The (un)structural biology of biomolecular liquid-liquid phase separation using NMR spectroscopy

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ABSTRACT

Liquid-liquid phase separation (LLPS) of proteins and nucleic acids is a phenomenon that underlies membraneless compartmentalization of the cell. The underlying molecular interactions that underpin biomolecular LLPS have been of increased interest due to the importance of membraneless organelles in facilitating various biological processes and the disease-association of several of the proteins that mediate LLPS. Proteins that are able to undergo LLPS often contain intrinsically disordered regions and remain dynamic in solution. Solution-state nuclear magnetic resonance (NMR) spectroscopy has emerged as a leading structural technique to characterize protein LLPS due to the variety and specificity of information that can be obtained about intrinsically disordered sequences. This review discusses practical aspects of studying LLPS by NMR, summarizes recent work on the molecular aspects of LLPS of various protein systems and discusses future opportunities for characterizing the molecular details of LLPS in order to modulate phase separation.

Introduction

Over the past decade, membraneless organelles have been characterized in cells as liquids (1–3). These dynamic assemblies are formed by the phenomenon of liquid-liquid phase separation (4). The underlying constituents of these assemblies are specific proteins and nucleic acids that are responsible for phase transitions. Many of the proteins that undergo physiologically relevant LLPS contain intrinsically disordered domains with low sequence complexity (5). Because these sequences do not contain persistent secondary/tertiary structure, solution-state biomolecular nuclear magnetic resonance (NMR) spectroscopy has become the leading biophysical technique to study intrinsically disordered proteins (IDP) and regions (IDR) associated with LLPS. In this review, we introduce protein LLPS and IDP NMR, we then describe the various types of samples used to probe protein LLPS by NMR, and, finally we highlight the NMR approaches for probing structure and motions of proteins that undergo LLPS and the information that each technique provides.

Protein liquid-liquid phase separation

In protein LLPS, proteins demix from the surrounding solvent to form a distinct, high concentration phase in equilibrium with a dispersed phase, depleted in protein. Protein phase separation is common under the high concentration conditions used for protein crystallization; however, proteins have only recently been shown to undergo phase separation at physiological concentrations and conditions (6). The sequence and interaction requirements for LLPS in a physiological context are still not well understood, although several sequence motifs have been identified in structurally

disordered, LLPS-prone proteins (Table 1) (7). A subset of disordered protein domains that facilitate LLPS are classified as prion-like, meaning that they have a sequence composition resembling yeast prion domains enriched in polar amino acids such as glutamine and asparagine (5). The RNA-binding proteins FUS, TDP-43 and hnRNPA2 contain prion-like low complexity domains that mediate phase separation; however, even within this group, the amino acids and molecular interactions that contribute to phase separation are varied (8–10). Charge-patterned sequences are also able to facilitate phase separation via complex coacervation, the co-demixing of oppositely charged biopolymers. In particular, positively-charged arginines in RGG motifs are able to interact with polyanions such as RNA to promote LLPS (11). Elastin-like peptides are enriched in hydrophobic amino acids and are also able to undergo LLPS (12). In addition, LLPS can be modulated by post-translational modifications which can change electrostatic, hydrophobic and π-interactions (13).

NMR spectroscopy of IDPs

The phase separation of proteins into liquid and solid states is of interest because of the formation of functional liquid condensates and amyloid-like compartments in cells (13–15). Many of these proteins are involved in diseases characterized by the presence of protein aggregates. X-ray crystallography and cryo-EM have been utilized to study amyloid fibrils that are related to disease; however, these techniques generate static 'snapshots' and are not able to give atomic-level information about regions that remain dynamic. NMR spectroscopy has emerged as a leading technique to measure transient formation of secondary structure, molecular motions and tumbling, and interactions of intrinsically disordered proteins.

Solution-state NMR spectroscopy can be used to provide detailed information on the structure and motions of individual components inside liquid-like assemblies. In brief, NMR spectroscopy relies on the interaction of atomic nuclei with a magnetic field to provide local information on the chemical environment of each nucleus. In an external magnetic field, nuclear spin magnetization resonates at a characteristic frequency, proportional to the strength of the magnetic field and gyromagnetic ratio of the nucleus. This resonant frequency is further influenced by atoms in close proximity either through bonds or space; the variation in the resonant frequency is often very small and measured in units of parts per million (called the chemical shift). In this way, NMR spectroscopy allows elucidation of the relationships through bonds and space between atoms within a molecule. Chemical shift perturbations of nuclei that correspond to the protein backbone can be used to monitor changes in secondary structure as well as intermolecular interactions. Interactions can further be probed by various techniques that allow for detection of short-range (<6Å, NOE) and long-range (10-25Å, PRE) interactions. NMR spectroscopy is also suited to characterizing molecular motions across different timescales and may be able to provide information on the phase transitions between liquid and solid states. It is important to note that for IDPs, these NMR observables represent a weighted average over the conformational ensemble.

The insights garnered by NMR studies can inform on the single chain properties of proteins that undergo LLPS and the interactions that are important for assembly. A detailed molecular picture of how different protein sequences mediate LLPS affords an understanding of the function of membraneless organelles, engineering of LLPS systems for use as novel biomaterials, and development of small molecules that can modulate self-assembly. In this review, we delve into practical aspects of studying protein LLPS using NMR. We explain the opportunities and limitations presented by each NMR method, which is important for those familiar with LLPS, as NMR data become more common and applied in LLPS studies, and for NMR experts, because LLPS systems present unique constraints. Critically, we present background on the experimental observables to help both communities understand the capabilities of NMR and critically evaluate data presented in the literature. Overall, we hope that the information presented herein will make the application and interpretation of NMR spectroscopy to LLPS more accessible for a broader audience.

Types of samples to study protein phase separation

Structural biologists often take a reductionist approach to studying the characteristics of biomolecules. The formation of membraneless organelles by LLPS is a complex phenomenon involving the presence of many different types of proteins, both those directly involved in LLPS and those that are clients for these assemblies, as well as nucleic acids. To gain insight into these assembles, many *in vitro* studies have focused on the protein domains that directly mediate phase separation as minimal models of biomolecular condensates. In addition, some *in vitro* studies have included molecular crowding agents to mimic the intracellular environment and induce LLPS; however, most NMR studies have excluded these molecules as they may confound results due to protein-crowding agent interactions.

The phases present in NMR samples of LLPS proteins determine the type of information that can be observed. As for any biomolecular NMR experiment, high quality sample preparation is integral to obtaining useful data. Protein assembly or aggregation typically presents problems in obtaining and interpreting solution NMR data. Because intermolecular interaction is a fundamental feature of LLPS proteins, care must be taken to gain reliable molecular insight into systems in which LLPS occurs. In this section, we review the classes of NMR sample preparations of LLPS proteins used thus far.

Dispersed phase

Insights into LLPS can be glimpsed through using samples in which the protein is at a concentration below that required for phase separation. Without prior knowledge of the boundaries of the phase diagram, it can be difficult to determine a concentration range appropriate for this type of sample. In addition, the critical concentration for some proteins to undergo LLPS can be below 1 μM which can prevent acquisition of data within a reasonable experimental time due to low signal-to-noise. If the protein system is amenable to this method, the dispersed phase (dilute phase) sample is easy to prepare and can be used to obtain information on the secondary structure of the protein, molecular motions and interactions in the dispersed phase.

Chemical shift perturbations (CSPs) are sensitive probes of interactions with residue-by-residue resolution. CSPs as a function of increasing protein concentrations can therefore inform on which residues are important for self-association (Fig. 1A). One study on the phase-separation-prone protein TDP-43 mapped a helical subregion (321-343) important for LLPS by observing chemical shift perturbations, which was then confirmed by testing the effect of mutations in this region on phase separation (9, 16). In contrast, other LLPS systems display small chemical shifts with increasing protein concentrations across the entirety of the protein sequence, suggesting that the interactions that stabilize phase separation are not localized to a particular region (10). In addition to chemical shift perturbations, other studies have estimated the saturation concentration for phase separation by measuring the concentration remaining in the dispersed phase from the signal intensities in standard one- and two-dimensional spectra (17, 18). The fraction of protein in the condensed phase contributes very little to the total signal due to enhanced relaxation rates in the condensed phase, leading to extreme signal broadening and leaving only the signals from the dispersed phase (9, 17–22). While dispersed phase samples are useful in characterizing certain aspects of protein systems that undergo LLPS, they are limited as conclusions about the condensed phase are indirect. Nonetheless, structural insights on proteins within the dispersed phase can provide quantitative information on the processes leading up to phase separation.

Biphasic sample

Several studies have been able to obtain structural information on protein LLPS systems using samples in which both dispersed and condensed phases are present – i.e. a suspension of "droplets" (8, 23). Due to the presence of two different protein populations, two sets of resonances with distinct chemical shifts are observed, reflecting the distinct chemical environments of the dispersed and condensed phases (Fig. 1B). It is possible to isolate signals

from the different species because phase separation into micro-sized condensates influences both translational diffusion and rotational tumbling; therefore, diffusion or relaxation editing can be used to select for signals corresponding to either the dispersed or condensed phase. For diffusion editing, pulsed field gradients (PFG) are used to create linear magnetic field variation across the sample. This results in a phase shift dependent on the position of the molecule. To rephase, magnetization is inverted with a 180° pulse and another pulsed field gradient of the same duration and strength is applied. Efficient rephasing only works if no diffusion has occurred, otherwise the intensity of the peak is dependent upon molecular diffusion and the strength and duration of the PFG. In this way, fast diffusing species like those in the dispersed phase can be removed, leaving only the signals in the dispersed phase. Conversely relaxation editing relies on the principle that large biomolecules have long rotational correlation times, leading to enhanced R_2 relaxation rates. Using standard experiments for the measurement of R_2, if the relaxation delay is long (i.e. several hundreds of ms), then fast decaying signals from large molecular weight species like the condensed phase can be removed. In the study of an elastin-like peptide, the spectra of the dispersed and condensed phases were separated by using either a relaxation filter to select for fast tumbling (to remove signals with $R_2 > 5$ s^{-1}) molecules such as those in the dispersed phase or a diffusion filter to select for slow diffusing (to remove signals with diffusion rates > 10^{-7} $cm^2 \cdot s^{-1}$) molecules such as those in the condensed phase (23).

There are several challenges associated with the use of a biphasic sample, namely that the sample is not stable over long periods of time and the signal intensity of the condensed phase is low. A biphasic sample type can be difficult to use for experiments that require long experiment times because the sample will change over time as the condensed phase settles due to gravity. In addition, broad line-widths due to the increased viscosity and slowed motions within the condensed phase make resonances associated with the condensed phase difficult to detect above the noise, requiring high protein concentrations (>1 mM) and conditions that maximize the protein concentration in the condensed phase, i.e. recording experiments far above the saturation concentration for LLPS. Thus far, molecular crowding agents (high molecular weight polyethylene glycol and dextran) have not been used in NMR studies of LLPS systems; however, experiment conducted in the presence of these compounds may with aid in driving more protein into the condensed phase and increasing signal intensity with appropriate controls.

Macroscopic condensed phase sample

To directly observe the condensed phase, several studies have taken advantage of sedimentation to fuse the dense droplets of the protein-rich condensed phase into macroscopic samples that fill the NMR coil observation volume (8, 10, 23–27) (Fig. 1C). To create macroscopic condensed phase samples, some approaches involve preparing high concentration samples that demix followed by allowing the condensed phase to settle due to gravity at the bottom of the NMR tube (25, 26), while others have expedited the process by centrifuging (<5000 g) the sample and transferring it into an NMR tube or directly centrifuging into an NMR tube (8, 10, 24). A macroscopic condensed phase can be challenging to make as it requires large amounts of purified protein (>150 mg for a 5 mm diameter NMR tube with a sample volume of ~400 µL). Using specialized NMR hardware, Sharpe and colleagues were able to decrease the sample volume requirements by using a 1 mm MicroProbe that enables use of sample volumes as low as 20 µL (23).

Despite the challenges of making this type of sample, it is currently the best way to achieve direct information on the structure, molecular diffusion, and interactions within a stable condensed phase. To demonstrate the relevance of this sample to studies of *in vitro* droplets typically visualized by microscopy, chemical shifts have been used to compare the environments of the different types of samples for the low complexity domain of RNA-binding protein FUS (FUS LC) (8). The chemical shifts of the macroscopic condensed phase overlaid well with the set of peaks in the biphasic sample arising from suspended droplets. Furthermore, the diffusion rate for FUS LC in the condensed phase by NMR (see below) matched that

estimated from fluorescence recovery after photobleaching kinetic microscopy experiments on spontaneously formed droplets at the same conditions. Together these observations suggest that the macroscopic condensed phase of FUS LC created for NMR retains all the biophysical properties of microscopic droplets. Interestingly, another study on the low complexity domain of the germline granule protein Ddx4, which is enriched in FG/RG sequence motifs, compared the macroscopic condensed phase of Ddx4 to a high concentration dispersed phase (~400 mg/mL) made from a Ddx4 variant unable to phase separate at tested conditions(25). This high concentration dispersed phase mimicked the enhanced viscosity effects of a macroscopic condensed phase but failed to recapitulate the extensive intermolecular interactions that are present within the condensed phase.

The macroscopic condensed phase may also be a good candidate for solid-state NMR (ssNMR) studies as not all systems remain liquid and stable for long periods of time at such high protein concentration. For example, the macroscopic condensed phase of the low complexity domain of FUS remains liquid for months (8); however, the macroscopic condensed phase of hnRNPA2 low complexity domain is solid at room temperature and requires heating to 65°C to liquify (10). ssNMR has proven useful to study the interplay between folded and disordered regions of LLPS systems (28, 29). A ssNMR study has been conducted on the two-component condensed phase of the nucleolar protein nucleophosmin (NPM1) and p14ARF tumor suppressor (28). The folded domain of NPM1 that mediates oligomerization into pentamers was detected within the condensed phase by cross polarization techniques (sensitive to solid regions) and found to form an immobilized scaffold while the disordered regions remained mobile. In the future, combining both solution-state and ssNMR may provide further insights into the interactions that mediate phase separation of full-length proteins. Solid-state NMR has also been used to study the amyloid fibrillar states of proteins that undergo LLPS as well as the transition between the liquid and hydrogel phases (29–31).

These three categories of NMR samples provide distinct information about characteristics of LLPS systems. In the future, it will be important to begin to recapitulate multi-component systems to understand the structural characteristics of biomolecular condensates. This may involve all of the different types of samples presented above and also the use of isotopic labeling techniques to differentiate multiple components.

Structure and contacts mediating LLPS

We now examine the NMR techniques appropriate for determining structure and contacts of phase separated proteins using solution NMR spectroscopy.

Chemical shifts

One of the requisite approaches in biomolecular NMR is to obtain chemical shift information for the protein backbone for each amino acid in a protein sequence. The 2D ^{1}H,^{15}N-heteronuclear single quantum coherence (HSQC) experiment provides information for each covalently bonded ^{1}H-^{15}N pair corresponding to the peptide backbone and amide containing side chains. Similarly, the ^{1}H,^{13}C-HSQC provides information for each ^{1}H-^{13}C pair corresponding to the Cα, Cβ, and side-chains of each amino acid. Because these experiments probe the peptide backbone and sidechains, they are sensitive to secondary structure and perturbations that can result from ligand binding or conformational change. The ^{1}H,^{15}N -HSQC of a disordered protein typically contains little $^{1}H_N$ signal dispersion, indicating that each residue is exposed to the solvent and experiences a similar chemical environment (32). Importantly, all of the ^{1}H,^{15}N-HSQC spectra of the low complexity domains of phase separation prone proteins have characteristics of IDPs in both the dispersed and condensed phases, as seen by the narrow signal dispersion in the ^{1}H dimension (9, 10, 23–25) (Figure 1B,C). The observed chemical shift represents the population-weighted average shift across the conformational ensemble (33) (assuming similar relaxation properties of the ensemble members) (34, 35). The repetitive sequence motifs found in many proteins that undergo LLPS also introduces spectral crowding

and overlap of resonances, making spectral interpretation difficult. However, advances in isotopic labeling and resonance assignment have made studying intrinsically disordered proteins (IDPs) easier (reviewed in (36)).

Changes in the position or intensity of the resonances corresponding to each residue have been used to map self-interactions and interactions with protein-binding partners and ligands of LLPS systems (9, 17, 18, 20, 24, 27, 37, 38). The proteasomal shuttle protein, ubiquilin (UBQLN), phase separates and localizes to stress granules (37). By observing chemical shift perturbations in the dispersed phase, Castañeda and coworkers mapped the domains responsible for phase separation of UBQLN and found that oligomerization of the folded domains coupled with interactions of intrinsically disordered regions mediate UBQLN LLPS. Alternatively, titrations can also be performed with protein binding partners to determine binding sites. For example, Burke et al. mapped the non-specific interactions between the low complexity domain of FUS and RNA polymerase II C-terminal heptad tail in the dispersed phase (24). Finally, interactions between LLPS-prone proteins and small molecules can be monitored using chemical shifts (20, 39). For example, the interaction of the low complexity domain of FUS and potential therapeutic small molecules was quantified—the chemotherapeutic mitoxantrone was found to interact with tyrosine residues present in the low complexity domain of FUS in the dispersed phase (39).

In addition to the information on intrinsic disorder and binding sites derived from amide chemical shifts, quantification of stable and transient protein secondary structure can be obtained by comparing the chemical shifts of each alpha and beta carbon to a true "random coil" reference. There are several libraries and methods available to aid in this analysis and take into account neighboring residue effects (32, 40, 41). Many of these methods have been used to evaluate the dispersed and condensed phases of LLPS-prone domains (9, 10, 24). We used secondary shift analysis to compare the different material states of the low complexity domain of FUS (8). The dispersed and condensed phases of FUS LC show secondary shifts consistent with disorder, while the fibrillar state has values with large deviations from random coil, consistent with β-sheet structure (30).

Because the condensed phase of many protein systems is highly viscous, the intrinsic line-widths that result are broadened due to the decreased molecular motions in the condensed phase (10, 20, 24, 25). To retain residue-by-residue resolution obtained in the dispersed state, one may change the apodization function used to process the data to increase resolution. In essentially all biomolecular NMR experiments, the raw time domain data are multiplied by a decreasing exponential or cosine-bell function to improve signal-to-noise and remove artifactual peak shapes that arise from Fourier transformation. In addition to typical line-broadening, line-sharpening was also used to process $^{1}H,^{15}N$-HSQC spectra of the macroscopic condensed phase of FUS LC; this choice of apodization function improved resolution but at the cost of decreased signal-to-noise, which is tolerated in very high concentration condensed samples (Figure 1C) (24). Another source of line-broadening for amide ^{1}H positions in samples of intrinsically disordered proteins (in dispersed and condensed phases) is hydrogen exchange with water, especially at physiological temperatures and pH ($T > 25°C$ and $pH > 7.0$). To circumvent the need to lower temperature or pH for optimal $^{1}H,^{15}N$-HSQC spectra, ^{13}C-direct detected experiments produce narrow, sharp peaks as there is no contribution to line broadening from water exchange while retaining a similar resolution to the $^{1}H,^{15}N$-HSQC. ^{13}C-direct detected experiments have been used to study LLPS-prone proteins in the dispersed and condensed phases (20, 27, 37). For example, Zweckstetter and colleagues used $^{13}CO/^{15}N$ correlation spectra (CON) in the dispersed phase to improve spectral resolution to determine the regions of microtubule-binding protein tau K18 that are responsible for phase separation (20). In addition, Forman-Kay and coworkers used CON spectra to observe stress/transport granule-associated FMRP/CAPRIN1 macroscopic condensed phases at physiological pH (7.4), which was essential for appropriate protonation of phosphorylated residues.

Nuclear Overhauser Spectroscopy (NOESY)

While chemical shift perturbations can detect transient population of secondary structural elements and intermolecular interaction sites, these observables do not provide direct information on intermolecular contacts. The NOE (nuclear Overhauser effect) is commonly used in solution NMR to measure internuclear distances (<6Å) via through-space dipolar-coupling of ^{1}H positions for structure determination. While NOEs cannot be used to directly quantitate distances in conformationally heterogenous IDPs due to the lack of inherent structure, they have been useful in LLPS systems to identify protein-protein interactions between residues that come in contact and hence are important to phase separation (8, 23, 25). In a basic NOE experiment, magnetization is transferred during the NOE mixing time via cross-relaxation between ^{1}H positions that are within close proximity, identifying molecular contacts (Fig. 2A). This experiment reports on all of the ^{1}H positions within close proximity to one another, meaning that it provides information on both the intramolecular and intermolecular interactions within a protein system.

To differentiate between intra- and intermolecular contributions, differential isotopic labeling schemes are often combined with heteronuclear filtering/editing ('selecting'). This method allows for differentiation between signals arising from isotopically labeled nuclei and natural abundance nuclei. Studies on an elastin-like peptide and on nuage granule protein Ddx4 implemented ^{13}C-filtered/edited experiments to isolate intermolecular interactions within the condensed phase (23, 25). In these experiments, magnetization of ^{1}H positions attached to ^{13}C and/or ^{15}N heteronuclei was removed in the filtering step, leaving only signals from ^{1}H positions attached to ^{12}C and ^{14}N heteronuclei (Fig. 2B). After the NOE mixing time, in the editing step magnetization of ^{1}H positions attached to ^{13}C and/or ^{15}N heteronuclei is selected and read out in an INEPT transfer (HSQC). If this experiment is conducted on a sample that contains 50% ^{15}N,^{13}C-labeled protein and 50% (^{14}N,^{12}C) unlabeled protein (i.e. a 1:1 mixture), then only the intermolecular contacts (i.e. between unlabeled residues and ^{15}N,^{13}C-labeled residues) can be detected. Because of natural abundance of ^{13}C present in the unlabeled sample and incomplete heavy isotope incorporation in the labeled sample (~1% and ~99%, respectively), artifacts due to incomplete isotopic labeling may arise. One strategy to avoid incomplete filtering artifacts is to perform doubly-edited HSQC-NOESY-HSQC experiments on samples containing 50% ^{13}C-labeled protein and 50% ^{15}N-labeled protein within the condensed phase (8). In this experiment, two INEPT transfers between ^{1}H positions and attached heteronuclei select for signals without relying on filtering. For example, in one version of the experiment, there are three steps: a heteronuclear editing step to select for magnetization starting on ^{15}N-attached ^{1}H nuclei, a conventional NOE step, and a second heteronuclear editing step to select for magnetization ending on ^{13}C-attached ^{1}H nuclei (Fig. 2C). While this experiment does decrease filtering artifacts, it can be challenging due to the loss of signal because of the multiple INEPT transfers. To combat the decreased signal, one may extend the NOE mixing time at the risk of increasing artifacts from spin diffusion (i.e. observation of NOEs between positions that do not directly interact but rather are mutually close to another ^{1}H position). However, the significant motions present in the condensed phases suggest that spin diffusion artifacts will not make major contributions to NOEs. Finally, it is important to note that because of the highly repetitive disordered sequence, the side chain chemical shifts are overlapped and hence these NOEs provide primarily residue-type information (e.g. tyrosine side chain positions interact with glutamine side chain amide positions).

Paramagnetic relaxation enhancement (PRE)

Because of the transient, weak nature of many of the contacts that are involved in LLPS, it may be difficult to detect self-interactions or interactions between binding partners using NMR chemical shifts or NOEs. Furthermore, because NOE-based experiments do not provide much sequence-position information (see above), additional techniques are needed. Alternatively, transient short-range interactions or persistent long-range interactions up to 25 Å can be probed using paramagnetic relaxation enhancement (PRE) NMR. PRE experiments require

conjugation of a paramagnetic probe which contains unpaired electrons (e.g. functionalized stabilized nitroxide radical or EDTA-Mn^{2+}) site-specifically, often to one endogenous or engineered cysteine residue. A reduced form (diamagnetic) of the spin label is used as a control. The PREs arise from dipolar interactions between a nucleus (often ^{1}H) and the unpaired electron(s) in the paramagnetic probe and are measured as the difference in the transverse relaxation rates between otherwise identical samples made with the paramagnetic or diamagnetic PRE probes (Fig. 2D). Because addition of the spin label requires protein engineering, it is important to ensure that addition of a cysteine site and conjugation of a label does not dramatically alter LLPS behavior and structure of the protein. In addition, non-specific interactions between the protein and spin label can occur. Hence, an experiment where free spin label is added to the protein serves as a useful control.

Several studies have used PREs to investigate transient intermolecular contacts and intramolecular collapse in the dispersed and condensed phases (8–10, 42). The intra- and intermolecular contacts disrupted by phosphorylation and phosphomimetic substitution in FUS LC were monitored by intra- and intermolecular PRE experiments, respectively (42). The interaction between the low complexity domains of hnRNPA2 and TDP-43 in the dispersed phase was characterized using intermolecular PREs (10). TDP-43 formed dynamic interactions across the entire LC domain of hnRNPA2, while the helical segment in TDP-43 seemed to participate more in contact formation. Importantly, studying the transient interactions of proteins within the dispersed phase may inform on which contacts exist within condensed phases. For example, we measured PREs within the condensed phase of FUS LC and found that the contacts formed within the phase did not favor a particular subregion of the sequence and are distributed throughout the domain (8). Interestingly, the PREs in the condensed phase followed a similar trend as PREs measured in the dispersed phase, with the N-terminal region of FUS LC exhibiting higher PREs than other regions (42). It is important to note that, in our experience, PREs within a condensed phase can be challenging to interpret due to the variability in partitioning of the PRE labeled protein and the extent of labeling, and hence the data is best interpreted qualitatively.

Protein motions and conformational changes in the dispersed and condensed phase

Solution state NMR spectroscopy has the advantage of being able to characterize motions of protein systems with atomistic resolution. A variety of techniques discussed below have been used to probe the timescale of molecular motions within the dispersed and condensed phases (Figure 3). These NMR methods also provide information on transiently populated structure (as seen by experiments probing ps-ns timescale motions) and exchange between conformational states (μs-s timescale motions).

Picosecond-to-nanosecond motions

NMR relaxation of the protein backbone is most easily probed using ^{15}N relaxation experiments that probe the reorientational motion of the amide bond vector. Together, R_1, R_2 and heteronuclear {^{1}H}-^{15}N NOE experiments probe the flexibility of each non-proline amino acid position on the ps-to-ns timescale to gain information about structure and motions. These observables can also be combined to examine the conformational exchange contributions to R_2 (see below). The spin relaxation parameters for proteins in the dilute and condensed phase remain predominantly uniform across the sequence, consistent with predominant, uniform disorder for these protein systems. In contrast to protein systems that remain intrinsically disordered across the entire domain, the low complexity domain of TDP-43 contains a short helical segment (~20 residues) that displays higher R_2 and heteronuclear NOE values in the dispersed phase (9). The transient formation of structure within the helical region rigidifies the amide bond vector and induces slower motions. In general, slowed reorientational motions are a feature of condensed phases due to increased viscosity and high protein concentrations, with an increase in R_2 and heteronuclear NOE values and alterations in R_1 (8, 10, 23–25). In summary, fast motions in the dispersed and condensed phases can be

probed and give insights into transiently populated structural features.

Microsecond-to-millisecond motions

To probe transient formation of structured conformations, the contribution of conformational exchange (R_{ex}) to R_2 can be determined by reduced spectral density mapping (RSDM) where R_1, R_2 and heteronuclear {^{1}H}-^{15}N NOE observables are analyzed together (43). For example, the contribution of conformational exchange within the condensed phase of FUS LC was evaluated using RSDM, and the R_{ex} term was determined to be effectively zero, suggesting that significant minor populations of structured states were not populated with μs to ms exchange rates (8). This is critical information as the presence of transient beta-sheet structure has been hypothesized to underlie phase separation, though these direct experiments failed to provide support for this hypothesis.

Probing motions on an intermediate timescale (τ ~100 μs-10 ms) including exchange between two conformational states, relaxation dispersion experiments provide information on the kinetics of assembly, chemical shift information of the minor state, and the relative distribution of the two populations. This family of techniques is particularly applicable to systems where the minor state is transiently populated and invisible to other NMR techniques. This is due to the small population of the minor state and lifetime line broadening which further decreases the resonance intensity due to the large k_{off} rate from the minor to the major state (44, 45). CPMG and $R_{1\rho}$ experiments enable quantification of the effect of conformational exchange on R_2, R_{ex}, by varying the repetition rate of 180° (π) refocusing pulses or the strength of a spin-lock radio frequency field, respectively (46). These techniques have been used to probe assembly and interactions in the dispersed and condensed states of TDP-43 and Ddx4 (9, 47). For the low complexity domain of TDP-43, the exchange between the monomeric state and the helix-mediated oligomer important for LLPS was quantified in dilute solution using CPMG relaxation dispersion. In this system, large differences in chemical shifts (up to 1 ppm) between the monomer and assembled state and the transverse relaxation rates of the assembled state are extracted from that relaxation dispersion analysis, consistent with enhancement and extension of helical structure (9). Kay and colleagues probed conformational exchange within a condensed phase of Ddx4 (47). They found that the R_2 rates are increased in a high concentration control as well as in the condensed phase, reflecting the increased concentration and viscosity. Interestingly, relaxation dispersion (<1 s^{-1}) was found and off-resonance $R_{1\rho}$ experiments were used to elucidate the exchange within the condensed phase. This technique is useful for elucidating exchange with larger rates between the two states than CPMG experiments but where ΔR_2 is much smaller than required for DEST between the two states (see below). In the condensed phase, the $R_{1\rho}$ data are consistent with a model where Ddx4 residues populate a minor state with higher transverse relaxation rates but small chemical shifts differences with the major state with an exchange rate of ~18 s^{-1}, suggesting that these interactions are weak and yet relatively long-lived. Crucially, the structural nature and significance of this minor state remain unknown, but perhaps represents probing of formation and breaking of the weak interactions that lead to LLPS. It is important to note that extraction of chemical shifts and exchange rate constants requires selection and application of an equilibrium chemical kinetic model for the exchange process. The simplest, two-state models (i.e. bound and unbound) have been able to describe the observables thus far, but the true nature of the exchange processes present in condensed phases remains poorly understood (47).

Slow motions (ms-to-s)

Observing and quantify slow conformational or phase exchange process that may occur in LLPS necessitates a different set of solution NMR approaches. Among possible slow exchange process may include the formation and breaking of contacts in the phase or formation of, or interaction with, hypothesized large, structured conformations including amyloid fibrils. Dark-state exchange saturation transfer (DEST) probes the slow exchange between large assemblies and monomeric species which may have utility in probing interaction or exchange between

different conformations within or between phases (48). High molecular weight assemblies have slow molecular tumbling which results in large increases in the transverse relaxation rate R_2 and therefore extreme line-broadening, making these species invisible in most NMR experiments. Thus, DEST takes advantage of the chemical exchange between an NMR-visible (i.e low molecular weight or monomeric) population and a high molecular weight species (e.g. a peptide bound to a large aggregate) to obtain dynamic information about the assembly (44). In this experiment, a weak saturating B_1 field is applied off-resonance from the monomeric signal, resulting in selective attenuation of only the invisible high molecular weight species. Importantly, the attenuation is proportional to the site-specific R_2 in the invisible species, providing residue-by-residue information on the invisible state. This signal attenuation is then transferred to the monomeric state by chemical exchange (i.e. unbinding) and read out as intensity changes in two-dimensional spectra. DEST can be useful in characterizing the regions mediating, and kinetics of, binding/unbinding of disordered domains in the dispersed phase to a solid and/or hydrogel phase also present in equilibrium/pseudo-equilibrium (48). DEST has been used to probe the interaction of C-terminal heptad-repeat tail of RNA polymerase II with TAF15 hydrogels (49), a simple model of transcriptional activation assemblies (50). Due to the aggregation-prone nature of many of the protein systems that undergo LLPS, DEST may be useful in characterizing the interactions between the dispersed phase and aggregate/fibrillar phase. However, no transient interaction between the disordered major species and potential oligomeric or fibrillar species in the liquid condensed phase of FUS LC was observed using DEST, providing no evidence for the population of large stable structures (e.g. amyloids or gels) within these LLPS samples (8). This observation is important because these experiments directly tested a previous hypothesis that amyloid fibril conformations are important for mediating LLPS.

The presence of two sets of chemical shifts, corresponding the dispersed and condensed phases, in biphasic samples indicate that these states are in slow exchange. In other words, the time for a given protein to transit from one phase to another is large on the chemical shift timescale, (i.e. approaching 1 s). ZZ-exchange NMR which can directly measure exchange rates between species in slow exchange may be appropriate to characterize the kinetics of exchange between the two phases. In addition, the timescale of proline isomerization in condensed phases, which may be significantly slowed compared to the dispersed phase, may be interesting future targets for measurement by ZZ-exchange (51).

Future directions

Solution-state NMR spectroscopy has emerged as a leading technique for characterizing intrinsically disordered systems, transient interactions, and conformational exchange. Probing all of these structural and motional features is essential for understanding the molecular details of systems that undergo liquid-liquid phase separation. Through various NMR experiments, common features of condensed phases are beginning to emerge: the maintenance of protein disorder, restricted motions due to high viscosities and protein concentrations, and transient, “fuzzy” interactions (52). In the future, it will be interesting to see how various mechanisms of phase separation between different systems can be characterized.

Currently, there remains a disconnect in the field between *in vitro* and *in vivo* studies of LLPS. Experimental techniques in cells typically measure bulk properties of membraneless organelles and often do not provide quantitative information (53). As a result, it has been difficult to connect the atomic level observations of NMR spectroscopy to biomolecular condensates present in cells; however, extending current NMR techniques and taking a multidisciplinary approach can allow the field to reconcile structural information about biomolecular condensates. Many questions remain open: What are the driving forces for LLPS? How do complex mixtures of biomolecules consisting of nucleic acids and proteins undergo LLPS? What confers specificity for components within distinct types of biomolecular condensates? How does biochemistry occur within biomolecular condensates? How are biomolecular condensates assembled and disassembled?

While most of these studies have been isolated to minimal models of protein LLPS (i.e. using disordered domains of larger proteins), it may be possible to look at full-length proteins with specific and segmental isotopic labeling, as well as with ssNMR, to begin to understand the interplay between oligomerization, association of IDRs, and LLPS (54, 55). In addition, to understand the behavior and interactions in multi-component proteinaceous phases, individual components can also be studied via NMR spectroscopy using differential labeling schemes. It is also possible to study LLPS systems in their endogenous environment using in-cell NMR techniques.

Finally, many of the methods discussed above can be used to characterize how small molecules interact with LLPS-prone proteins as well as identify how they disrupt the weak, multivalent interactions that are important for phase separation in order to develop treatments for neurodegenerative disease where phase separation may play a role.

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Protein	**MLO**	**Biological function**	**LLPS protein-protein interaction domain**	**Sequence motifs/structural features**
TDP-43	Stress granules, mRNA transport granules	RNA metabolism	276-414	Gly-rich polar transient α-helix (320-343)
FUS	Stress granules, paraspeckles, DNA-damage foci, transcriptional granules	RNA metabolism	1-163 164-267 372-422 453-507	[S/G/X]Y[S/G/X] repeats RG/RGG motifs
hnRNPA2	Stress granules, mRNA transport granules	RNA metabolism	190-341	Glycine-rich RG/RGG motifs [G/X][N/X]FG repeats
Elastin-like peptides	Extracellular matrix	Elastic matrix in vertebrate tissues	Hydrophobic domain	$(GVPGV)_7$
Ddx4	Developmental granules	DEAD-box RNA helicase	1-236	Charge-patterning [F/R]G repeats
UBQLN2	Stress granules	Proteasomal shuttle protein	379-486	Hydrophobic amino acids
Tau	Stress granules	Microtubule binding protein	1-441	Charge-patterning KXGS repeats
FMRP	Stress granules, mRNA transport granules	RNA metabolism	445-632	Charge-patterning RG/RGG motifs

Table 1. Subset of proteins able to undergo LLPS.

Figure 1

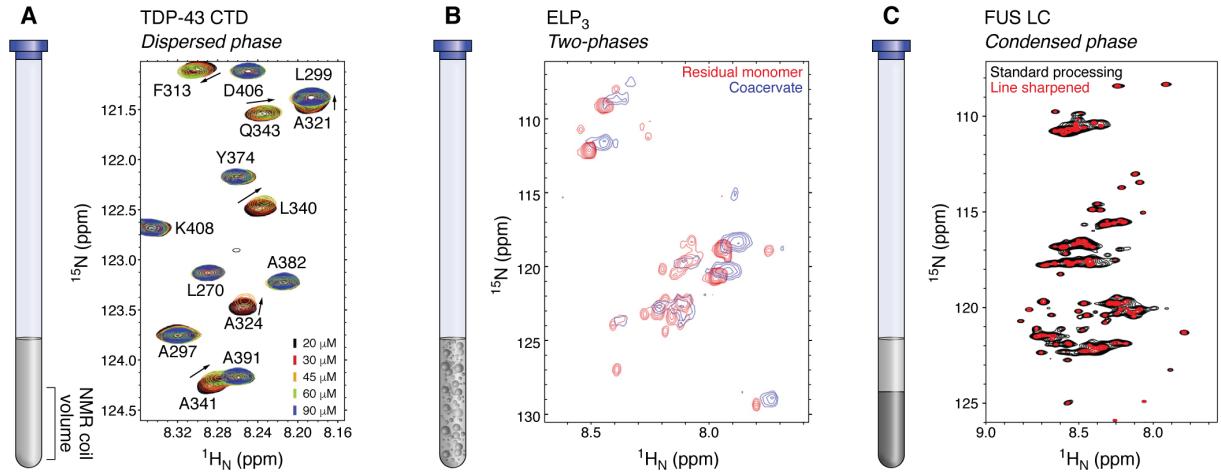

Figure 1. Methods to study the condensed phase by NMR spectroscopy. A) The dispersed phase can be used to garner information about the condensed phase indirectly. A titration of the phase separation prone C-terminal region of TDP-43 shows chemical shift perturbations of certain residues that are involved in LLPS (adapted from (9)). B) A biphasic sample containing the dispersed and condensed phases can be used to study properties of both. Spectra of an elastin-like peptide recorded with an R_2 relaxation rate filter or a pulsed field gradient diffusion rate filter select for signals arising from either the dispersed or condensed phases, respectively (adapted from (23)). C) The condensed phase can be studied directly by creating a macroscopic phase that fills the coil volume of the NMR spectrometer. Spectra of the condensed phase of the low complexity domain of FUS produce one set of broad resonances (adapted from (24)).

Figure 2

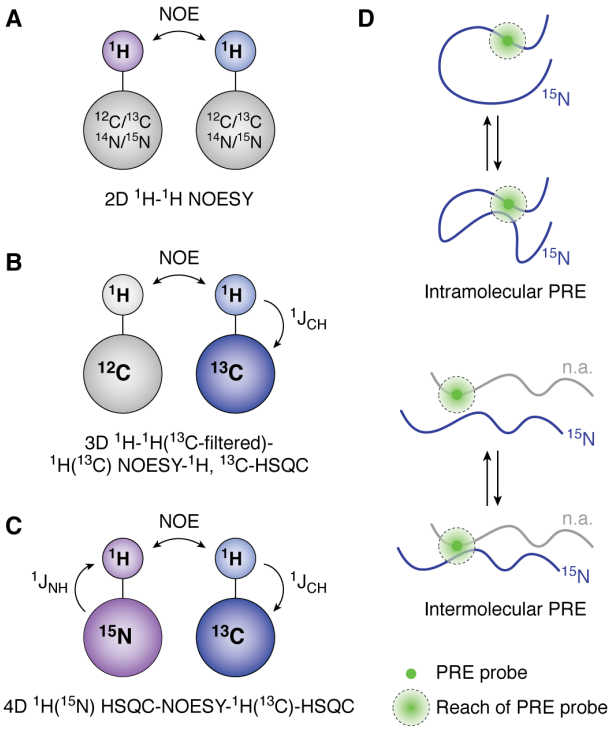

Figure 2. Structural analysis of LLPS systems using NOESY and PREs. A) Basic ^{1}H-^{1}H NOESY experiment transfers magnetization between protons in close proximity through space. This is used to study both intra- and intermolecular contacts within protein systems. B) ^{13}C/^{12}C-filtered/edited NOESY experiments utilize the basic ^{1}H-^{1}H NOESY but select for differentially isotopic-labeled protein through the HSQC transfer. C) To increase selectivity, a HSQC-NOESY-HSQC experiment can be run on a sample containing both ^{15}N-labeled protein and ^{13}C-labeled protein. D) Intra- and intermolecular paramagnetic relaxation enhancement experiments measure the frequency of contact within a single molecule to investigate collapse (intramolecular PRE) or between two molecules to detect interactions (intermolecular PRE).

Figure 3

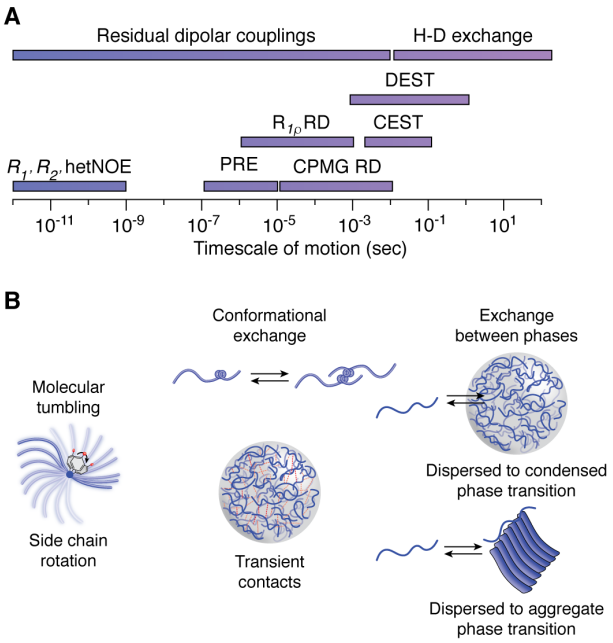

Figure 3. NMR timescale of motion for studying the dynamics of LLPS systems. A) Various types of NMR experiments can probe for processes from the picosecond to second timescale. B) Fast motions (ps-ns) that involve overall molecular tumbling and fluctuations of the peptide backbone and sidechain rotations can be measured using R_1, R_2, and heteronuclear NOE experiments. Intermediate motion processes (µs-ms) that involve conformational exchange and transient contacts can be measured by a variety of experiments such as paramagnetic relaxation enhancement, CPMG relaxation dispersion, and $R_{1\rho}$. Slower processes (ms-s) such as the exchange between liquid and solid phases can be probed using saturation transfer techniques and well as hydrogen-deuterium exchange.