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*Curr Opin Struct Biol*. Author manuscript; available in PMC 2022 October 01.

Published in final edited form as:

*Curr Opin Struct Biol*. 2021 October ; 70: 78–86. doi:10.1016/j.sbi.2021.04.004.

# Biophysical studies of phase separation integrating experimental and computational methods

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## Abstract

Biomolecular phase separation in the formation of membraneless organelles and biomolecular condensates has recently gained tremendous attention due to the importance of these assemblies in physiology, disease, and engineering applications. Understanding and directing biomolecular phase separation requires a multi-scale view of the biophysical properties of these phases. Yet, many classic tools to characterize biomolecular properties do not apply in these condensed phases. Here, we discuss insights obtained from spectroscopic methods, in particular NMR spectroscopy, in understanding the molecular and atomic interactions that underlie the formation of protein-rich condensates. We also review approaches closely coupling NMR data with computational methods especially coarse-grained and all-atom molecular simulations, which provide insight into molecular features of phase separation. Finally, we point to future developments, particularly visualizing biophysical properties of condensates in cells.

## Introduction

Biomolecular phase separation has generated tremendous interest recently, having been found or attributed to play a role in an ever-growing list of biological processes [1,2]. Consequently, the importance of understanding the biophysical basis of phase separation is now clear [3]. A repeating theme in work linking phase separation to cellular function is: 1) the identification of key components contributing to the formation of a particular

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membraneless organelle or phase separated structures in cells and organisms using the tools of cell biology, and 2) demonstrating that biomolecular condensates can be minimally reconstituted *in vitro* using one or a few of these critical components. This reconstitution provides the important opportunity to analyze in detail the components and interactions that give rise to phase separation. Yet, unusual biophysical properties common to many phase separated condensates, including component density, sample heterogeneity, and disorder – preclude application of many common biophysical approaches to understand the structural and mechanistic details of phase separation.

## Integrative biophysical tools to study biomolecular phase separation

Liquid-liquid phase separation of biomolecules requires molecules that can form multiple simultaneous contacts (necessary to stabilize a network to define a condensed phase) that lack rigid long-range order (otherwise the assemblies would be solid) [4]. Hence, disordered proteins and domains are often important contributors to phase separation as both mediators of phase separation or simply as linkers between folded domains that mediate the contacts. As liquids, these condensed phases are not directly amenable to x-ray crystallography or single-particle cryoelectron microscopy. Therefore, both solution and solid-state NMR spectroscopies have emerged as important techniques to probe the structural details of phase separation with atomic or residue-by-residue resolution [5]. Yet, NMR experiments report on average behavior, hence details on heterogeneous populations and ensembles are difficult to interrogate directly. Furthermore, because NMR experiments are uniquely sensitive probes of molecular motions, the magnitude and timescales of rotational and conformational changes impact NMR spectra – but these same features complicate quantitative interpretation. Such motions can obscure highly dynamic or static conformers or regions, depending on the particular NMR technique. Therefore, complementary approaches including molecular simulation and other spectroscopies can provide frameworks for interpreting the NMR data.

Specifically, all-atom molecular dynamics (MD) simulations using physics-based models have emerged as an essential tool in linking the laboratory measurements with molecular and atomic details with high spatiotemporal resolution [6]. Critical to using MD simulations is validation of the model (e.g. protein and water force field) for this new class of systems that are distinct from the state (folded protein and even dilute disordered proteins) and sequence compositions for which these models were originally parameterized. An explosion of activity in the last decade in protein force field refinement, new simulation algorithms[7], and the availability of modern computer hardware to generate microsecond-long trajectories[8] has made feasible near quantitative agreement of simulated properties with experimental observables such as chemical shifts, relaxation order parameters, and radius of gyration, among others. Applying these approaches to proteins that phase separate, residue-level NMR data on low-complexity (LC) disordered proteins that are implicated in cellular phase separation was used recently to assess and tune the backbone dihedral potential parameters for polar residues to balance the propensity of helical and extended structures [9*]. Going forward, these protein models will help develop a molecular mechanistic understanding of atomic interactions[10] and other important questions in condensates such as water/ion distribution and dynamics[11**]. These approaches are also

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used to interpret the NMR spectroscopy data on protein structure, interactions, and dynamics in dilute and dense protein phases when experimental observations alone cannot distinguish between different possible scenarios.

*In situ* molecular spectroscopy of phase separated condensates is an area where NMR spectroscopy is challenged due to the inherently heterogeneous nature of the sample (e.g. condensates within a homogenous bulk phase). Several solution state NMR studies of condensates have been performed with “macro-droplets” [12], while others have explicitly tested and demonstrated the correspondence between these samples and two-phase systems [13–15]. (We note that the interpretation that our previous work demonstrating direct observation of chemical shift differences in the condensed phase is due to interaction with glass [15] is not consistent with the quantification of peak intensity and spectral analysis we and others performed.) Still, spatially or spectroscopically probing droplets and particularly droplet interfaces is not straightforward. Optical spectroscopies, such as vibrational or fluorescence spectroscopy, offer an alternative set of approaches. Combined with imaging platforms to spatially resolve condensate phases *in situ*, they can provide fingerprints of molecular structural and chemical interactions with sub-micron spatial resolution. Below, we focus on vibrational spectroscopies (Fourier transform infrared (FTIR) spectroscopy and Raman spectroscopy) and fluorescence spectroscopy (fluorescence correlation spectroscopy (FCS)) that report on the intrinsic physical chemical environment and contacts in the phases.

Following on the success of previous work combining complementary techniques to study disordered protein conformational properties[16], here we review biophysical questions in phase separation, focusing on proteins, and integrative approaches to revealing the molecular details of phase separation (Figure 1).

## Probing Conformational Properties and Structure

### NMR spectroscopy:

An essential question in the biophysical characterization of phase separation is the structure of the components both in the dispersed and condensed phases. Many phase separating proteins contain sequence-repetitive low complexity domains (LCs) that are predicted to be disordered but also prone to assembly [17]. Therefore, it is not surprising that solution NMR spectroscopy (Figure 1), which provides a residue-by-residue picture of the dynamic ensemble of conformations including the secondary structure of these proteins, have shown that these domains are disordered when in the dispersed phase (i.e. not phase separated). Based on pioneering earlier work showing the formation of amyloid “hydrogels” (Box 1) from tagged forms of these domains and the correlative impact of mutations on amyloid formation and, later, phase separation[18–20], some studies have claimed that these disordered domains phase separate primarily due to β-sheet contacts. Yet, solution NMR studies *directly observing* the liquid condensed phases have found no support for assertions of increased β-sheet ordering. On the contrary, despite different sequence compositions and driving forces for phase separation, the LC domains of FUS, DDX4, hnRNPA2, and elastin-like peptides all retain predominant disorder within liquid condensed phases [12–14*,21]. Similarly, the acidic disordered domains of nucleophosmin (NPM1) remain disordered even in the context of a viscoelastic condensed phase formed with an arginine-rich peptide that

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contributes to nucleolar formation [22**]. We note that solution NMR is not sensitive to slowly moving species or minority populations. For our work, we have overcome this challenge for FUS LC with exchange-based NMR and coherent Raman scattering (CRS) to probe for conformational exchange and slow-moving states, which further confirmed no evidence of structured conformations in the liquid condensed phase [14]. Still for some proteins including FUS, it is clear that gradual conversion of liquid phases into more static structures can involve β-sheet formation depending on the conditions and missense mutations[23**]. Additionally, electron paramagnetic resonance (EPR) of site-specifically labeled samples has recently been applied to the question of disordered domain structure in droplets to probe chain motions and dimensions upon phase separation[15].

While we have focused on disordered domains until now, folded domains, especially those that oligomerize, can also contribute to scaffolds for phase separation[25]. Thus, a logical question is if the structure of folded domains changes due to phase separation? The NPM1 contains a folded, pentamerizing N-terminal domain that facilitates phase separation of NPM1's acidic disordered domains with R-rich motif peptides and RNA [26]. After phase separation, the NPM1 pentamers remain spaced as seen by neutron scattering. Solid-state NMR of phase separated of NPM1 showed that the folded domain of NPM retains the same structural fingerprint (i.e. folded pentamer) in a condensed phase [22]. Can phase separation influence folded conformations? The Dcp1/Dcp2 mRNA decapping complex can phase separate with the enhancer of decapping 3 (Edc3) to form mRNA processing body and speed mRNA decapping [27]. In these phases, the LSm domain of Edc3, which interacts with Dcp2, remains folded but is dynamically tethered via an RNA-binding disordered region to the dimerization domain[28]. Recent findings suggest that Dcp1/Dcp2 form an inactive conformation inhibiting decapping when at high concentration in condensed phases but co-condensation of Dcp1 and Dcp2 with Edc3 activates decapping within the phase-separated compartment by inducing a conformation change in the catalytic domain[29*]. Curiously, Edc3 is dimeric and hence in this model interactions with multiple copies of Dcp2 replace the autoinhibitory multivalent Dcp2 self-interactions, effectively "rewiring" the interactions that stabilize condensation.

### Vibrational spectroscopy:

Though missing the atomic resolution of NMR, vibrational spectroscopy also reports on molecular structure and interactions with the added advantage that these features can be quantified in samples with heterogenous structure and motions. Linear FTIR has been used to follow pressure-induced self-assembly of γD-crystallin with crowding agents [30] and thermally-triggered phase separation of fatty-acid-modified elastin-like polypeptides [31]. Specifically, FTIR was used demonstrate the changes in solute-protein and protein-protein interactions along the process of phase separation and material maturation. Coherent, two-dimensional FTIR (2D IR), which can resolve overlapping peaks and correlations of molecular vibrations over femto- to picosecond timescales (often considered an optical analog of 2D NMR), was recently applied to study LLPS of dipeptide repeats of proline-arginine (PR) chains [32]. In this study, $PR_{20}$ chains phase separated into droplets when crowded with PEG and phase separated $PR_{20}$ displayed spectral signatures consistent with

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backbone configurations associated with polyproline I and II helices compared to a random coil state when in the dispersed phase.

Imaging spectroscopies such as atomic force microscopy IR (AFM-IR) [33**] and coherent Raman scattering (CRS) [34] can discriminate between the continuous phase and protein-rich phase separated droplets. AFM-IR offers the promise of molecular spectroscopy with ~50 nm spatial resolution but is challenging in water; CRS does not have limitations in water but offers only optical (~300 nm) resolution. St George-Hyslop and Knowles used AFM-IR to show that methylation state of FUS changes the thermodynamic state, average protein secondary structure, and mechanics of FUS condensates [35]. Our own studies, using CRS, have recently shown that various promiscuous interactions stabilize liquid FUS LC droplets, which on average showed the same overall secondary structure as dispersed FUS LC [14*]. As complementary methods to NMR, vibrational spectroscopies are useful methods to study heterogeneous phase separation samples *in situ*.

### Molecular simulations:

Rauscher and Pomes conducted one of the first all-atom simulations of the liquid-like state of a short elastin-like peptide[36], highlighting conformational disorder within the assembled state consistent with experiment [13]. In this case, hydrophobic interactions were found to contribute to intermolecular contacts of hydrated peptides within the protein-rich phase. Some groups have also used coarse-grained (CG) models to access longer timescales including the process of phase separation as well as examine partitioning and surface properties [37,38]. To complement these approaches, we recently used our CG model based protein-rich phase configurations to generate fully atomistic models with explicit solvent/ions and probed structure and motions inside the condensed phase and on the droplet interface[11**] (Figure 3). These recent investigations suggest that these simulations can provide meaningful information that is difficult to access by experiment is available on protein structure, contacts, and motions. To realize the potential of this new frontier, accessing longer timescales in order to ensure adequate sampling of states and observe the full range of structural transitions will require further advances in both hardware and software.

## Molecular interactions in condensed phases

One of the central goals of many studies integrating experimental and computational results is to identify the fundamental rules (aka “molecular grammar”) based on atomic interactions that dictate the sequence-dependent phase separation of proteins [39–41]. Although perhaps starting from different initial hypotheses regarding the primary interactions (Is the dominant contribution cation-$\pi$ vs. $sp^2/\pi$ interactions to phase separation over non-specific hydrophobic effect and hydrogen bonding?), many studies have arrived at similar conclusions about the role of certain amino acids in promoting the phase separation, leading to some confusion about several important issues. Most importantly, are few residue types solely responsible for driving the phase separation as expected for associative polymers or do most amino acids in the sequence contribute to the phase separation to varying degrees (primary, secondary, tertiary, etc. drivers)[39,42,43]? Mutagenesis experiments, changing

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one residue type at a time in the low complexity domains have highlighted tyrosine and arginine as particularly important in polar-rich sequence phase separation [40,44,45]. Yet, other residues including glutamine/serine [14,46] and charged residues [21,44,47] contribute to phase separation in some systems while even hydrophobicity plays a role in some contexts [48]. In our view, together these approaches have generated consequential insights about the primary drivers of phase separation (e.g. Tyr and Arg over Phe and Lys for many polar-rich domains in vertebrates) and the role of other stabilizing interactions, e.g., involving residues such as glutamine and threonine in the case of FUS LC and stabilization of small helical motifs in case of TDP-43 CTD[49].

Still, these approaches cannot easily show what ensemble of contacts these residues types make. Therefore, NMR experiments that directly interrogate contacts have played an important role. In the dispersed phase, the contacts contributing to phase separation may form transient weak intramolecular interactions due to the repetitive nature of the sequences. Using this approach, the contribution of aromatic (tyrosine to phenylalanine) contacts was directly observed for hnRNPA1 LC using nuclear Overhauser effect experiments, where amino acids in proximity can be detected even if the interaction is transient [45]. Going further, in condensed phase samples where the density of proteins is high and hence provides higher signal to noise, a full network of contacts between many different residue types has been observed for several disordered domains often using a combination of mixing peptides with distinct isotopic labeling patterns and heteronuclear edited (and filtered) NMR experiments[13,14,21,50]. These observations are in accordance with both fully atomistic and coarse-grained simulations demonstrating a broad array of contacts both in initial contacts mimicking the interactions leading to phase separation and within condensed phases[51]. Therefore, these insights have informed efforts to understands the role of disease-associated mutations and post-translational modifications on phase separation[52–54].

It is important to note that although residue-residue contributions are directly observed by NMR and validated by mutagenesis, it is not trivial to parse out the energetic balance of specific and overlapping interaction modes that these residues make such as cation-$\pi$, $sp^2/\pi$, hydrophobicity, and hydrogen bonding using available experimental techniques and computational models[55]. On the experimental side, it will be beneficial to conduct phase separation studies using non-natural amino acids to more precisely perturb the interaction modes in hope of disentangling their contributions, including further work such as the impact of fluorination of aromatic residues on phase separation[56]. On the simulation side, all-atom simulation studies could be complemented with more complex and polarizable models[57] or ab initio models that could more accurately represent, for example, explicit $\pi$-interactions and the behavior of surface water to provide confidence that they capture the interaction modes and to further probe the unusual chemical environment created by phase separation[58].

## Molecular motions in condensed phases

Phase separated condensates contain high concentrations of biomolecules, and hence, it is not a surprise that local and global motions of molecules are distinct in the condensed

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phase compared to studies in bulk. NMR has been extensively used to demonstrate that local motions on the ns/ps timescale are slowed but the chain remains highly mobile in liquid condensed phases of disordered proteins[12–14,21,59**]. Curiously, signatures of slower timescale (ms) conformational transitions have been observed for condensed phases of DDX4, yet the origin of these remain unknown[60]. Translational protein diffusion as measured by diffusion NMR is slowed by orders of magnitude[14*,21], consistent with results from fluorescent recovery after photobleaching readily accessible via microscopy and consistent with a dense (percolated) phase[4].

Fluorescence spectroscopies, specifically FCS and FRET, have a long history in biophysical measurements. Unique from NMR or vibrational spectroscopy, these methods do not directly probe molecular interactions in protein, using fluorophores as handles to measure fluorophore environment, motions or fluorophore-fluorophore distances; however, FCS and FRET offer unparalleled single-molecule sensitivity and exquisite spatial selectivity. Here, we focus on FCS as it is detected as a traditional spectroscopic measurement as a measure of molecular motion in condensed phases. FCS was used to show how RNA-FUS interactions modified the tracer mobility of condensed phases, identifying two populations of FUS upon RNA-FUS interaction[61]. Similarly, the effect of RNA concentration on morphology of hollow phase-separated protamine condensates and the mobility in the rim regions was also probed by FCS. The rim, where protein and RNA co-condense, showed significantly reduced dynamics compared to the lumen [62]. In an alternative system, FCS was used to probe protein-protein interactions between FG nucleoporins (Nups) and nuclear transport receptors, identifying changes in Nup mobility with nuclear transport receptor addition [63]. Finally, as FCS provides a measurement of mobility, it has also been used to quantify the microrheological properties in LAF-1 condensates formed with different macromolecular constituents [64]. The versatility and relative ease of FCS make it a very attractive method for physical-chemical analysis of phase separation, and one that provides highly complementary data for phase field models of condensate dynamics[65].

## Conclusion

Taken together, magnetic resonance and optical spectroscopies combined with molecular simulation offer a complementary set of tools to probe the biophysical details of phase separation with atomistic and molecular resolution. As more complex condensates are reconstituted and in order to realize the ultimate goal of probing the structure, interactions, and molecular motions in condensates in living cells, cooperation between these techniques becomes even more important.

## Acknowledgement

This work was supported in part by the National Institutes of Health R01NS116176 (N.L.F. and J.M.), R01GM120537 (J.M.), National Science Foundation grants DMR2004796 (J.M), the SPP2191 of the Deutsche Forschungsgemeinschaft (DFG) PA2526/3-1 (S.H.P.), the Human Frontier in Science Program (RGP0045/2018 to N.L.F. and S.H.P.). We thank Vinald Francis for designing Figure 2.

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**Box 1:**

### Phase separation, gels, “hydrogels”.

It is important to distinguish between the rapid phase separation into liquid droplets from the slow conversion of liquid phases to “hydrogels”, which has been seen for FUS LC, nucleoporin FGs, hnRNPA2 LC. In phase separation, condensed phase droplets – with varying viscosities – forms, depleting the dispersed solution of the biopolymer components. For NMR samples of condensed phases, 15 to 50 ml of solution is often created in order to generate <500 μl of >200 mg/ml (up to 25 mM) condensed phase [5,21,22**]. By contrast, amyloid-based “hydrogels” are formed gradually (over days) and uniformly (the entire sample changes from a liquid to a hydrogel, phase separation is not required) from samples of ~mM concentration of proteins (that are often tagged to prevent avid phase separation). We surmise that the addition of solubility tags modifies the self-assembly process, resulting in different final states. Taking this into account, it is possible that additional semantic confusion regarding phase separation, “hydrogels”, and “gels” arises for the following reasons: 1) *liquid* condensed phases are sometimes referred to as “gels” as they are loosely-held networks that can achieve a percolated state, 2) very high concentration samples of liquid forming components (i.e. beyond the high concentration arm of the binodal) undergo “gelation without phase separation” in this sense that protein-dense liquids are “gels” [4], 3) some proteins with disordered regions phase separate into viscoelastic condensed phases that do not readily flow (i.e. they “gel” according to a different common usage of the word) based on the conditions, temperature, and valency/strength of interactions[12,22] yet are distinct from amyloid hydrogels, and 4) some liquid-liquid phase separated condensates “age” into static and even amyloid structures that may also be referred to as “gels” [24]. These distinctions are important because, although the same residues may contribute to both phase separation and amyloid formation, the physical features and hence molecular structures are different.

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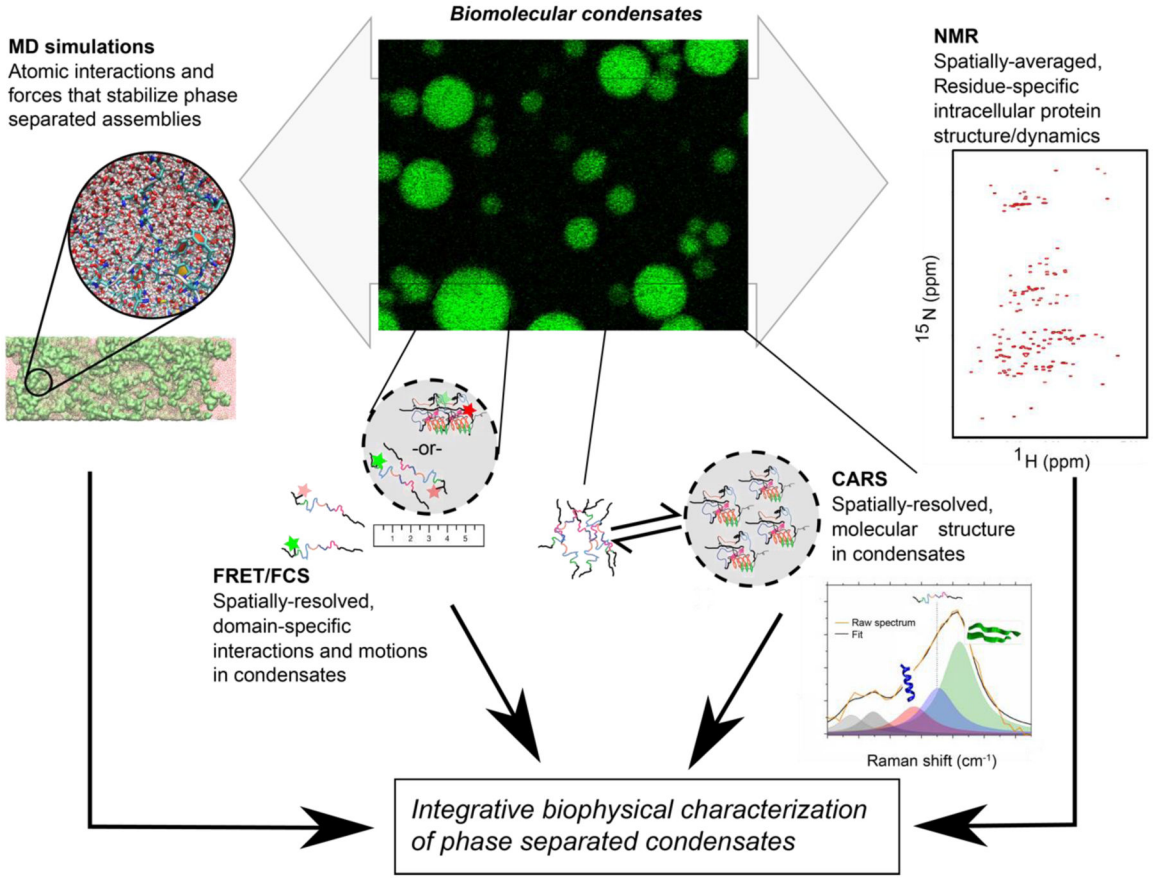


**Figure 1: Integrative approach to biophysical characterization of phase separation.**

Integration of information from NMR (or EPR) and optical and vibrational spectroscopies combined with insight from molecular simulations are a powerful combination to probe structure, interactions, and molecular motions in phase separated biomolecular condensates.

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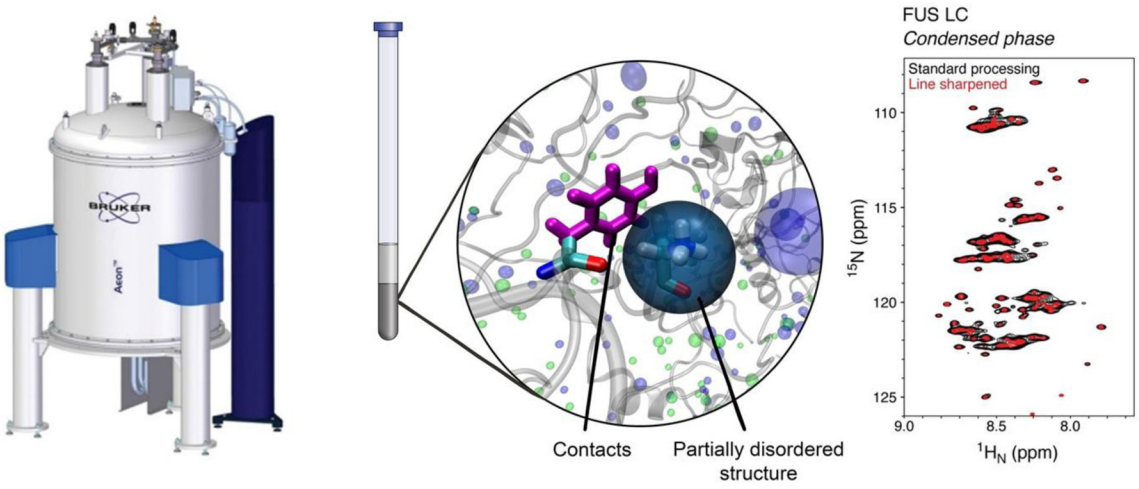


**Figure 2. NMR spectroscopy of phase separation.**

NMR spectroscopy of samples where the condensed phase (gray, center) fills the observation (coil) volume enables direct interrogation of structure and disorder in proteins and their contacts (center) with residue-by-residue resolution (right). Image concept by Vinald Francis.

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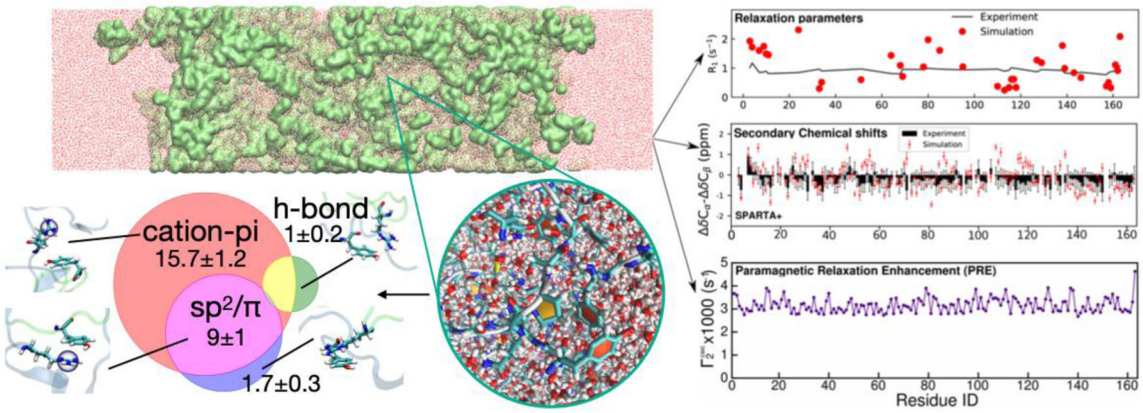


**Figure 3: All atom simulation of phase separation.**

Creation and simulation of a fully atomistic “slabs” (top left) by conversion of coarse-grained models of phase separation enables atomistic analysis of contact modes (bottom left), molecular structure and solvent/ion properties (bottom center), and predicted NMR observables (right).