipper structure in amyloid plaques, they interact weakly through polar atoms and aromatic side chains, bury smaller surface areas, and have lower binding energy.

Liquid-liquid phase separation (LLPS): A homogenous liquid phase demixes into two distinct liquid phases, namely, one light phase and one concentrated phase. This term is also widely referred as 'coacervation' when comprising macromolecular components.

Low complexity domains (LCDs) : Domains of IDPs with low sequence complexity and biased composition of amino acids, namely, typically enriched in specific polar and charged amino acids, whilst interspersed with aromatic residues (especially tyrosine and phenylalanine). They are also referred as 'prion-like domains (PLDs)', which is often considered as necessary and sufficient for driving LLPS of IDPs intracellularly.

Maturation: A transition process of MLOs from liquid-like droplets to solid-like aggregates, together with the loss of metastability. This process is thermodynamically spontaneous, which can take place simply over time or be accelerated by pathological changes. This term has also been referred to as 'hardening', 'aging' or 'solidification'.

Membraneless organelles (MLOs) : Non-membrane-bound organelles that usually form via liquid-liquid phase separation in cells. They have also been referred to as many other names including biomolecular condensates, cellular bodies, speckles, puncta and granules.

Metastability /Metastable: A system is thermodynamically metastable when staying at a local minimum of free energy, with a tendency to spontaneously transit into more stable state, namely, a global minimum of free energy. The metastable nature of a thermodynamic system is metastability.

Neurodegenerative diseases : A group of diseases engendered by the progressive loss of structure and function of neurons, which is also known as degenerative nerve diseases. Common examples include Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease, Huntington's disease, multiple system atrophy and frontotemporal dementia.

Steric zipper: The dry and tightly self-complementing structure formed within double β-sheets of amyloid-like fibrils. This name was coined by David Eisenberg's group in 2005 because the interdigitating side chains of β-sheets resemble the teeth of a zipper.

Supersaturated: A protein is supersaturated when its concentration is higher than the solubility under normal conditions.

Outstanding Questions

How can membraneless organelles attain both supersaturation and high selectivity of a plethora of biomolecules?

Is it possible to predict phase behavior of IDPs in different environment simply from the information of sequence?

Why do 'upper critical solution temperature' and 'lower critical solution temperature' phase behaviors often exist with intracellular and extracellular intrinsically disordered proteins, respectively? Is there any biological significance or reason?

How and to what extent can cells prevent the pathological aggregation from metastable membraneless organelles?

Is the maturation process in cells inevitable?

How to rescue the stable amyloid-like assemblies by therapeutic intervention? Will this be an effective pathway for the treatment of neurodegenerative diseases and cancer?

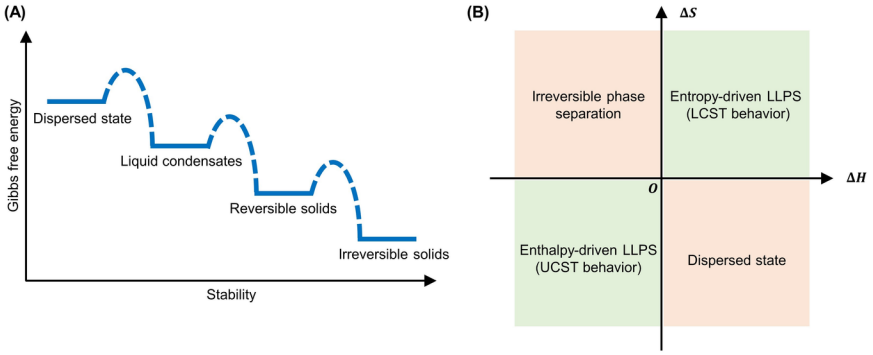

Figure 1. Energy depiction. (A) Schematic illustration of landscape of Gibbs free energy and stability associated with different possible materials states. (B) Enthalpy and entropy change of phase transition from dispersed state to phase-separated state. Four possible scenarios are shown as four quadrants of an enthalpy–entropy coordinate.

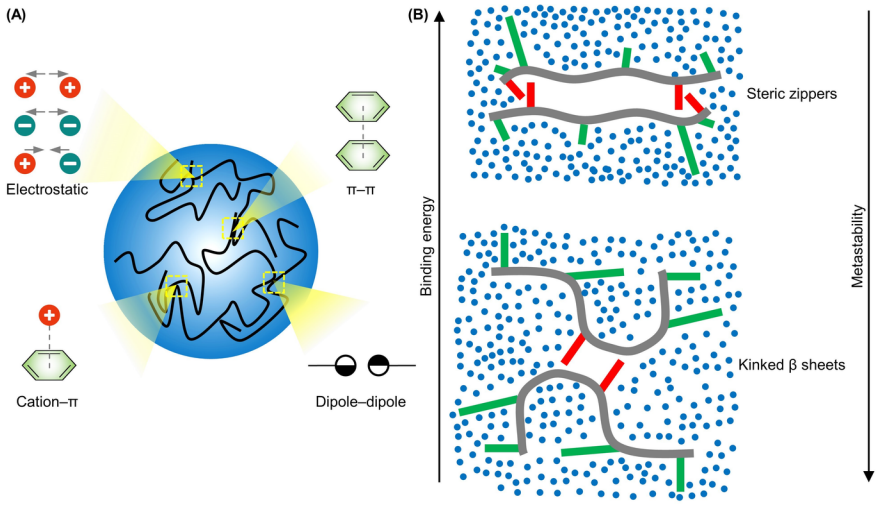

Figure 2. Metastable molecular interactions and molecular structures in LLPS. (A) Typical molecular interactions in MLOs. (B) Schematic of steric zippers in amyloids and kinked β sheets in IDPs. Side chains in and out of interfaces are colored in red and green, respectively. Peptide backbones are in grey. Water molecules (in blue circles) are drawn for illustrating dry (hydrophobic) and hydrous (hydrophilic) interfaces of steric zippers and kinked β sheets, respectively.

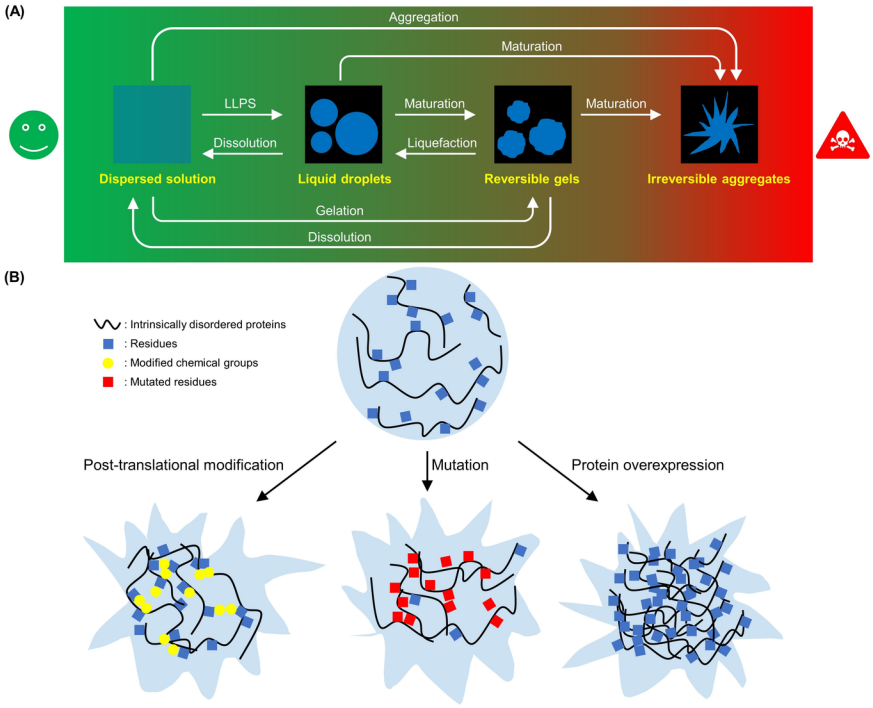

Figure 3. MLOs are metastable biological assemblages associated with disease. (A) Metastable liquid droplets can be changeable/interchangeable to other phase states. (B) The maturation process can be accelerated by disease-associated changes.

Table 1. Structural parameters of kinked β sheets compared with a steric zipper.

Proteins	Peptide motifs	S_c	A_b	Structure formed	Ref
FUS	^{37}SYSGYS^{42}	0.91	81	Kinked β sheets	[74]
	^{54}SYSSYGQS^{61}	0.81	120		
	^{77}STGGYG^{82}	0.82	111		
hnRNPA1	^{243}GYNGFG^{248}	0.86	120		
nup98	^{116}GFGNFGTS^{123}	0.86	114		
Amyloid-β	^{27}NKGAII^{32}	0.86	178	Steric zipper	

Table 2. Metastability of fibrillogenic peptides of LCDs and amyloids.

Proteins	Proteins	Peptide motifs	Metastability	Melting temperature of fibrils	Refs
hnRNP	hnRNPA1	^{209}GFGGNDNFG^{217} (hnRAC1)	Yes	4–25 °C	[17]
		^{246}GFGNDGSNF^{254} (hnRAC2)		4–37 °C	
		^{260}YNDFGNY^{266} (hnRAC3)		4–25 °C	
	hnRNPA2	^{227}GFGDGYNGYG^{236}		4–37 °C	
		^{270}GYGGGYDNYGG^{280}		4–37 °C	
	hnRNPDL	^{365}YGGDQNY^{371}		4–50 °C	
	hnRNPH	^{442}SDFQSN^{447}		Above 50 °C	
	hnRNPK	^{367}SGYDYS^{372}		Above 50 °C	
	hnRNPR	^{615}GYNNDN^{620}		Above 50 °C	
FUS	FUS	^{37}SYSGYS^{42} (RAC1)		4–50 °C	[55]
		^{54}SYSSYG^{59} (RAC2)		4–20 °C	
Amyloid-β	Amyloid-β	^{16}KLVFFA^{21}	No	No melting (up to 50 °C)	[55]

References

1. Wheeler RJ, Hyman AA. Controlling compartmentalization by non-membrane-bound organelles. *Philos Trans R Soc B Biol Sci* . 2018;373(1747):20170193. doi:10.1098/rstb.2017.0193

2. Alberti S. Phase separation in biology. *Curr Biol* . 2017;27(20):R1097-R1102. doi:10.1016/j.cub.2017.08.069

3. Shin Y, Brangwynne CP. Liquid phase condensation in cell physiology and disease. *Science* . 2017;357(6357):eaaf4382. doi:10.1126/science.aaf4382

4. Alberti S, Dormann D. Liquid–Liquid Phase Separation in Disease.*Annu Rev Genet* . 2019;53(1):171-194. doi:10.1146/annurev-genet-112618-043527

5. Uversky VN. Protein intrinsic disorder-based liquid–liquid phase transitions in biological systems: Complex coacervates and membrane-less organelles. *Adv Colloid Interface Sci* . 2017;239(6400):97-114. doi:10.1016/j.cis.2016.05.012

6. Ranganathan S, Shakhnovich EI. Dynamic metastable long-living droplets formed by sticker-spacer proteins. *Elife* . 2020;9:1-25. doi:10.7554/eLife.56159

7. Sehgal PB, Westley J, Lerea KM, DiSenso-Browne S, Etlinger JD. Biomolecular condensates in cell biology and virology: Phase-separated membraneless organelles (MLOs). *Anal Biochem* . 2020;597:113691. doi:10.1016/j.ab.2020.113691

8. Alberti S, Gladfelter A, Mittag T. Considerations and Challenges in Studying Liquid-Liquid Phase Separation and Biomolecular Condensates.*Cell* . 2019;176(3):419-434. doi:10.1016/j.cell.2018.12.035

9. Lin Y, Protter DSW, Rosen MK, Parker R. Formation and Maturation of Phase-Separated Liquid Droplets by RNA-Binding Proteins. *Mol Cell* . 2015;60(2):208-219. doi:10.1016/j.molcel.2015.08.018

10. Peskett TR, Rau F, O'Driscoll J, Patani R, Lowe AR, Saibil HR. A Liquid to Solid Phase Transition Underlying Pathological Huntingtin Exon1 Aggregation. *Mol Cell* . 2018;70(4):588-601.e6. doi:10.1016/j.molcel.2018.04.007

11. Qamar S, Wang GZ, Randle SJ, et al. FUS Phase Separation Is Modulated by a Molecular Chaperone and Methylation of Arginine Cation-π Interactions. *Cell* . 2018;173(3):720-734.e15. doi:10.1016/j.cell.2018.03.056

12. Patel A, Lee HO, Jawerth L, et al. A Liquid-to-Solid Phase Transition of the ALS Protein FUS Accelerated by Disease Mutation. *Cell* . 2015;162(5):1066-1077. doi:10.1016/j.cell.2015.07.047

13. Kanaan NM, Hamel C, Grabinski T, Combs B. Liquid-liquid phase separation induces pathogenic tau conformations in vitro. *Nat Commun* . 2020;11(1):1-16. doi:10.1038/s41467-020-16580-3

14. Babinchak WM, Surewicz WK. Liquid–Liquid Phase Separation and Its Mechanistic Role in Pathological Protein Aggregation. *J Mol Biol* . 2020;432(7):1910-1925. doi:10.1016/j.jmb.2020.03.004

15. Wheeler RJ. Therapeutics-how to treat phase separation-associated diseases. *Emerg Top Life Sci* . 2020;4(3):331-342. doi:10.1042/ETLS20190176

16. Lafontaine DLJ, Riback JA, Bascetin R, Brangwynne CP. The nucleolus as a multiphase liquid condensate. *Nat Rev Mol Cell Biol* . 2021;22(3):165-182. doi:10.1038/s41580-020-0272-6

17. Gui X, Luo F, Li Y, et al. Structural basis for reversible amyloids of hnRNPA1 elucidates their role in stress granule assembly. *Nat Commun* . 2019;10(1):1-12. doi:10.1038/s41467-019-09902-7

18. Yuan C, Levin A, Chen W, et al. Nucleation and Growth of Amino Acid and Peptide Supramolecular Polymers through Liquid–Liquid Phase Separation. *Angew Chemie Int Ed* . 2019;131(50):18284-18291. doi:10.1002/ange.201911782

19. Molliex A, Temirov J, Lee J, et al. Phase Separation by Low Complexity Domains Promotes Stress Granule Assembly and Drives Pathological Fibrillization. *Cell* . 2015;163(1):123-133. doi:10.1016/j.cell.2015.09.015

20. Hernández-Vega A, Braun M, Scharrel L, et al. Local Nucleation of Microtubule Bundles through Tubulin Concentration into a Condensed Tau Phase. *Cell Rep* . 2017;20(10):2304-2312. doi:10.1016/j.celrep.2017.08.042

21. Cinar H, Fetahaj Z, Cinar S, Vernon RM, Chan HS, Winter RHA. Temperature, Hydrostatic Pressure, and Osmolyte Effects on Liquid–Liquid Phase Separation in Protein Condensates: Physical Chemistry and Biological Implications. *Chem – A Eur J* . 2019;25(57):13049-13069. doi:10.1002/chem.201902210

22. Brangwynne CP, Tompa P, Pappu R V. Polymer physics of intracellular phase transitions. *Nat Phys* . 2015;11(11):899-904. doi:10.1038/nphys3532

23. Zhou HX, Nguemaha V, Mazarakos K, Qin S. Why Do Disordered and Structured Proteins Behave Differently in Phase Separation? *Trends Biochem Sci* . 2018;43(7):499-516. doi:10.1016/j.tibs.2018.03.007

24. Ruff KM, Roberts S, Chilkoti A, Pappu R V. Advances in Understanding Stimulus-Responsive Phase Behavior of Intrinsically Disordered Protein Polymers. *J Mol Biol* . 2018;430(23):4619-4635. doi:10.1016/j.jmb.2018.06.031

25. Ten Wolde PR, Frenkel D. Enhancement of protein crystal nucleation by critical density fluctuations. *Science* . 1997;277(5334):1975-1978. doi:10.1126/science.277.5334.1975

26. Galkin O, Vekilov PG. Control of protein crystal nucleation around the metastable liquid-liquid phase boundary. *Proc Natl Acad Sci U S A* . 2000;97(12):6277-6281. doi:10.1073/pnas.110000497

27. Mohammadi P, Aranko AS, Lemetti L, et al. Phase transitions as intermediate steps in the formation of molecularly engineered protein fibers. *Commun Biol* . 2018;1(1):1-12. doi:10.1038/s42003-018-0090-y

28. Wegmann S, Eftekharzadeh B, Tepper K, et al. Tau protein liquid–liquid phase separation can initiate tau aggregation. *EMBO J* . 2018;37(7):e98049. doi:10.15252/embj.201798049

29. Vogler TO, Wheeler JR, Nguyen ED, et al. TDP-43 and RNA form amyloid-like myo-granules in regenerating muscle. *Nature* . 2018;563(7732):508-513. doi:10.1038/s41586-018-0665-2

30. Zhang X, Lin Y, Eschmann NA, et al. RNA stores tau reversibly in complex coacervates. *PLoS Biol* . 2017;15(7). doi:10.1371/journal.pbio.2002183

31. McManus JJ, Charbonneau P, Zaccarelli E, Asherie N. The physics of protein self-assembly. *Curr Opin Colloid Interface Sci* . 2016;22:73-79. doi:10.1016/j.cocis.2016.02.011

32. Ray S, Singh N, Kumar R, et al. α-Synuclein aggregation nucleates through liquid–liquid phase separation. *Nat Chem* . 2020;12(8):705-716. doi:10.1038/s41557-020-0465-9

33. Alberti S, Carra S. Quality Control of Membraneless Organelles.*J Mol Biol* . 2018;430(23):4711-4729. doi:10.1016/j.jmb.2018.05.013

34. Wu H, Fuxreiter M. The Structure and Dynamics of Higher-Order Assemblies: Amyloids, Signalosomes, and Granules. *Cell* . 2016;165(5):1055-1066. doi:10.1016/j.cell.2016.05.004

35. Alberti S, Hyman AA. Are aberrant phase transitions a driver of cellular aging? *BioEssays* . 2016;38(10):959-968. doi:10.1002/bies.201600042

36. Boeynaems S, Alberti S, Fawzi NL, et al. Protein Phase Separation: A New Phase in Cell Biology. *Trends Cell Biol* . 2018;28(6):420-435. doi:10.1016/j.tcb.2018.02.004

37. Yewdall NA, André AAM, Lu T, Spruijt E. Coacervates as models of membraneless organelles. *Curr Opin Colloid Interface Sci* . 2021;52:101416. doi:10.1016/j.cocis.2020.101416

38. Bah A, Forman-Kay JD. Modulation of intrinsically disordered protein function by post-translational modifications. *J Biol Chem* . 2016;291(13):6696-6705. doi:10.1074/jbc.R115.695056

39. Monahan Z, Ryan VH, Janke AM, et al. Phosphorylation of the FUS low-complexity domain disrupts phase separation, aggregation, and toxicity. *EMBO J* . 2017;36(20):2951-2967. doi:10.15252/embj.201696394

40. Mann JR, Gleixner AM, Mauna JC, et al. RNA Binding Antagonizes Neurotoxic Phase Transitions of TDP-43. *Neuron* . 2019;102(2):321-338.e8. doi:10.1016/j.neuron.2019.01.048

41. Ganassi M, Mateju D, Bigi I, et al. A Surveillance Function of the HSPB8-BAG3-HSP70 Chaperone Complex Ensures Stress Granule Integrity and Dynamism. *Mol Cell* . 2016;63(5):796-810. doi:10.1016/j.molcel.2016.07.021

42. Liu Z, Zhang S, Gu J, et al. Hsp27 chaperones FUS phase separation under the modulation of stress-induced phosphorylation. *Nat Struct Mol Biol* . 2020;27(4):363–372. doi:10.1038/s41594-020-0399-3

43. Guo L, Kim HJ, Wang H, et al. Nuclear-Import Receptors Reverse Aberrant Phase Transitions of RNA-Binding Proteins with Prion-like Domains. *Cell* . 2018;173(3):677-692.e20. doi:10.1016/j.cell.2018.03.002

44. Frottin F, Schueder F, Tiwary S, et al. The nucleolus functions as a phase-separated protein quality control compartment. *Science* . 2019;365(6451):342-347. doi:10.1126/science.aaw9157

45. Mateju D, Franzmann TM, Patel A, et al. An aberrant phase transition of stress granules triggered by misfolded protein and prevented by chaperone function. *EMBO J* . 2017;36(12):1669-1687. doi:10.15252/embj.201695957

46. Patel A, Malinovska L, Saha S, et al. ATP as a biological hydrotrope. *Science* . 2017;356(6339):753-756. doi:10.1126/science.aaf6846

47. Kroschwald S, Maharana S, Mateju D, et al. Promiscuous interactions and protein disaggregases determine the material state of stress-inducible RNP granules. *Elife* . 2015;4:e06807. doi:10.7554/eLife.06807

48. Gomes E, Shorter J. The molecular language of membraneless organelles. *J Biol Chem* . 2019;294(18):7115-7127. doi:10.1074/jbc.tm118.001192

49. Reichheld SE, Muiznieks LD, Keeley FW, Sharpe S. Direct observation of structure and dynamics during phase separation of an elastomeric protein. *Proc Natl Acad Sci U S A* . 2017;114(22):E4408-E4415. doi:10.1073/pnas.1701877114

50. Cai H, Gabryelczyk B, Manimekalai MSS, Gruber G, Salentinig S, Miserez A. Self-coacervation of modular squid beak proteins-a comparative study. *Soft Matter* . 2017;13(42):7740-7752. doi:10.1039/c7sm01352c

51. Tan Y, Hoon S, Guerette PA, et al. Infiltration of chitin by protein coacervates defines the squid beak mechanical gradient. *Nat Chem Biol* . 2015;11(7):488-495. doi:10.1038/nchembio.1833

52. Gabryelczyk B, Cai H, Shi X, et al. Hydrogen bond guidance and aromatic stacking drive liquid-liquid phase separation of intrinsically disordered histidine-rich peptides. *Nat Commun* . 2019;10(1):1-12. doi:10.1038/s41467-019-13469-8

53. Nott TJ, Petsalaki E, Farber P, et al. Phase Transition of a Disordered Nuage Protein Generates Environmentally Responsive Membraneless Organelles. *Mol Cell* . 2015;57(5):936-947. doi:10.1016/j.molcel.2015.01.013

54. Schuster BS, Reed EH, Parthasarathy R, et al. Controllable protein phase separation and modular recruitment to form responsive membraneless organelles. *Nat Commun* . 2018;9(1):1-12. doi:10.1038/s41467-018-05403-1

55. Luo F, Gui X, Zhou H, et al. Atomic structures of FUS LC domain segments reveal bases for reversible amyloid fibril formation. *Nat Struct Mol Biol* . 2018;25(4):341-346. doi:10.1038/s41594-018-0050-8

56. Li HR, Chiang WC, Chou PC, Wang WJ, Huang J rong. TAR DNA-binding protein 43 (TDP-43) liquid-liquid phase separation is mediated by just a few aromatic residues. *J Biol Chem* . 2018;293(16):6090-6098. doi:10.1074/jbc.AC117.001037

57. Gallivan JP, Dougherty DA. A computational study of cation-π interactions vs salt bridges in aqueous media: Implications for protein engineering. *J Am Chem Soc* . 2000;122(5):870-874. doi:10.1021/ja991755c

58. Liu FF, Liu Z, Bai S, Dong XY, Suna Y. Exploring the inter-molecular interactions in amyloid-β protofibril with molecular dynamics simulations and molecular mechanics Poisson-Boltzmann surface area free energy calculations. *J Chem Phys* . 2012;136(14):145101. doi:10.1063/1.3702195

59. Padrick SB, Miranker AD. Islet amyloid: Phase partitioning and secondary nucleation are central to the mechanism of fibrillogenesis.*Biochemistry* . 2002;41(14):4694-4703. doi:10.1021/bi0160462

60. Falkenberg C V., Blinov ML, Loew LM. Pleomorphic ensembles: Formation of large clusters composed of weakly interacting multivalent molecules. *Biophys J* . 2013;105(11):2451-2460. doi:10.1016/j.bpj.2013.10.016

61. Uversky VN. Proteins without unique 3D structures: Biotechnological applications of intrinsically unstable/disordered proteins.*Biotechnol J* . 2015;10(3):356-366. doi:10.1002/biot.201400374

62. Uversky VN. Paradoxes and wonders of intrinsic disorder: Stability of instability. *Intrinsically Disord Proteins* . 2017;5(1):e1327757. doi:10.1080/21690707.2017.1327757

63. Kostylev MA, Tuttle MD, Lee S, et al. Liquid and Hydrogel Phases of PrP C Linked to Conformation Shifts and Triggered by Alzheimer's Amyloid-β Oligomers. *Mol Cell* . 2018;72(3):426-443.e12. doi:10.1016/j.molcel.2018.10.009

64. Habchi J, Tompa P, Longhi S, Uversky VN. Introducing protein intrinsic disorder. *Chem Rev* . 2014;114(13):6561-6588. doi:10.1021/cr400514h

65. Darling AL, Liu Y, Oldfield CJ, Uversky VN. Intrinsically Disordered Proteome of Human Membrane-Less Organelles. *Proteomics* . 2018;18(5-6):1700193. doi:10.1002/pmic.201700193

66. Pejaver V, Hsu WL, Xin F, Dunker AK, Uversky VN, Radivojac P. The structural and functional signatures of proteins that undergo multiple events of post-translational modification. *Protein Sci* . 2014;23(8):1077-1093. doi:10.1002/pro.2494

67. Li P, Banjade S, Cheng HC, et al. Phase transitions in the assembly of multivalent signalling proteins. *Nature* . 2012;483(7389):336-340. doi:10.1038/nature10879

68. Su X, Ditlev JA, Hui E, et al. Phase separation of signaling molecules promotes T cell receptor signal transduction. *Science* . 2016;352(6285):595-599. doi:10.1126/science.aad9964

69. Mitrea DM, Cika JA, Guy CS, et al. Nucleophosmin integrates within the nucleolus via multi-modal interactions with proteins displaying R-rich linear motifs and rRNA. *Elife* . 2016;5(FEBRUARY2016). doi:10.7554/eLife.13571.001

70. Banani SF, Rice AM, Peeples WB, et al. Compositional Control of Phase-Separated Cellular Bodies. *Cell* . 2016;166(3):651-663. doi:10.1016/j.cell.2016.06.010

71. Nelson R, Sawaya MR, Balbirnie M, et al. Structure of the cross-β spine of amyloid-like fibrils. *Nature* . 2005;435(7043):773-778. doi:10.1038/nature03680

72. Sawaya MR, Sambashivan S, Nelson R, et al. Atomic structures of amyloid cross-β spines reveal varied steric zippers. *Nature* . 2007;447(7143):453-457. doi:10.1038/nature05695

73. Eisenberg D, Jucker M. The amyloid state of proteins in human diseases. *Cell* . 2012;148(6):1188-1203. doi:10.1016/j.cell.2012.02.022

74. Hughes MP, Sawaya MR, Boyer DR, et al. Atomic structures of low-complexity protein segments reveal kinked β sheets that assemble networks. *Science* . 2018;359(6376):698-701. doi:10.1126/science.aan6398

75. Peran I, Mittag T. Molecular structure in biomolecular condensates. *Curr Opin Struct Biol* . 2020;60:17-26. doi:10.1016/j.sbi.2019.09.007

76. Lu J, Cao Q, Hughes MP, et al. CryoEM structure of the low-complexity domain of hnRNPA2 and its conversion to pathogenic amyloid. *Nat Commun* . 2020;11(1). doi:10.1038/s41467-020-17905-y

77. Hughes MP, Goldschmidt L, Eisenberg DS. Prevalence and species distribution of the low-complexity, amyloid-like, reversible, kinked segment structural motif in amyloid-like fibrils. *J Biol Chem* . 2021;297(4):101194. doi:10.1016/j.jbc.2021.101194

78. Kato M, Han TW, Xie S, et al. Cell-free formation of RNA granules: Low complexity sequence domains form dynamic fibers within hydrogels. *Cell* . 2012;149(4):753-767. doi:10.1016/j.cell.2012.04.017

79. Burke KA, Janke AM, Rhine CL, Fawzi NL. Residue-by-Residue View of In Vitro FUS Granules that Bind the C-Terminal Domain of RNA Polymerase II. *Mol Cell* . 2015;60(2):231-241. doi:10.1016/j.molcel.2015.09.006

80. Murray DT, Kato M, Lin Y, et al. Structure of FUS Protein Fibrils and Its Relevance to Self-Assembly and Phase Separation of Low-Complexity Domains. *Cell* . 2017;171(3):615-627.e16. doi:10.1016/j.cell.2017.08.048

81. Murthy AC, Dignon GL, Kan Y, et al. Molecular interactions underlying liquid-liquid phase separation of the FUS low-complexity domain. *Nat Struct Mol Biol* . 2019;26(7):637-648. doi:10.1038/s41594-019-0250-x

82. Ding X, Sun F, Chen J, et al. Amyloid-Forming Segment Induces Aggregation of FUS-LC Domain from Phase Separation Modulated by Site-Specific Phosphorylation. *J Mol Biol* . 2020;432(2):467-483. doi:10.1016/j.jmb.2019.11.017

83. Ambadipudi S, Biernat J, Riedel D, Mandelkow E, Zweckstetter M. Liquid-liquid phase separation of the microtubule-binding repeats of the Alzheimer-related protein Tau. *Nat Commun* . 2017;8(1):275. doi:10.1038/s41467-017-00480-0

84. Guenther EL, Cao Q, Trinh H, et al. Atomic structures of TDP-43 LCD segments and insights into reversible or pathogenic aggregation.*Nat Struct Mol Biol* . 2018;25(6):463-471. doi:10.1038/s41594-018-0064-2

85. Ryan VH, Dignon GL, Zerze GH, et al. Mechanistic View of hnRNPA2 Low-Complexity Domain Structure, Interactions, and Phase Separation Altered by Mutation and Arginine Methylation. *Mol Cell* . 2018;69(3):465-479.e7. doi:10.1016/j.molcel.2017.12.022

86. Conicella AE, Zerze GH, Mittal J, et al. ALS Mutations Disrupt Phase Separation Mediated by α-Helical Structure in the TDP-43 Low-Complexity C-Terminal Domain. *Structure* . 2016;24(9):1537-1549. doi:10.1016/j.str.2016.07.007

87. Quiroz FG, Chilkoti A. Sequence heuristics to encode phase behaviour in intrinsically disordered protein polymers. *Nat Mater* . 2015;14(11):1164-1171. doi:10.1038/nmat4418

88. Panas MD, Schulte T, Thaa B, et al. Viral and Cellular Proteins Containing FGDF Motifs Bind G3BP to Block Stress Granule Formation.*PLoS Pathog* . 2015;11(2):e1004659. doi:10.1371/journal.ppat.1004659

89. Conicella AE, Dignon GL, Zerze GH, et al. TDP-43 α-helical structure tunes liquid–liquid phase separation and function. *Proc Natl Acad Sci U S A* . 2020;117(11):5883-5894. doi:10.1073/pnas.1912055117

90. Murray DT, Zhou X, Kato M, Xiang S, Tycko R, McKnight SL. Structural characterization of the D290V mutation site in hnRNPA2 low-complexity-domain polymers. *Proc Natl Acad Sci U S A* . 2018;115(42):E9782-E9791. doi:10.1073/pnas.1806174115

91. Deng Q, Holler CJ, Taylor G, et al. FUS is phosphorylated by DNA-PK and accumulates in the cytoplasm after DNA damage. *J Neurosci* . 2014;34(23):7802-7813. doi:10.1523/JNEUROSCI.0172-14.2014

92. Quiroz FG, Li NK, Roberts S, et al. Intrinsically disordered proteins access a range of hysteretic phase separation behaviors.*Sci Adv* . 2019;5(10):5177-5195. doi:10.1126/sciadv.aax5177

93. Liu J, Zhorabek F, Dai X, Huang J, Chau Y. Minimalist Design of an Intrinsically Disordered Protein-Mimicking Scaffold for an Artificial Membraneless Organelle. *ACS Cent Sci* . 2022;8(4):493–500. doi:10.1021/ACSCENTSCI.1C01021

94. Liu J, Zhorabek F, Chau Y. Nucleic Acids Modulate Liquidity and Dynamics of Artificial Membraneless Organelles. *ACS Macro Lett* . 2022;11(4):562-567. doi:10.1021/ACSMACROLETT.2C00167

95. Liu J, Zhorabek F, Zhang T, Lam JWY, Tang BZ, Chau Y. Multifaceted Cargo Recruitment and Release from Artificial Membraneless Organelle.*Small* . 2022;(In press).

96. Liu J, Zhorabek F, Chau Y. Biomaterial design inspired by membraneless organelles. *arXiv Prepr arXiv210410927* . April 2021. http://arxiv.org/abs/2104.10927. Accessed April 27, 2021.

97. Wallace EWJ, Kear-Scott JL, Pilipenko E V., et al. Reversible, Specific, Active Aggregates of Endogenous Proteins Assemble upon Heat Stress. *Cell* . 2015;162(6):1286-1298. doi:10.1016/j.cell.2015.08.041

98. Kroschwald S, Munder MC, Maharana S, et al. Different Material States of Pub1 Condensates Define Distinct Modes of Stress Adaptation and Recovery. *Cell Rep* . 2018;23(11):3327-3339. doi:10.1016/j.celrep.2018.05.041

99. Franzmann TM, Jahnel M, Pozniakovsky A, et al. Phase separation of a yeast prion protein promotes cellular fitness. *Science* . 2018;359(6371):eaao5654. doi:10.1126/science.aao5654

100. Sheu-Gruttadauria J, MacRae IJ. Phase Transitions in the Assembly and Function of Human miRISC. *Cell* . 2018;173(4):946-957.e16. doi:10.1016/j.cell.2018.02.051

101. Shin Y, Berry J, Pannucci N, Haataja MP, Toettcher JE, Brangwynne CP. Spatiotemporal Control of Intracellular Phase Transitions Using Light-Activated optoDroplets. *Cell* . 2017;168(1-2):159-171.e14. doi:10.1016/j.cell.2016.11.054

102. Reed EH, Schuster BS, Good MC, Hammer DA. SPLIT: Stable Protein Coacervation Using a Light Induced Transition. *ACS Synth Biol* . 2020;9(3):500-507. doi:10.1021/acssynbio.9b00503

103. Banani SF, Lee HO, Hyman AA, Rosen MK. Biomolecular condensates: Organizers of cellular biochemistry. *Nat Rev Mol Cell Biol* . 2017;18(5):285-298. doi:10.1038/nrm.2017.7

104. Woodruff JB, Hyman AA, Boke E. Organization and Function of Non-dynamic Biomolecular Condensates. *Trends Biochem Sci* . 2018;43(2):81-94. doi:10.1016/j.tibs.2017.11.005

105. Jawerth L, Fischer-Friedrich E, Saha S, et al. Protein condensates as aging Maxwell fluids. *Science* . 2020;370(6522):1317-1323. doi:10.1126/science.aaw4951

106. Zhang H. The glassiness of hardening protein droplets: Protein condensates can age to form glasses that increase in viscosity but retain elasticity. *Science* . 2020;370(6522):1271-1272. doi:10.1126/science.abe9745

107. Dogra P, Joshi A, Majumdar A, Mukhopadhyay S. Intermolecular Charge-Transfer Modulates Liquid-Liquid Phase Separation and Liquid-to-Solid Maturation of an Intrinsically Disordered pH-Responsive Domain. *J Am Chem Soc* . 2019;141(51):20380-20389. doi:10.1021/jacs.9b10892

108. Ciryam P, Tartaglia GG, Morimoto RI, Dobson CM, Vendruscolo M. Widespread Aggregation and Neurodegenerative Diseases Are Associated with Supersaturated Proteins. *Cell Rep* . 2013;5(3):781-790. doi:10.1016/j.celrep.2013.09.043

109. Ciryam P, Kundra R, Morimoto RI, Dobson CM, Vendruscolo M. Supersaturation is a major driving force for protein aggregation in neurodegenerative diseases. *Trends Pharmacol Sci* . 2015;36(2):72-77. doi:10.1016/j.tips.2014.12.004

110. Ramaswami M, Taylor JP, Parker R. Altered ribostasis: RNA-protein granules in degenerative disorders. *Cell* . 2013;154(4):727-736. doi:10.1016/j.cell.2013.07.038

111. Murakami T, Qamar S, Lin JQ, et al. ALS/FTD Mutation-Induced Phase Transition of FUS Liquid Droplets and Reversible Hydrogels into Irreversible Hydrogels Impairs RNP Granule Function. *Neuron* . 2015;88(4):678-690. doi:10.1016/j.neuron.2015.10.030

112. Nedelsky NB, Taylor JP. Bridging biophysics and neurology: aberrant phase transitions in neurodegenerative disease. *Nat Rev Neurol* . 2019;15(5):272-286. doi:10.1038/s41582-019-0157-5

113. Elbaum-Garfinkle S. Matter over mind: Liquid phase separation and neurodegeneration. *J Biol Chem* . 2019;294(18):7160-7168. doi:10.1074/jbc.REV118.001188

114. von Mikecz A. Pathology and function of nuclear amyloid: Protein homeostasis matters. *Nucleus* . 2014;5(4):311-317. doi:10.4161/nucl.29404

115. Chung CG, Lee H, Lee SB. Mechanisms of protein toxicity in neurodegenerative diseases. *Cell Mol Life Sci 2018 7517* . 2018;75(17):3159-3180. doi:10.1007/S00018-018-2854-4

116. Mori K, Weng SM, Arzberger T, et al. The C9orf72 GGGGCC repeat is translated into aggregating dipeptide-repeat proteins in FTLD/ALS.*Science* . 2013;339(6125):1335-1338. doi:10.1126/science.1232927

117. Kwon I, Xiang S, Kato M, et al. Poly-dipeptides encoded by the C9orf72 repeats bind nucleoli, impede RNA biogenesis, and kill cells.*Science* . 2014;345(6201):1139-1145. doi:10.1126/science.1254917

118. Lee KH, Zhang P, Kim HJ, et al. C9orf72 Dipeptide Repeats Impair the Assembly, Dynamics, and Function of Membrane-Less Organelles.*Cell* . 2016;167(3):774-788.e17. doi:10.1016/j.cell.2016.10.002

119. Li YR, King OD, Shorter J, Gitler AD. Stress granules as crucibles of ALS pathogenesis. *J Cell Biol* . 2013;201(3):361-372. doi:10.1083/jcb.201302044

120. Ryan VH, Fawzi NL. Physiological, Pathological, and Targetable Membraneless Organelles in Neurons. *Trends Neurosci* . 2019;42(10):693-708. doi:10.1016/j.tins.2019.08.005

121. Ferreon JC, Jain A, Choi KJ, et al. Acetylation disfavors tau phase separation. *Int J Mol Sci* . 2018;19(5):1360. doi:10.3390/ijms19051360

122. López-Erauskin J, Tadokoro T, Baughn MW, et al. ALS/FTD-Linked Mutation in FUS Suppresses Intra-axonal Protein Synthesis and Drives Disease Without Nuclear Loss-of-Function of FUS. *Neuron* . 2018;100(4):816-830.e7. doi:10.1016/j.neuron.2018.09.044

123. Kim HJ, Kim NC, Wang YD, et al. Mutations in prion-like domains in hnRNPA2B1 and hnRNPA1 cause multisystem proteinopathy and ALS.*Nature* . 2013;495(7442):467-473. doi:10.1038/nature11922

124. Mackenzie IR, Nicholson AM, Sarkar M, et al. TIA1 Mutations in Amyotrophic Lateral Sclerosis and Frontotemporal Dementia Promote Phase Separation and Alter Stress Granule Dynamics. *Neuron* . 2017;95(4):808-816.e9. doi:10.1016/j.neuron.2017.07.025

125. Jain A, Vale RD. RNA phase transitions in repeat expansion disorders. *Nature* . 2017;546(7657):243-247. doi:10.1038/nature22386

126. Bouchard JJ, Otero JH, Scott DC, et al. Cancer Mutations of the Tumor Suppressor SPOP Disrupt the Formation of Active, Phase-Separated Compartments. *Mol Cell* . 2018;72(1):19-36.e8. doi:10.1016/j.molcel.2018.08.027

127. Morelli FF, Verbeek DS, Bertacchini J, et al. Aberrant Compartment Formation by HSPB2 Mislocalizes Lamin A and Compromises Nuclear Integrity and Function. *Cell Rep* . 2017;20(9):2100-2115. doi:10.1016/j.celrep.2017.08.018