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Structural biology of RNA-binding proteins in the context of phase separation: What NMR and EPR can bring?

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Abstract

Liquid–liquid phase separation of RNA-binding proteins underlies the formation of membraneless organelles, whose composition is dynamic and whose existence may be transient. These organelles are involved in regulation of RNA processing and translation and, if they behave abnormally, in pathologies. Because disorder phenomena are essential in their formation and dynamics, established methodology is insufficient for characterizing their structure. In this review, we consider the current and potential contribution of NMR and EPR spectroscopy to the understanding of structure and dynamics of phase-separating RNA-binding proteins in, both, their dispersed and condensed state *in vitro*. We discuss which experiments are applicable under what conditions and which information can be obtained from them. Because for these phenomena, the accessible information depends crucially on metastable phase equilibria, we also consider aspects of sample preparation for NMR and EPR experiments.

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Liquid–liquid phase separation (LLPS) underlies the formation of membraneless organelles in cells, allowing them to restrict biochemical reactions in space and time [1]. These compartments can be found both in the cytoplasm and in the nucleus, and they respond to environmental cues such as cell cycle stage, growth stimuli, and stress conditions [2]. Proteins with intrinsically disordered regions are common constituents, and promiscuous interactions among these regions are drivers of LLPS [3,4].

The formation of membraneless organelles is driven not only by promiscuous interactions between intrinsically disordered regions. Ribonucleoprotein granules, for instance, are membraneless organelles enriched in RNA and RNA-binding proteins (RBPs). RBPs are often composed of both structured RNA-binding domains and intrinsically disordered regions [5]. This yields a complex network of multivalent protein–protein, protein–RNA, and RNA–RNA interactions that is responsible for ribonucleoprotein granule formation [6,7].

Phase separation can be investigated *in vitro* through the formation of liquid droplets [8]. We review recent findings using new nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR) approaches to study the intramolecular and intermolecular interactions, structure, and protein–RNA interactions of RBPs in their condensed (liquid droplets) and dispersed forms.

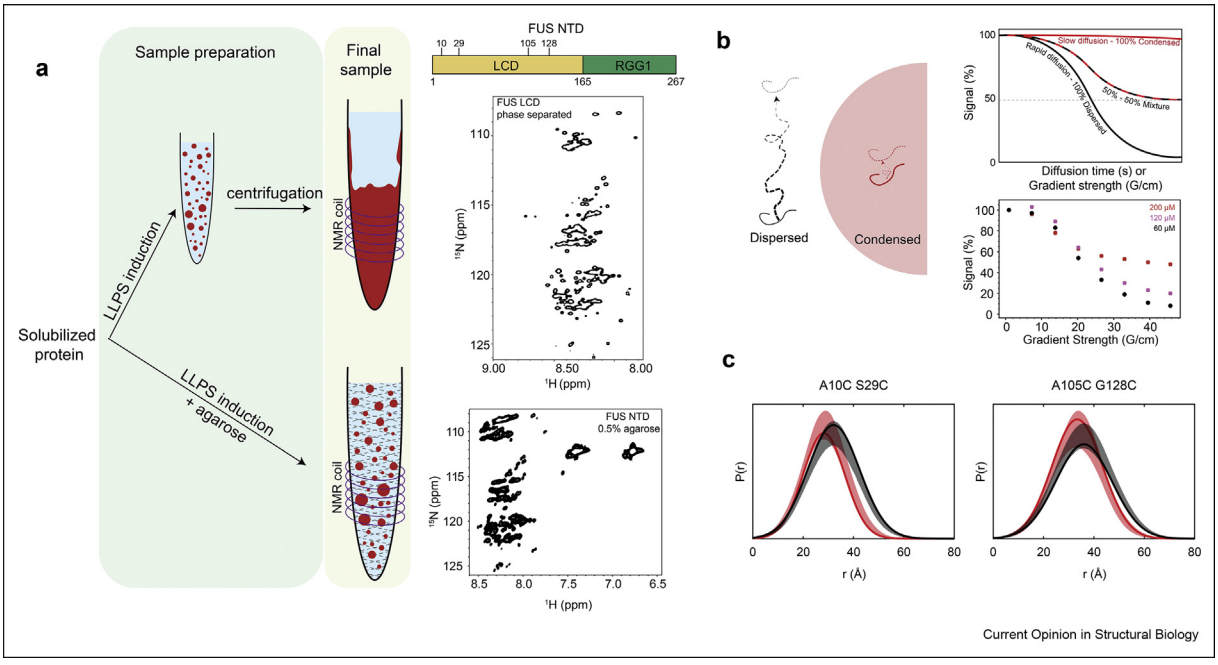
NMR and EPR spectra of RBPs in droplet form

Conditions, which favor inter-protein interactions over interactions between protein and the solvent, result in demixing and formation of an additional liquid phase. Multivalent interactions between biopolymers (RNA, protein, and even DNA) reduce the average distance between them, thus producing a much denser phase than the aqueous one [9]. Initially, this rationale delayed the study of such condensed phases by solution NMR as it was anticipated that such viscous environment would reduce the mobility of the molecules to an extent that signals would be broadened beyond detection. Pioneering work from Burke et al. [10] showed that multidimensional NMR spectra of proteins can be measured in the condensed phase. Strikingly, even though proteins inside the liquid droplets diffuse by orders of magnitude slower than outside, local dynamics in the low-complexity region of the RBP FUS in the

condensed phase was found to proceed on pico- to nanosecond timescales, leading to reasonably sharp signals. In addition, sedimentation of all droplets into a single continuous condensed phase allowed measurement of two-dimensional ^{1}H-^{15}N HSQC and ^{13}C-^{15}N spectra of additional phase-separating proteins, namely DDX4, hnRNPA2, and CAPRIN1 [10–14]. In all cases, the spectra of the condensed phase differed only slightly from those of the dispersed phase, indicating no significant apparent change in the unfolded nature of the fragments tested (Figure 1a). Some line broadening was observed in line with the expected slower dynamics as compared with the dispersed state. Very recently, we developed a methodology to observe intact droplets of the protein FUS by NMR (Figure 1a and b). By measuring spectra of FUS droplets in the presence of a few percent of agarose, protein signals from both the dispersed and condensed phase can be detected simultaneously [15**].

Although EPR spectroscopy has been rarely used so far for studying LLPS, this spectroscopic method holds great potential. As opposed to NMR, size or mobility of the macromolecules is not a limiting factor. To date, EPR has been used to probe local changes in protein dynamics, conformation, and water accessibility on LLPS [15–20]. Detailed structural information can be obtained by pulsed EPR measurements, but this requires cryogenic temperatures. Although this raises concerns about structural changes on changing temperature, we could recently demonstrate that the state of the dispersed and condensed phases of the N-terminal domain of FUS can be arrested by freeze quenching in the absence of a low-molecular-weight cryoprotectant [15]. Furthermore, double electron–electron resonance measurements of distance distributions between spin labels revealed that in the condensed phase, FUS undergoes a compaction (Figure 1c). Although similar information can be obtained by paramagnetic relaxation enhancement (PRE) NMR experiments [21], in contrast to double electron–electron resonance measurements where dilute conditions are accessible, it is difficult with PRE experiments to clearly differentiate between intermolecular or intramolecular contact, so that enhanced PRE on LLPS can be interpreted as either a compaction or intermolecular interactions. Overall, although initial experiments indicated that RBPs in the droplets do not adapt any secondary structure, in recent work, intramolecular compaction in the droplet form was seen by EPR [15].

Figure 1



NMR and EPR of biphasic samples. **(a)** Different methods for NMR sample preparation to study the condensed phase of RBPs with examples of corresponding spectra. Proteins that undergo LLPS are stored routinely in conditions that prevent liquid-droplet formation. On change of buffer conditions (i.e. dilution of urea, change in pH, addition/reduction of salt), droplets are formed, which are either centrifuged to a single condensed phase or stabilized by the addition of agarose and gelation. With the first approach, the resulting NMR spectra report exclusively on the macroscopic condensed phase, whereas the latter approach observes, both, the dispersed and condensed phases [10,15]. **(b)** Protein in the dispersed phase, outside of the droplets, diffuses significantly faster than that in the condensed phase. This difference in the diffusion coefficient allows the spectroscopic separation of the species and the relative quantification with diffusion ordered spectroscopy (DOSY) at various concentrations of the FUS N-terminal domain. **(c)** Spin labeling of proteins at specific sites allows accurately measuring the distance distribution between the sites in co-existing dispersed and condensed phases by EPR [15].

Interactions that drive LLPS

The nature of the interactions that drive LLPS remained elusive until recently. Most of the protein regions studied are intrinsically disordered, with no evidence of formation of defined structure during phase separation. This suggests that the interactions that underlie the demixing must be transient or at least unspecific. Indeed, a combination of unspecific hydrophobic, cation−π, and hydrogen bonds was shown to stabilize the phase-separated state of the low-complexity domain (LCD) of FUS and DDX4 [11,13] (Figure 2a and b). The phase-separating properties of the LCD of FUS are further enhanced in the presence of the arginine- and glycine-rich RGG1 domain, which the authors attributed to cation−π interactions between the aromatic residues in the LCD and the Arg/Lys residues in the RGG1 domain [22]. In the case of hnRNPA1, intermolecular NOEs were reported between aromatic residues in the dispersed phase, suggesting their involvement in LLPS [23**].

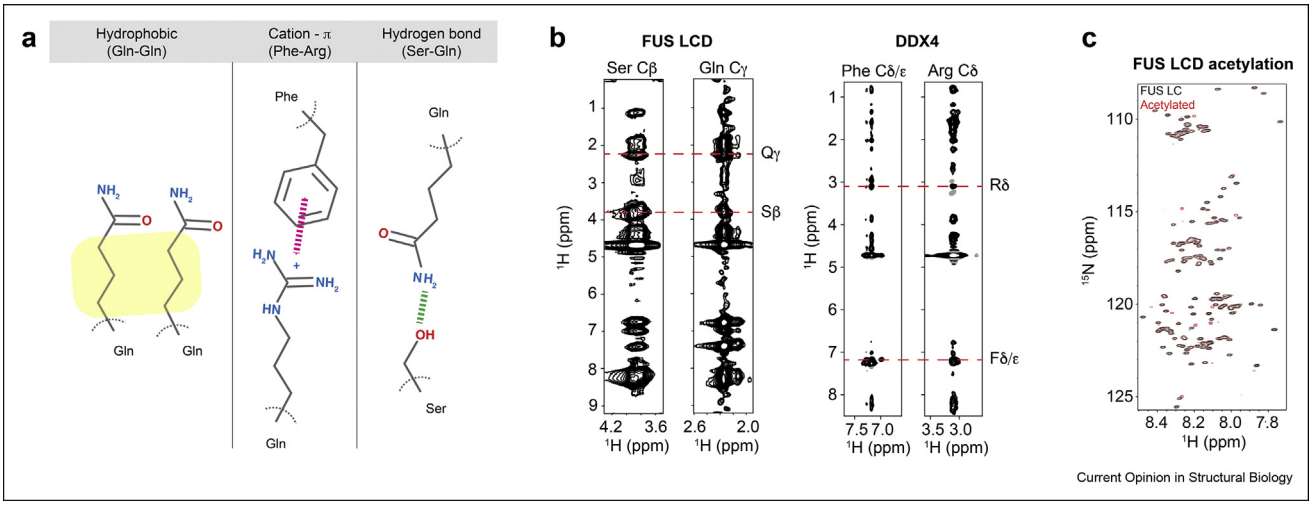
Besides the promiscuous unspecific interactions of the unstructured regions, there are examples also of specific interactions playing a role in structure-formation and phase-separation behavior. Although the C-terminal domain of TDP-43 is largely unstructured, a stretch of approximately 30 amino acids shows high propensity to form a helix [24]. The appearance of an additional immobilized component within this α-helical region under LLPS conditions points to the formation of oligomers stabilized by intermolecular interactions involving this region [16*]. In addition, point mutations that stabilized the helix greatly enhanced intermolecular interactions and subsequently decreased the exchange rate between the two phases. Interestingly, these point mutations seem to affect additionally the splicing *in vitro*; however, it is unknown yet how this is related with helix stabilization or the altered phase-separation properties [25].

Most of the literature on LLPS of RBPs reports on unstructured regions of proteins. However, it cannot be excluded from first principles that fully folded proteins participate in the interactions that drive phase separation. Indeed, recently it was shown that dimerization of the N-terminal folded domain of TDP-43 contributes to the formation of liquid droplets *in vitro* [26]. The NMR structure of this asymmetric dimer resembles the one determined by crystallography earlier [27]. Disrupting the dimer interface leads to a decreased propensity to form liquid droplets *in vitro*, hence implying that an oligomeric state of the protein is present in the condensed phase [26]. Evidently, no threshold has been identified yet for nucleation of macroscopic droplets by higher oligomeric species. However, the mechanism reported in this study supports the idea of a stepwise transition to a condensed phase via oligomerization.

Precise quantification of the condensed phase with NMR

Spectroscopic techniques allow for direct quantitative analysis. Assuming that the observed signal can be separated into its constituents and that the dynamics involved are well understood, it would be relatively simple to determine protein concentration or the absolute amount of protein inside the condensed phase. In this context, it is important to define the meaningful measured quantity, as it depends on the state of the

Figure 2



Intermolecular interaction and modification of proteins in the condensed form as probed by NMR. **(a)** Different types of intermolecular interactions found in condensed phases of RBPs with residue-specific examples. **(b)** NOESY spectra showing intermolecular interactions observed in condensed macroscopic phases [11,13]. **(c)** ^{1}H-^{15}N HSQC spectra of the acetylated (at the N-terminus) FUS LCD in the condensed form reveal that the overall structure of the protein remains unaltered on modification [36].

sample. The currently most commonly used protocol to study phase-separated proteins by solution NMR is to sediment all individual droplets to a monophasic continuous condensed phase (Figure 1a). By definition, this approach cannot quantify the protein mass in the droplet phase as no droplets are present. More importantly, the approach also cannot directly access the partition coefficient between the phases, as the two phases have orders of magnitude smaller contact interface than when the same volume of the condensed phase is distributed into many micrometer-sized droplets. For the same reason, it is impossible to measure the exchange rate between the two phases. In contrast, biphasic samples allow for quantification of partitioning. Guseva et al. [28] have measured the amount of the measles nucleoprotein and phosphoprotein associated with droplets by subtracting the observed ^{1}H-^{15}N HSQC peak intensities in samples with and without droplets. Alternatively, it is possible to quantify the concentration of the protein inside the droplets by measuring translational diffusion with pulsed-field gradient NMR at various total protein concentrations and temperatures. Thereby, any effects due to increased viscosity can be corrected, and the data can be fitted to obtain concentration [29,30]. Notably, concentration of tropoelastin peptides in droplets was found to be independent of total peptide concentration, highlighting that concentration inside droplets may be insensitive to the conditions under which phase separation occurs. Hence, a more meaningful way to compare partitioning in more viscous phases is by direct quantification of the slowly diffusing species inside the droplets by pulsed-field gradient—NMR in a stable biphasic sample. For the unstructured regions of FUS and DDX4, sufficiently fast protein backbone dynamics allows for such measurements as shown in Figure 1b [15].

Post-translation effects on LLPS as studied by NMR

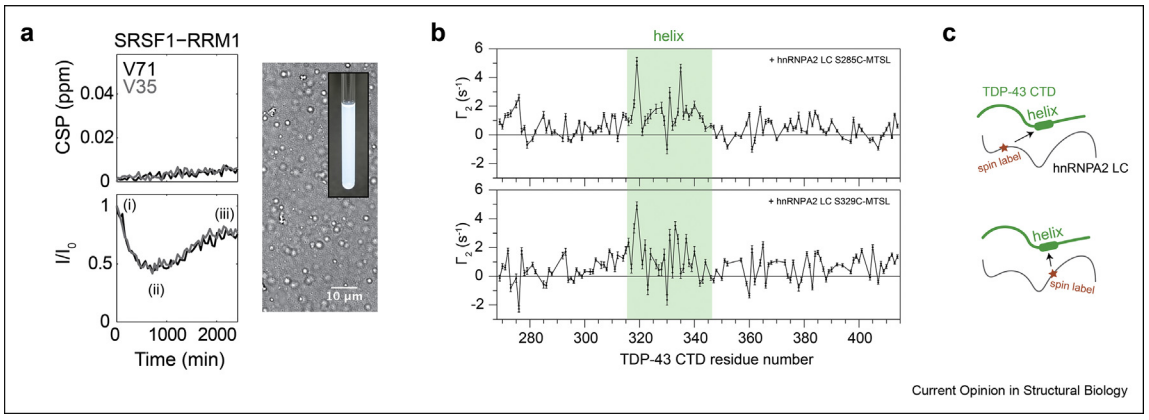
Post-translational modifications have been promptly identified as controlling mechanisms in many cellular pathways [31]. In the case of LLPS, phosphorylation and methylation are the two most established regulatory modifications, and NMR can investigate the behavior of modified proteins in comparison with the unmodified ones. Interestingly, even though they target different types of amino acids and cause opposite changes of side chain polarity and hydrophobicity, both modifications result in reduced liquid droplet formation for FUS, TDP43, hnRNPA2, and DDX4 [26,32—35]. Although NMR of the TDP43 N-terminal domain with a single phosphomimetic mutation at the oligomerization interface revealed no apparent change in the overall structure compared with the wild-type protein, this mutation was sufficient to impair liquid droplet formation [26]. In the case of the FUS LCD, phosphorylation and acetylation did not alter the disordered structure of the protein, as shown by NMR HSQC and relaxation data in dispersed samples. However, PRE experiments revealed phosphomimetic substitutions to inhibit transient intramolecular and intermolecular interactions which are directly linked with LLPS propensity [33,36] (Figure 2c). Interestingly, tyrosine phosphorylation alters LLPS of the hnRNPA2 LCD by leading to an inverse salt dependence, whereas arginine methylation decreases LLPS [14,35]. In the case of tau, phosphorylation of the K18 construct was shown to promote LLPS [37].

Effects of nucleic acid binding on LLPS of RBPs as observed by NMR and EPR

The nucleic acids DNA, RNA, and ATP affect LLPS in various ways. Nucleic acids can modulate LLPS by interacting with either folded or unfolded protein domains. In the case of the N-terminal domain of TDP-43, both the well-folded ubiquitin-like fold and the disordered region are required for ssDNA-induced LLPS [38]. Although oligonucleotides generally modulate LLPS of FUS and its isolated domains by cation—π interactions with arginine or lysine, the outcome may differ depending on which construct is investigated. Nucleic acids enhance LLPS of FUS and induce LLPS of its C-terminal domain, but lead to a monotonic decrease in phase separation of the N-terminal domain with increasing concentration [10,22]. Weak interactions of hnRNPA2 with RNAs derived from the A2 recognition element hinder LLPS [39]. The LCD interacts with RNA in the dispersed state, suggesting that this interaction prevents the LCD—LCD interactions crucial for LLPS. More surprisingly, we observed by time-resolved NMR that the RNA-binding domains can induce phase separation on RNA binding without any LCD present; RBP-RNA droplets can be dissolved when enough RNA is present (Figure 3a) [40].

Particular amino acids can modulate the interaction with RNA and consequently the properties of the resulting liquid droplets. NMR experiments on polypeptides suggest that lysine interacts more weakly with RNA than arginine, which results in liquid droplets that are more dynamic [37]. In line with these observations, continuous wave EPR experiments on a lysine-rich construct of tau revealed no immediate changes in the local protein dynamics on RNA-induced LLPS [17,20]. Interestingly, lysine acetylation of an isoform of tau reverses LLPS by decreasing the affinity to tRNA [37]. The type of interactions that drive droplet formation can in fact induce different modes of droplet maturation, as highlighted in two recent studies by Han et al. [18,19]. While electrostatically driven LLPS of a construct of tau by addition of RNA or heparin is

Figure 3



RNA and protein interactions with proteins in droplets. **(a)** *In vitro* transcription inside the NMR tube in the presence of various RBPs allows the study of the effect of RNA on LLPS in real time. In the case of SRSF1 RRM1, analysis of valine methyl peak intensities from the beginning of the reaction (i) reveals a drop (ii) and then recovery (iii) of the intensities. This behavior correlates with the emergence and subsequent dissolution of the droplets formed during the reaction [40]. **(b)** PRE data of the spin-labeled hnRNPA2 LCD complexed with the isotope-labeled TDP-43 LCD. The α-helical segment between residues 320 and 340 is identified as the region of interaction on the TDP-43 LCD [14]. **(c)** Schematic representation of hnRNPA2 LCD–TDP-43 LCD interaction involving the helical segment of TDP-43 with the hnRNPA2 LCD protein, considering that no specific interaction site was detected on the latter protein.

reversible and does not promote aggregation, hydrophobically driven LLPS under high-salt conditions leads to protein dehydration and triggers fibril formation, which is irreversible [19].

Intermolecular interactions of RBPs and other proteins affecting LLPS as probed by NMR

Experiments on phase-separated samples that contain multiple proteins are necessary to better understand the multivalent protein–protein interactions that take place in membraneless organelles. NMR investigations in the dispersed state allow a precise mapping of the interaction and revealed that the hnRNPA2 LCD interacts weakly with the C-terminal domain of TDP-43 with some specificity involving mainly the α-helical region of TDP-43 (Figure 3b and c) [14*]. The SH3 domain of Fyn kinase may be involved in the interaction with the hnRNPA2 LCD, but a direct identification of the interaction site was precluded by the formation of NMR-invisible species [41]. While the hnRNPA2 LCD interacts weakly with the disordered loops and helical faces of TOG domain 1, the interaction with the hnRNPF prion-like domain seems to be unspecific [35].

Post-translational modifications of certain residues are a frequently used mechanism for regulating protein–protein interactions. It was recently shown by NMR that arginine-rich regions of CAPRIN1 ^{13}C, ^{15}N CON correlations respond differently to the interacting partner FMRP or its phosphorylated version when present in the co-phase-separated state [12**]. Tubulin and α-synuclein are recruited into tau droplets, and phosphorylation of tau mediates the interactions with both proteins by targeting the proline-rich P2 region [42,43]. The C-terminal domain of α-synuclein interacts strongly with the P2 region and weakly with the repeat regions of tau, and hence, phosphorylated tau disfavors the recruitment of α-synuclein into preformed tau droplets [43]. Both phosphorylated tau and unmodified tau bind tubulin only through the repeat regions in the crowded conditions. Therefore, phosphorylation of tau's P2 region does not prevent the recruitment of tubulin into preformed tau droplets, but interestingly prevents the formation of microtubules [42].

Cells can also regulate the content of bimolecular condensates by specific protein–protein interactions. Binding of the nuclear import factor karyopherin-β2 to the proline-tyrosine nuclear localization signal of FUS facilitates weak interactions with other regions of FUS that are involved in FUS self-association and thus inhibits LLPS [44]. Conversely, the C-terminal domain of RPB1, the largest subunit of human RNA polymerase II, can induce LLPS of the FUS LCD [10].

Summary and future challenges

As shown previously, solution NMR and EPR have proven to be informative methods for revealing the molecular basis of droplet formation, whereas solid-state NMR is suitable for studying the solid RBP condensates. The field is still in its infancy, and many open questions remain on biogenesis, structure, and ultimately function of RBP condensates. NMR and EPR will be important methods for addressing these questions because they can be applied in the liquid state

and their versatility allows for studying structure, interactions, and dynamics. The next step will be to observe the condensed and the dispersed state of proteins, RNAs, and their complexes to compare directly their structure and dynamics and also how the two phases can exchange and mature over time.

Conflict of interest statement

Nothing declared.

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** of outstanding interest

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