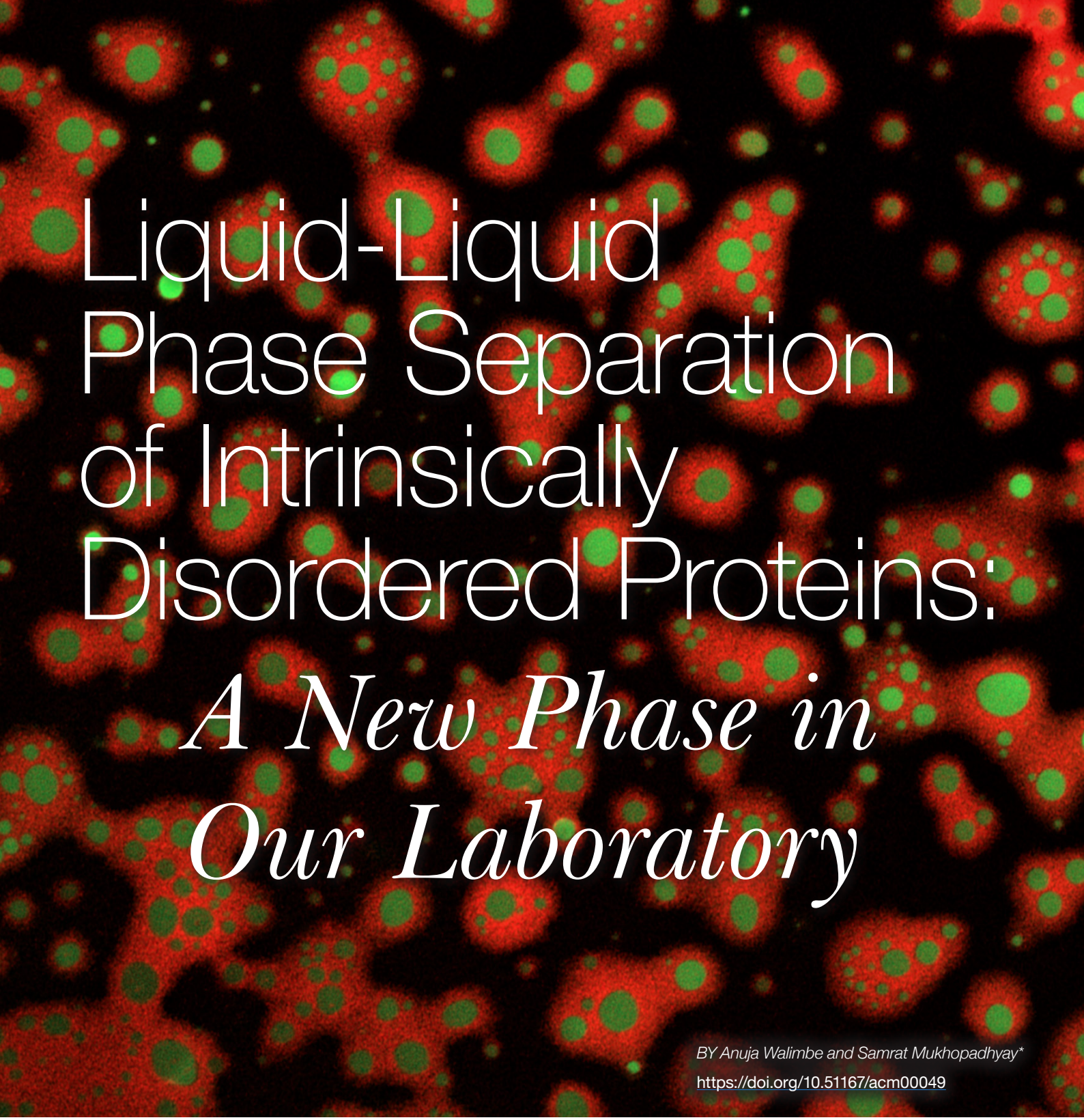

Liquid-Liquid Phase Separation of Intrinsically Disordered Proteins: *A New Phase in Our Laboratory*

*BY Anuja Walimbe and Samrat Mukhopadhyay**

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Our laboratory at the Indian Institute of Science Education and Research (IISER) Mohali is involved in studying the intriguing conformational characteristics of intrinsically disordered proteins (IDPs) that do not fold up into a well-defined 3D structure and exist as rapidly fluctuating conformational ensemble. Our research over the past decade or so has dealt with the IDPs and partially unfolded proteins and their conversion into amyloid aggregates that are associated with deadly neurodegenerative diseases such as Alzheimer's, Parkinson's, Huntington's, and prion diseases. We were particularly interested in characterizing the monomeric and oligomeric precursors of amyloid fibrils. This work has been summarized in an invited Feature Article.[1] The current article primarily deals with the newer research direction of our lab in liquid-liquid phase separation of IDPs. Here we summarize our recent exciting discoveries in phase separation of range of disease-related IDPs. Below we provide a background of the emerging field of biomolecular condensates and describe our published results.

Background

Eukaryotic cells comprise well-defined, membrane-bound organelles that ensure compartmentalization and proper functioning of cells. A growing body of exciting research has revealed that there is an alternate way to achieve compartmentalization and spatiotemporal organization of cellular constituents. This is via liquid-liquid phase separation of biomolecules into non-canonical, membrane-less organelles[2-4]. These membrane-less compartments or biomolecular condensates are non-stoichiometric, dynamic, mesoscopic assemblies rich in proteins and nucleic acids and include stress granules, germ granules, P bodies, Cajal bodies, nuclear paraspeckles, nucleolus, and so forth[4-6]. Emerging evidence suggests that intrinsically disordered proteins/regions (IDPs/IDRs) harboring low-complexity, prion-like domains serve as the best candidates to undergo phase separation into these liquid-like membrane-less organelles. IDPs/IDRs, without or with nucleic acids, participate in a multitude of weak, non-covalent, transient, multivalent interactions resulting into the formation of a dynamic network which sequesters biomolecules into these membrane-less compartments[7,8]. Along with the transient driving forces, these liquid-like, mesoscopic assemblies can be tuned by other factors including interacting protein partners, nucleic acids, or environmental cues[2,4]. In addition to these liquid-like assemblies, proteins are also capable of forming highly compact, structured assemblies or amyloid aggregates[9]. Aberrant transition of these condensates into solid-like-aggregates is linked with a number of fatal neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), frontotemporal lobar dementia (FTLD), so forth[2,4,9]. However, the molecular origin and the sequence of events that regulates intracellular phase transitions into these liquid-like compartments and liquid-to-solid phase transition remain elusive. Our group has been interested in elucidating the fundamental molecular mechanism of such biological phase transitions of IDPs and IDRs.

Conformational shapeshifting coupled with fluctuations and solvation drive phase separation[10]

One of our initial phase separation studies focused on the human tau protein's intrinsically disordered, amyloidogenic fragment, namely tau K18. Misfolding and pathological inclusions of tau are associated with several neurodegenerative diseases, including Alzheimer's disease. Full-length tau and tau K18 are known to undergo phase separation to form liquid-like droplets capable of transitioning into pathological aggregates. Previous structural studies on tau K18 monomer have established the presence of ordered regions, including α-helices and β-turns, suggesting a collapsed conformation. With this in mind, our first objective was to study the change in the conformational distribution upon undergoing condensation into droplets. In this direction, we employed a molecular spectroscopic probe pyrene, as a reporter of intramolecular distances. Pyrene dimer upon excitation shows a longer wavelength emission in addition to the monomeric excited state emission band. Monomeric tau K18 displayed a strong excimer band suggesting structural compaction in accordance with the previous structural studies. Upon droplet formation, the excimer emission band reduced gradually, suggesting a conformational expansion of the polypeptide chain in the condensed phase (Fig. 1A, B). Thus, we could utilize a sensitive proximity probe, pyrene, as a readout of transition from a compact globule state to extended coils, which can facilitate the formation of multiple, transient, noncovalent interactions within the droplet phase.

Next, in order to study the environment in the droplet interior, we performed fluorescence quenching experiments to measure solvent accessibility within the condensates. Lifetime measurements were made in order to obtain the biomolecular quenching rate constants and Stern-Volmer quenching constants (Fig. 1C). Based on the Stern-Volmer plots, the dispersed state exhibited very low solvent accessibility, as suspected for a polypeptide chain under compaction. On the contrary, solvent accessibility in the droplet interior was much higher than for monomeric tau K18, possibly due to extensive solvent recruitment and chain solvation within the droplets. Intending to study the conformational dynamics of the expanded polypeptide chain in the aqueous-organic environment inside the droplets, we performed steady-state anisotropy experiments which showed a significant decrease in the anisotropy value from the monomeric to the droplet state indicating an increased rotational flexibility of the polypeptide chains in droplets. Additionally, we carried out picosecond time-resolved fluorescence anisotropy experiments which permitted us to recover two separate rotational correlation times (ϕfast and ϕslow). The slow rotational correlation time in monomeric tau K18 indicated a compact globule state whereas upon condensation into droplets, ϕslow decreased further indicating an expansion of the polypeptide chain resulting in extensive segmental rotational mobility as seen from the faster depolarization kinetics (Fig. 1D, E). Hence, upon undergoing phase separation, the polypeptide chain exhibits segmental chain fluctuations involving backbone torsional relaxation in expanded polypeptide chains (Fig. 1F). Thus, using steady-state and time-resolved fluorescence tools, this work captures the dynamics of the polypeptide chain and chain solvation which can act as crucial regulators and determinants of condensate properties.

Anuja Walimbe

Anuja Walimbe was born in Pune, India. After her B.Sc. (Microbiology major) from Savitribai Phule Pune University, she joined the integrated Ph.D. (M.S. and Ph.D.) program at the Indian Institute of Science Education and Research (IISER) Mohali in 2019. She is currently a Ph.D. student working with Samrat Mukhopadhyay. She is interested in biomolecular condensates that are formed via liquid-liquid phase separation of intrinsically disordered proteins and nucleic acids. She is currently working on the biophysical aspect of phase separation of eukaryotic intrinsically disordered proteins associated with nuclear condensates using a diverse array of spectroscopic and microscopic tools.

She is a recipient of the Prime Minister's Research Fellowship.

Samrat Mukhopadhyay

Samrat Mukhopadhyay was born in Kolkata, India, and received his B.Sc. in Chemistry (Honors) from Jadavpur University, Kolkata, he joined the integrated Ph.D. program at the Indian Institute of Science, Bangalore. During his Ph.D. work (2000-2004) in organic chemistry with Uday Maitra he was interested in protein folding and misfolding, which led to a short stint as a Visiting Fellow at the Tata Institute of Fundamental Research (TIFR), Mumbai, where he worked with G. Krishnamoorthy and Jayant Udgaonkar. His postdoctoral work with Ashok Deniz at the Scripps Research Institute, California,

(2005-2008) focused on single-molecule biophysics in collaboration with Susan Lindquist of the Whitehead Institute, MIT. In late 2008 he joined the newly established science university, Indian Institute of Science Education and Research (IISER) Mohali, where he became a full Professor. His laboratory is interested in intrinsically disordered proteins that undergo amyloid formation and phase separation associated with physiology and disease. He is a member of the editorial board of the Biophysical Journal (Cell Press) and editorial advisory boards of the ACS Journal of Physical Chemistry, and Essays in Biochemistry (Biochemical Society, London, UK). He is also an Ambassador of the Biophysical Society (USA).

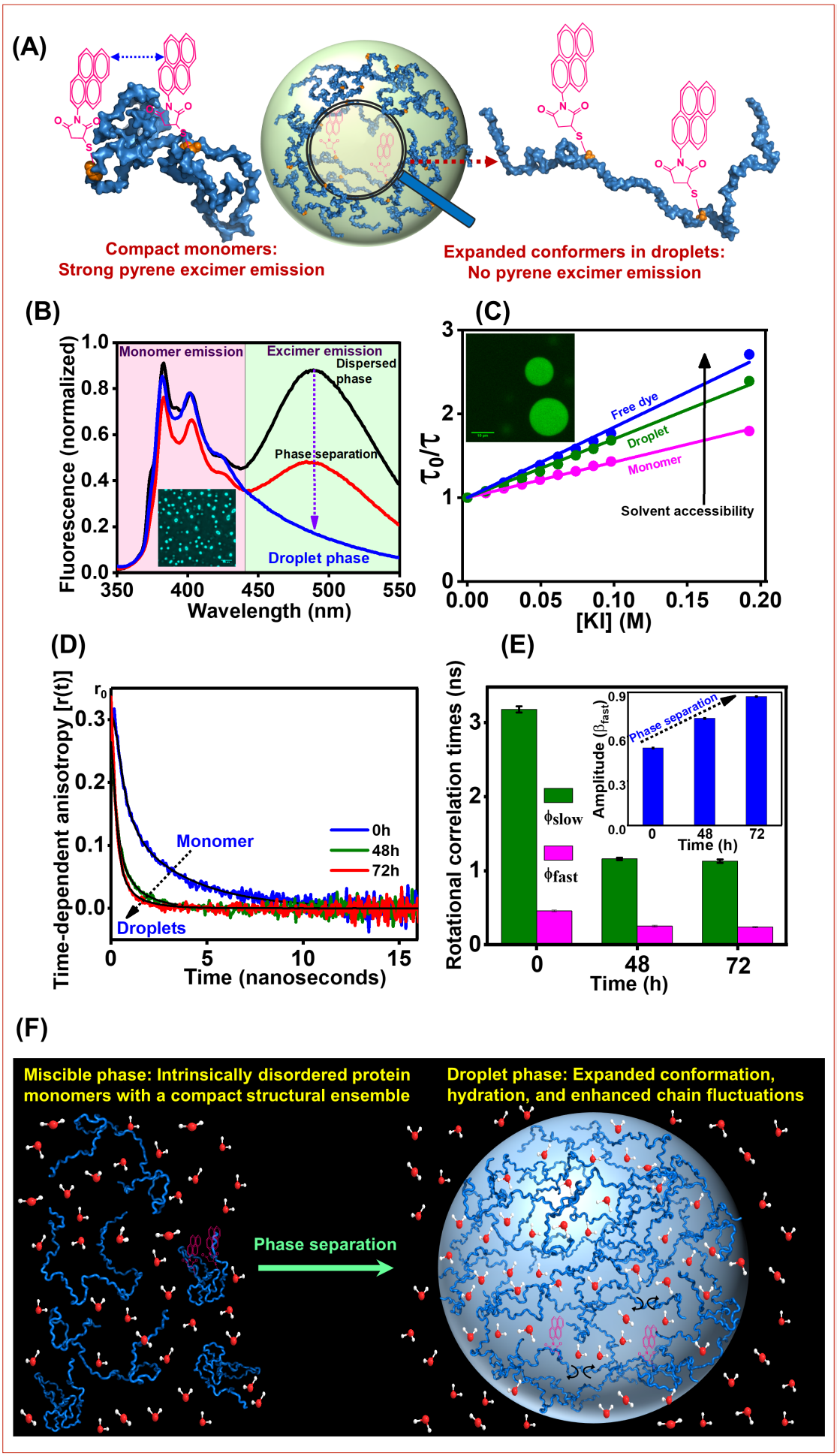


Figure 1. (A) Schematic representation showing chain expansion during phase separation observed using pyrene excimer. **(B)** Fluorescence spectra of pyrene-labeled tauK18 in dispersed and droplet phases. **(C)** Stern-Volmer plots for free dye F5M and tau K18 monomer and in the droplet state. **(D)** Fluorescence depolarization kinetics of F5M-labeled tau K18 during phase separation. **(E)** Rotational correlation times as a function of phase separation. **(F)** Schematic illustration of conformational expansion, fluctuations, and solvation during phase separation (Adapted with permission from Ref. 10. Copyright American Chemical Society).

Intermolecular charge-transfer in phase transitions of an intrinsically disordered domain[11]

The melanosomal protein or Pmel17 is a functional amyloid that undergoes amyloid assembly within the cell in order perform its physiological role of melanogenesis. The oligopeptide repeat domain (RPT) of Pmel17 is known to form the amyloid core essential for amyloidogenesis of Pmel17 at acidic pH (4-5.5) but not at cytosolic pH. Based on its low complexity sequence composition and the resulting intrinsic disorder, we hypothesized that RPT could undergo phase separation under physiological conditions. Indeed, upon incubation at cytosolic pH, we observed the formation of dynamic, liquid-like, spherical droplets (Fig. 2A). Our next objective was to probe the environment experienced by the polypeptide chain within these droplets. To this end, we performed quenching experiments and observed significant solvent recruitment and chain solvation within the condensates. Further, in order to study the chain dynamics of these polypeptide chains, we performed steady-state anisotropy experiments in combination with picosecond time-resolved experiments which suggested huge conformational fluctuations in addition to the rapid diffusion of the polypeptide chain within the droplet environment (Fig. 2B, C).

Next, to determine the role of electrostatic interactions in this phase transition we began with studying the effect of ionic strength on droplet formation. We observed an increase in phase separation with increasing salt at lower salt concentrations followed by complete inhibition of droplet formation at higher salt concentrations, highlighting the impact of ionic strength on RPT phase separation. In order to distinguish between phase separation and aggregation of RPT, we performed tryptophan anisotropy experiments during which we noticed an intriguing longer-wavelength fluorescence emission exclusively in the condensed phase. This intrinsic blue fluorescence is known to originate from extensive intermolecular charge-transfer facilitated by the water molecules-mediated hydrogen-bonded network of the polypeptide chain backbone. The intensity of this blue emission band was found to be phase separation and ionic

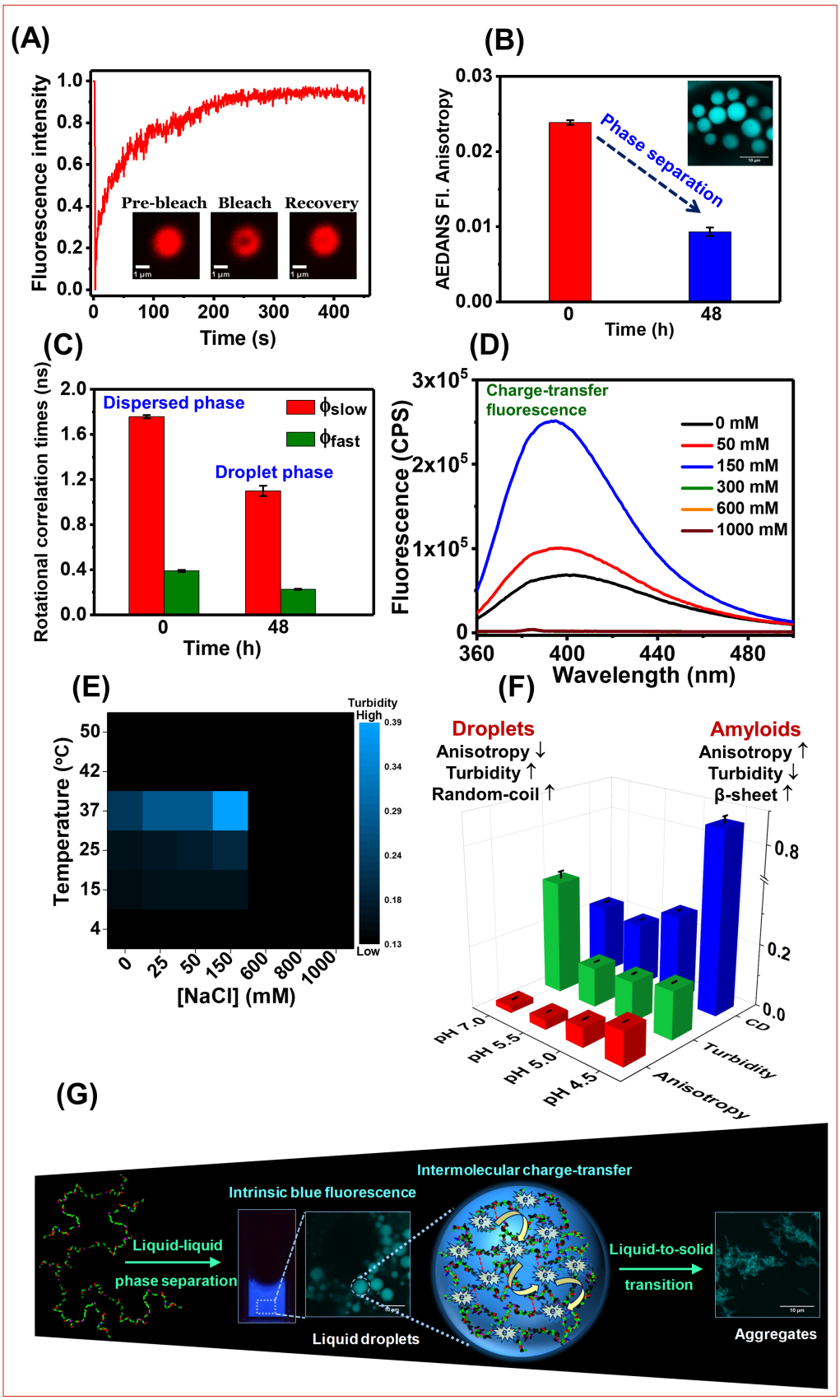


Figure 2. (A) FRAP kinetics for RPT droplets showing fast near-complete recovery. **(B)** Decrease in the steady-state fluorescence anisotropy of IAEDANS-labeled RPT. (C) Rotational correlation times in monomeric and in droplet phase. **(D)** Changes in the intrinsic blue fluorescence due to charge transfer. **(E)** Phase diagram of RPT as a function of temperature and salt concentrations. **(F)** Phase separation versus aggregation as a function of pH as monitored through CD, turbidity, and anisotropy measurements. **(G)** Schematic illustration of charge-transfer-mediated phase transitions (Adapted with permission from Ref. 11. Copyright American Chemical Society).

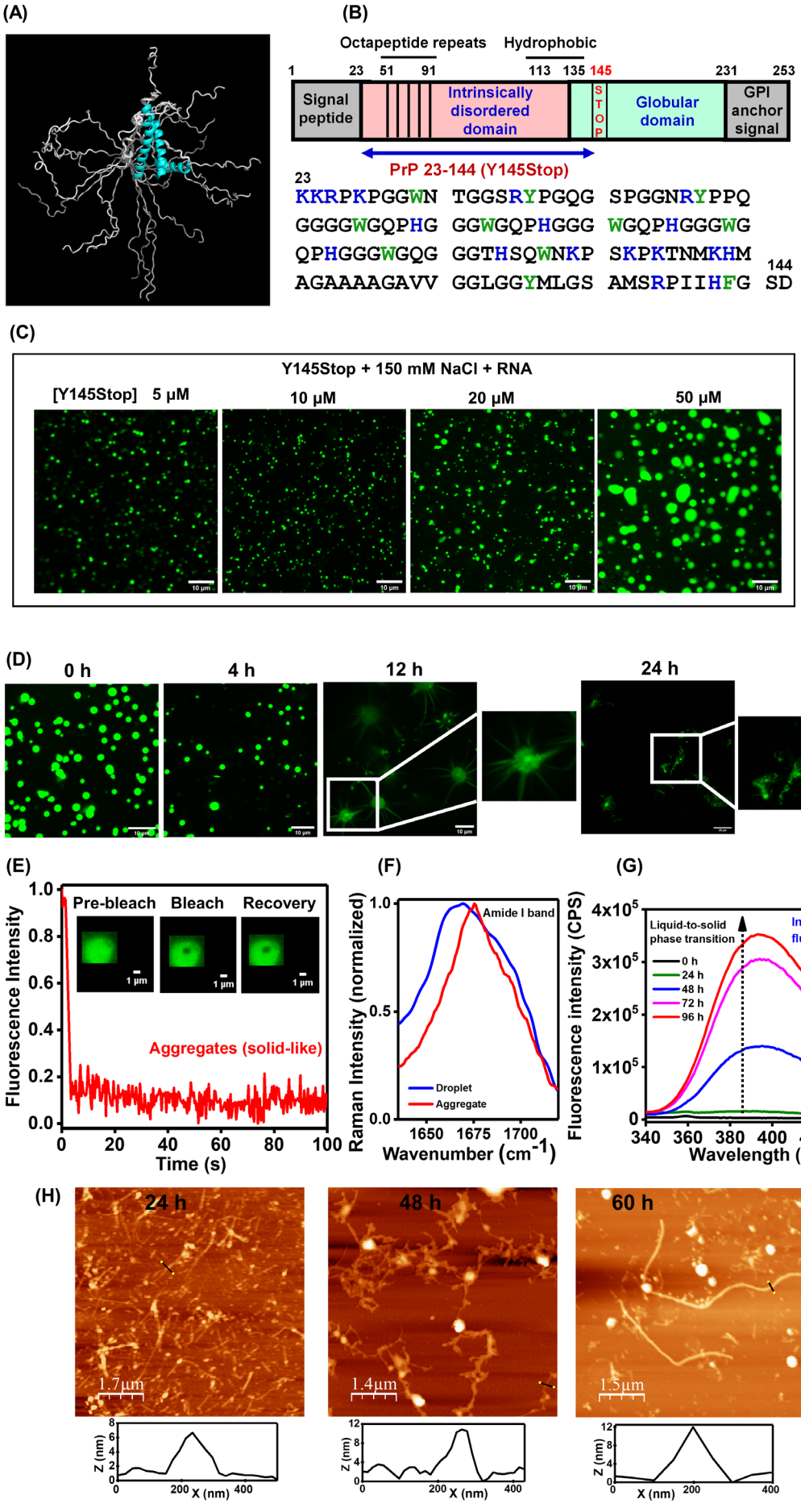


Figure 3. (A) Structure of PrP showing intrinsically disordered N-terminal domain and C-terminal folded domain. **(B)** Amino acid sequence of PrP indicating Y145Stop mutation. **(C)** Confocal imaging of Y145Stop droplets in presence of RNA. **(D-H)** Liquid-to-solid phase transitions of Y145Stop liquid droplets studied using confocal **(D)**, FRAP **(E)** vibrational Raman **(F)**, charge transfer fluorescence **(G)**, and AFM **(H)** (Adapted from Ref. 12).

strength dependent (Fig. 2D). Disrupting the weak hydrophobic interactions eventually led to complete inhibition of droplet formation. Thus, RPT phase separation is driven by a complex interplay of transient intermolecular charge-transfer, electrostatic, and hydrophobic interactions. Next, we set out to investigate the temperature dependence of RPT phase separation. Phase diagram of RPT as a function of varying temperature and salt concentration (Fig. 2E) revealed an interesting competition between electrostatic interactions along with intermolecular charge-transfer, which dominate at lower temperatures and the hydrophobic interactions, which dominate at higher temperatures. Thus, RPT phase separation displays a dual LCST (lower critical solution transition) and UCST (upper critical solution transition) phase behavior.

Considering the various pH conditions experienced by RPT within the cell, we next aimed to study the effect of varying pH on RPT phase separation. Droplet formation was inhibited at the melanosomal pH (4.5-5.5). Similarly, a switch from amyloid to droplet phase was enabled by a sudden increment in pH as indicated by the decrease in β-sheet content toward neutral pH (Fig. 2F). These observations corroborated our anisotropy measurements which showed a decrease in anisotropy with increasing pH, corresponding to the increased chain flexibility in droplets. We then directed our efforts toward investigating the liquid-to-solid phase transition of RPT. Here, we incubated droplets of RPT for prolonged time and monitored the formation of solid-like aggregates through increased blue fluorescence intensity and slow, incomplete FRAP recovery kinetics. The increasing FRET efficiency between tryptophan (donor) and charge-transfer species (acceptor) with increasing incubation time indicated the chain compaction upon liquid-to-solid phase transition. Thus, our studies reveal the role of RPT as a pH-sensitive modulator of the amyloid aggregation and phase separation pathway (Fig. 2G). Based on our observations, we hypothesized that phase separation of RPT can kepp the melanosomal fragment of Pmel17 in a concentrated but liquid form in the cytosol and facilitate melanogenesis by initiating amyloid formation at melanosomal pH.

Phase separation of an intrinsically disordered pathological variant of the human prion protein[12]

Misfolding and aggregation of the human prion protein are associated with a range of fatal neurodegenerative diseases termed transmissible spongiform encephalopathies. Prion protein (PrP) is a 253 residue GPI anchored membrane protein and consists of an intrinsically disordered N-terminal domain (23-120) and a structured C-terminal domain (121-230) flanked by a cleavable signal peptide (1-22) and a GPI-anchor signal (231-253). A naturally occurring, C-terminally truncated disease variant of the full-length PrP namely Y145Stop, is accumulated within the cell in stress conditions in the form of amyloid deposits (Fig. 3A,B). In accordance with the high phase separation propensity predicted by the bioinformatics tools (FuzDrop and catGRANULE), we were able to establish the formation of dynamic, liquid-like droplets of Y145Stop in the presence of salt at near-physiological conditions. In the cytoplasmic ribonucleoprotein granules, RNA is known to be associated with PrP, hence we wanted to probe effect of RNA on the phase behavior of Y145Stop. A significant reduction in the saturation concentration (Csat) and droplet formation was observed in the presence of RNA (Fig 3C); however, the interior for these RNA-induced droplets was less liquid-like as compared to the Y145Stop only droplets. These droplets exhibit a characteristic reentrant phase behavior in the presence of RNA. Our single-droplet Raman spectroscopy measurements suggested the presence of disorder and an enhanced conformational distribution within the condensed phase.

Based on a high net positive charge, we suspected a significant role of electrostatics screening in driving phase separation. As suspected, with an increasing salt concentration and pH, we observed phase separation at lower protein concentrations owing to the higher charge screening or a reduction in net charge on the polypeptide chain. The presence of droplets even at high salt concentrations indicated possible contribution of hydrophobic interactions in driving phase separation. Additionally, we created mutants either with reduced or increased hydrophobicity by replacing alanine residue with glycines or valines, respectively, and monitored their LLPS behavior. The mutant with reduced hydrophobicity showed phase behavior similar to the wild-type Y145Stop with a slightly increased fluidity. On the contrary, the alanine to valine mutant exhibited an enhanced phase separation propensity as observed by fluorescence imaging and

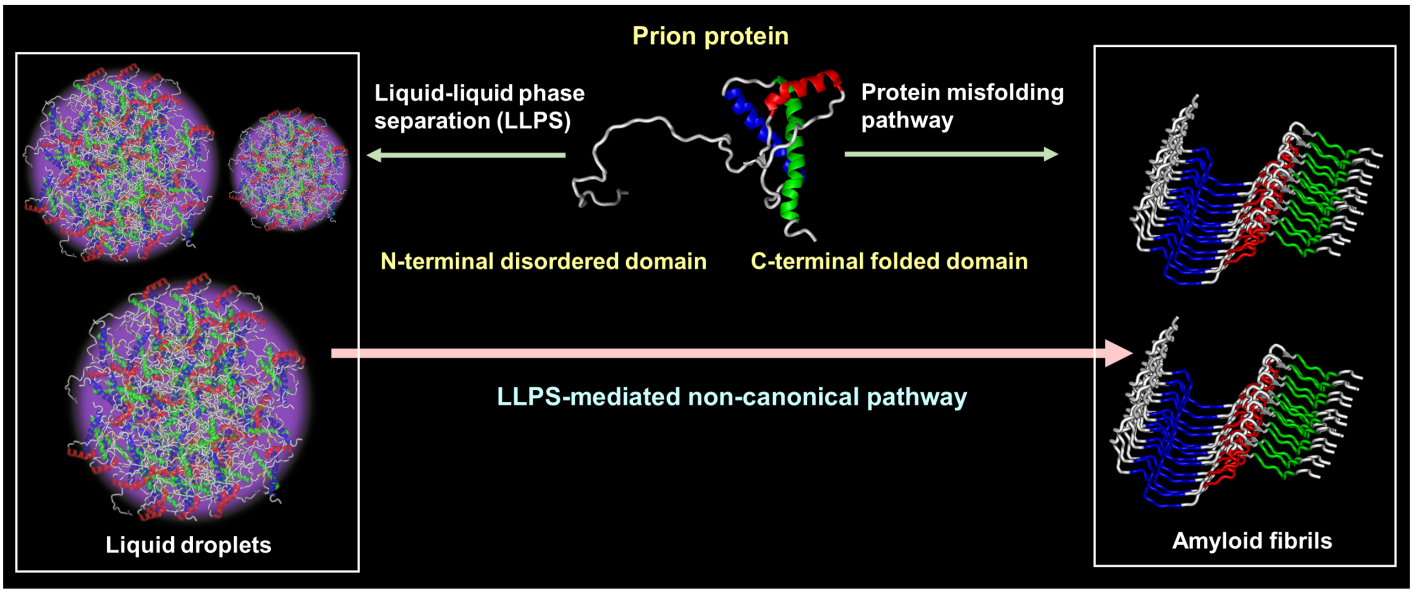


Figure 4. Two pathways of prion amyloid formation: Conventional protein misfolding pathway and non-canonical phase separation-mediated pathway (Reproduced with permission from Ref 13. Copyright Elsevier).

reduced FRAP recovery, establishing the role of hydrophobicity in phase transition. From our temperature dependent studies, Y145Stop demonstrated an upper critical solution transition (UCST) phase behavior arising from the possible involvement of aromatic residues in π-π and cation-π interactions. Lastly, we headed to study the maturation of these condensates into more gel-like or solid-like aggregates upon aging (Fig. 3D). The solid-like nature of these assemblies after maturation (~ 5 h) was confirmed through confocal imaging, where non-spherical, irregular assemblies with no significant FRAP recovery were observed (Fig. 3E). Raman spectroscopy measurements could capture formation of these solid-like amyloid aggregates as also indicated by the increase in the cross-β architecture (Fig. 3F), the evolution of intrinsic blue fluorescence (Fig. 3G), and the presence of fibrils as monitored by atomic force microscopy (AFM) (Fig. 3H). These aggregates were capable of seeding aggregation of droplets of Y145Stop in a dose-dependent manner and exhibited an autocatalytic, self-templating behavior recapitulating the prion-like behavior. Thus, our studies shed light on the phase separation and interplay of molecular determinants driving and modulating phase separation and liquid-to-solid phase transition of Y145Stop, a disease-associated truncation mutant of the human PrP. Our results also demonstrated that amyloid formation could be achieved by two alternate pathways – classical protein misfolding or non-canonical phase separation mediated pathways (Figure 4).[13]

Synergistic heterotypic interactions drive amyloid aggregation through complex coacervation of prion and α-synuclein[14]

Overlapping neuropathological features involving abnormal co-deposits of unrelated proteins involved in amyloid formation are emerging as pathological hallmarks of numerous neurodegenerative diseases including Creutzfeldt-Jakob disease (CJD), AD, PD, ALS and so on. Lewy bodies comprising abnormal aggregates of α-synuclein are detected in cases of CJD, a prion misfolding disease. Given the pathological co-localization of prion and α-synuclein within cells, we aimed to unravel the mechanism of synergistic interactions between PrP and α-synuclein through phase separation. We performed phase separation assays and observed co-phase separation of prion and α-synuclein into highly dynamic, heterotypic condensates upon incubation. α-synuclein (~ -8) and PrP (~ +10) being oppositely charged at our phase separation conditions, we suspected the possibility of charge neutralization as a criterion for co-phase separation. We observed a reentrant phase behavior where phase separation was exhibited only in a narrow concentration regime (Fig. 5A, B), the possible reason behind this being charge inversion in extreme stoichiometry regimes. Similarly, higher salt concentrations lead to droplet dissolution emphasizing the role of ionic strength in driving this complex coacervation. Temperature-dependent phase separation studies revealed a lower critical solution transition (LCST) behavior characteristic of LLPS driven by electrostatic interactions.

To determine the involvement of the disordered and oppositely charged domains, we created truncation mutants of PrP and α-synuclein and studied their phase behavior with respect to the full-length proteins. Our results revealed the crucial role of N-terminal prion and C-terminal α-synuclein domains in driving the electrostatically driven multicomponent condensation. The formation of electrostatic nanocomplexes was proposed to drive complex coacervation. To determine the presence of such complexes within our system, we performed steady-state anisotropy in conjunction with picosecond time-resolved anisotropy experiments. Steady-state anisotropy measurements indicated local ordering involving the N-terminal domain of PrP and C-terminal domain of α-synuclein (Fig 5C,D). To discern the dynamical events occurring at various timescales, we proceeded with picosecond time-resolved fluorescence anisotropy measurements which enabled us to capture distinct modes of rotational relaxation experienced by the polypeptide chain. Fluorescence anisotropy decay revealed local ordering due to the formation of nano-blobs arising from the electrostatic clustering of the C-terminal of α-synuclein and N-terminal of prion inside the droplets. These nanoscopic clusters might undergo making and breaking of interactions at a slower timescale providing these condensates with liquid-like nature on longer time and length scales.

Nucleic acids are the other major component in cellular biomolecular condensates and PrP is known to interact with RNA within the cell. In the presence of RNA, ternary condensates with uniform protein distribution were formed at lower concentrations which transformed into multiphasic, hollow, vesicle-like condensates at higher RNA concentrations and eventually dissolved with increasing RNA, exhibiting a typical reentrant phase behavior. In the presence of RNA, competing interactions between α-synuclein and RNA with prion lead to a weak partitioning of α-synuclein in these condensates, as observed from a decrease in the steady state anisotropy for α-synuclein. On the contrary, anisotropy for PrP increased within RNA–rich hollow condensates suggesting spatial ordering within these. Thus, our results indicated a significant role of RNA in modulating the compositional specificity of α-synuclein and PrP condensates in a context-dependent manner. To investigate the role of phase separation in mediating heterotypic aggregation of PrP and α-synuclein, we monitored these condensates as a function of time and observed the formation of aggregates with a much shorter lag phase in comparison to aggregation through non-phase separating conditions. Thus, complex coacervation of α-synuclein and PrP is capable of mediating rapid conversion and liquid-to-solid transition of these condensates into amyloid-like aggregates. This work reports on the influence of the synergistic interactions of PrP and α-synuclein in driving the formation of heterotypic condensates and their maturation into solid-like aggregates (Fig 5E).

Illuminating biomolecular condensates by single-droplet surface-enhanced Raman scattering[15]

The interplay of molecular driving forces of biomolecular condensation through phase separation remains elusive. Many existing spectroscopic and microscopic techniques are not capable of providing the wealth of molecular information within individual droplets. Vibrational Raman spectroscopy is one such technique that can provide us with molecular information within individual condensates. However, as a result of the low Raman scattering cross-section, aqueous biological samples need a high laser power which is often detrimental to the samples. To overcome this limitation, we utilized surface-enhanced Raman scattering (SERS) using metallic nanostructures capable of enhancing Raman signals of protein molecules present within the plasmonic enhancement field created around these nanostructures (Fig. 6A). In this work, we used a model phase-separating protein namely, Fused in Sarcoma (FUS) containing a prion-like, low complexity domain and a nucleic acid binding domain.

We performed single-droplet normal Raman spectroscopy as a prelude to SERS experiments and obtained typical Raman bands arising from the polypeptide backbone and side-chain vibrations. The amide bands (Amide I and III) suggested a high disorder and conformational heterogeneity within the condensed phase. Additional marker bands for aromatic side chains indicated the presence of π-π or cation-π interactions in the droplets. Following

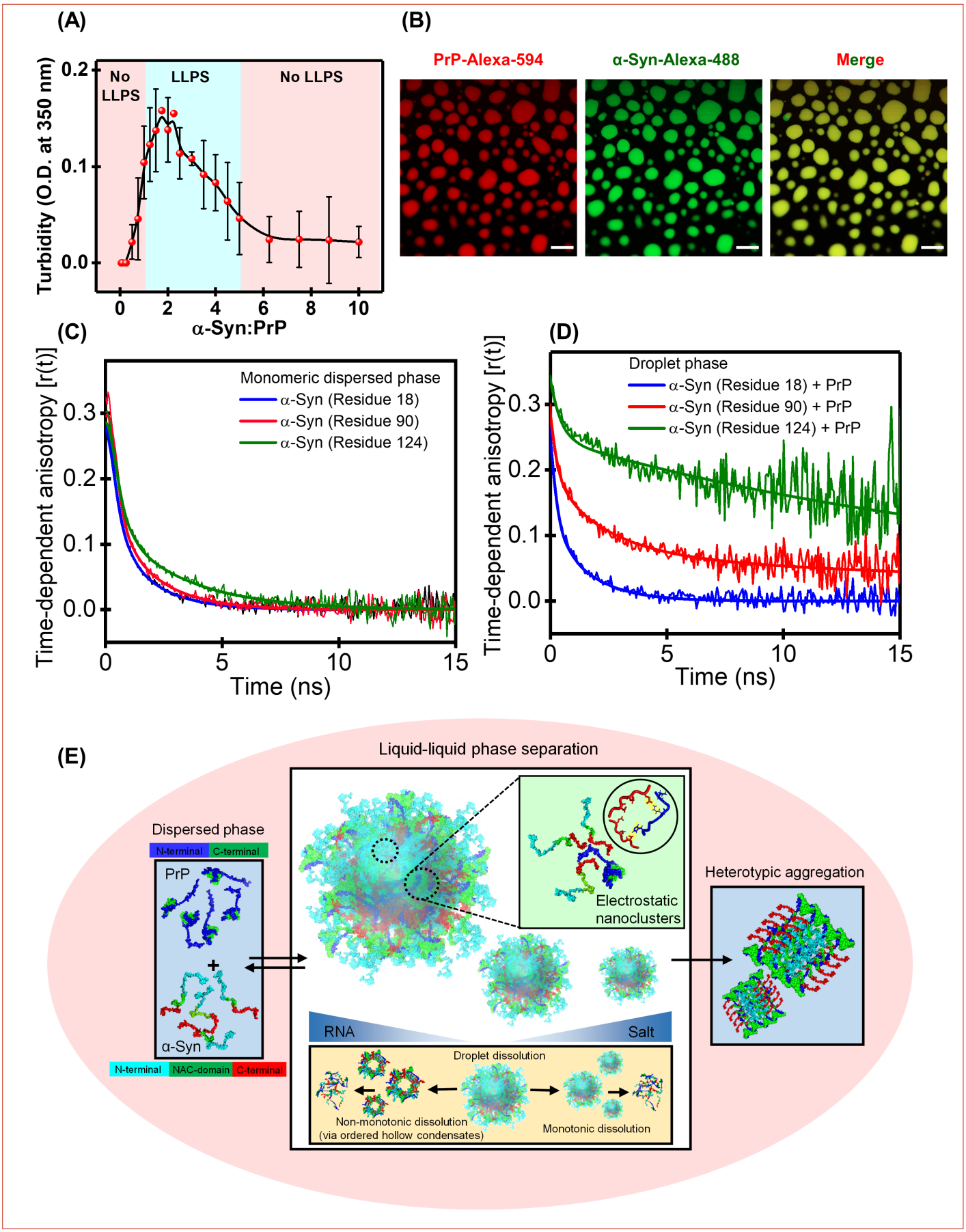


Figure 5. (A) Turbidity plot showing reentrant phase separation behavior of α-syn:PrP. **(B)** Confocal images of complex coacervates of PrP (Alexa-594) and α-synuclein (Alexa-488) showing complete colocalization. Fluorescence anisotropy decay of single-cysteine α-synuclein labeled using IAEDANS in monomeric dispersed **(C)** and in phase-separated states **(D)**. **(E)** Schematic illustration of complex phase transitions of α-syn and PrP (Adapted from Ref. 14).

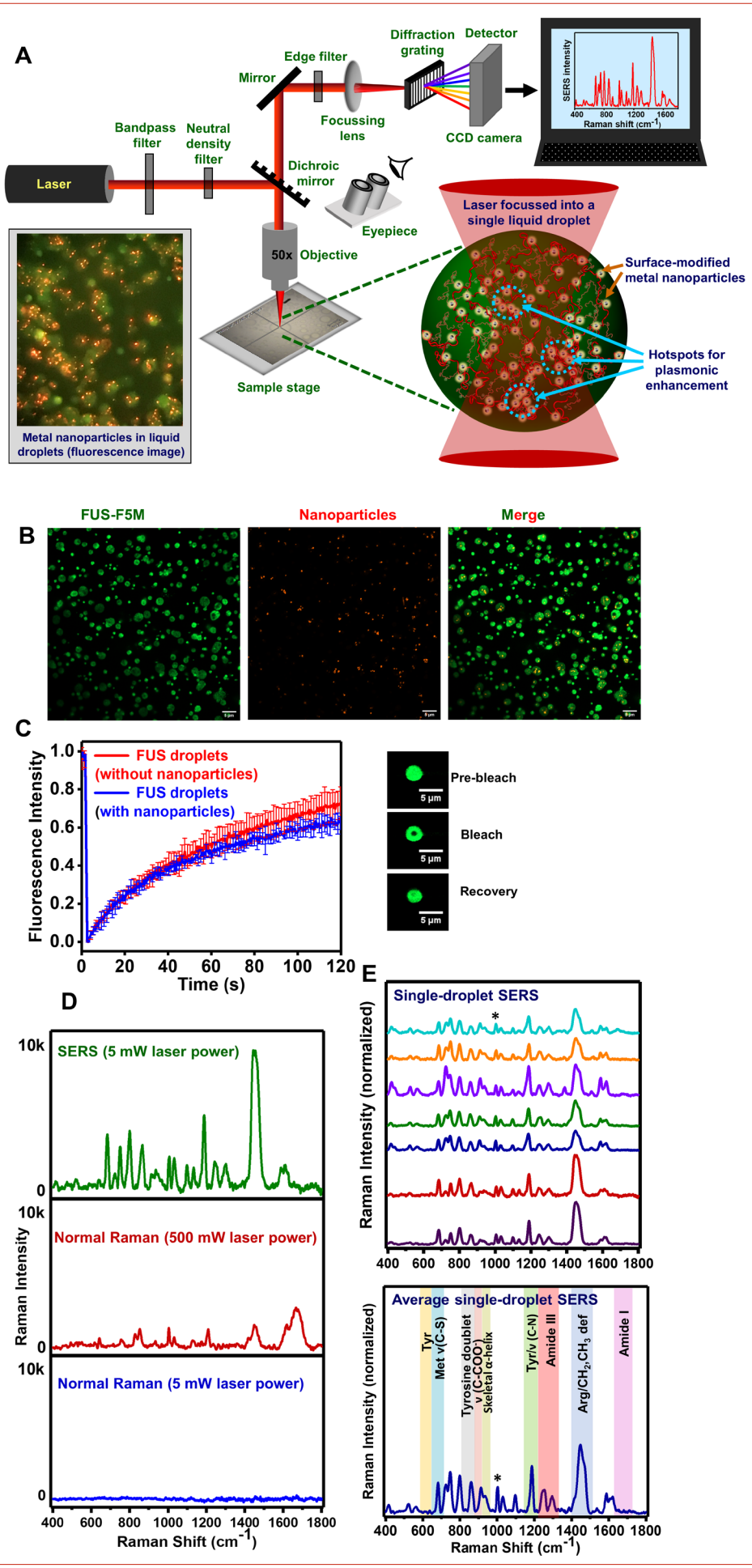
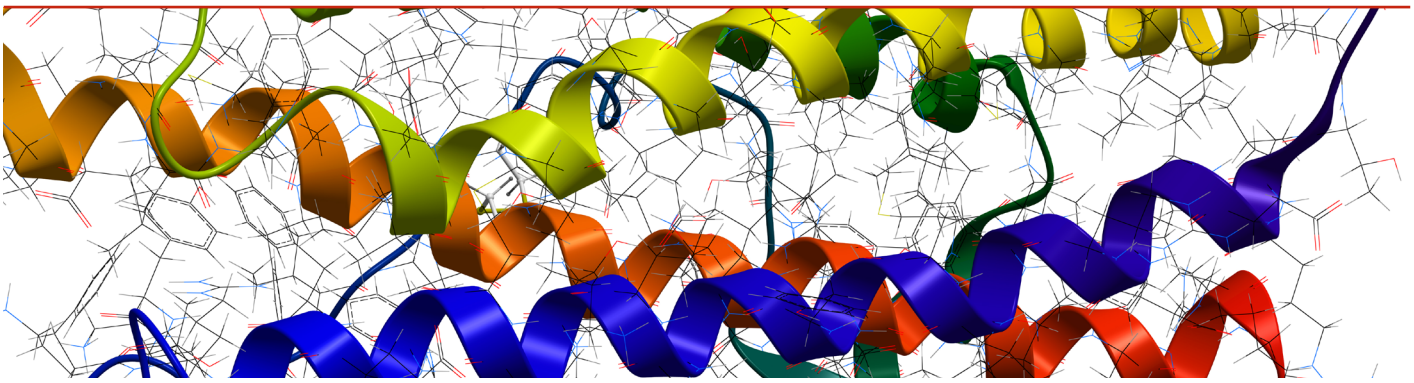


Figure 6. (A) A schematic representation of our single-droplet Raman spectroscopy setup with an eyepiece image capturing the encapsulation of surface-modified silver nanoparticles inside F5M-labeled FUS droplets. **(B)** Encapsulation of silver nanoparticles within FUS condensates. **(C)** FRAP kinetics showing no significant changes in the internal mobility in the presence of silver nanoparticles. **(D)** Normal Raman and SERS spectra showing the enhancement. **(E)** Single-droplet SERS spectra of FUS condensates (Adapted from Ref. 15).

single-droplet Raman spectroscopy, we next set out to perform SERS experiments. Based on the net positive charge on FUS, we prepared negatively charged silver nanoparticles, coated them with iodide and performed single-droplet SERS measurements in the presence of low concentration of iodide modified silver nanoparticles. Using confocal imaging, we established that the surface-modified silver nanoparticles got encapsulated into FUS liquid droplets (Fig. 6B). Our FRAP measurements indicated little or no changes in the internal material property of FUS condensates in the presence of nanoparticles (Fig. 6C). We performed SERS experiments with a much lower laser power (5 mW) and were able to obtain Raman signals with an enhancement in the order of ≥ 1 0^4 (Fig 5D). In these experiments, we captured Raman bands of a large number of side chain vibrations as well as an increase in the overall structural content in droplets (Fig. 6E). RNA being a well-studied modulator of phase behavior and condensate properties, we also performed single-droplet SERS experiments to unravel the sequence of events upon the addition of polyU RNA. Taken together, by employing ultrasensitive SERS methodology, we were able to access the wealth of molecular information in a droplet-by-droplet fashion for both FUS homotypic and FUS-RNA heterotypic condensates. This tool can be further extended to study other phase separating systems involved in physiology and disease.

Conclusion and Future Directions

Liquid-liquid phase separation has emerged as an important mechanism controlling spatiotemporal organization in biology. Whereas, liquid-to-solid phase transitions of IDPs/IDRs are thought to be associated with a range of debilitating human diseases. Therefore, delving deeper into the molecular determinants will help us understand the fundamental molecular mechanisms and will allow us to develop therapeutic strategies to combat aberrant phase transitions associated with disease. As conformational fluctuations and chain flexibility have emerged as crucial factors for phase separation and for maintaining the dynamic liquid-like nature, our recent findings on internal friction of IDPs[16,17] will be of great importance in understanding conformational characteristics of proteins within biomolecular condensates. Our future investigations will aim at unveiling the complex interplay of protein-protein and protein-nucleic acid interactions as well as the impact of pathological mutations and post-translational modifications in biological phase transitions involved in cell physiology and disease.



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