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Solution NMR goes big: Atomic resolution studies of protein components of molecular machines and phase-separated condensates

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The tools of structural biology have undergone remarkable advances in the past decade. These include new computational and experimental approaches that have enabled studies at a level of detail – and ease – that were not previously possible. Yet, significant deficiencies in our understanding of biomolecular function remain and new challenges must be overcome to go beyond static pictures towards a description of function in terms of structural dynamics. Solution Nuclear Magnetic Resonance (NMR) spectroscopy has emerged as a powerful technique for atomic resolution studies of the dynamics of a wide range of biomolecules, including molecular machines and the components of phase-separated condensates. Here we highlight some of the very recent advances in these areas that have been driven by NMR.

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Introduction

The emergence of computational-based approaches for structural biology studies of biomolecules, principally through the development of artificial intelligence (AI)-based methods, has had a profound impact on the nature of the problems that can now be rapidly addressed [1]. On the experimental side, there have been equally important advances that have been fueled by improvements to methodologies that allow, for example, facile structure determination of large complexes by cryo-electron microscopy (cryo-EM). It is widely appreciated that the next major challenge lies in understanding the role of biomolecular dynamics in driving function. The pervasiveness of motion becomes clear when one considers that nearly 60 % of human proteins contain structured domains tethered by long intrinsically disordered regions (IDRs) of polypeptide [2], but, of course, even folded regions of proteins are often dynamic in ways that are critical for function. The inherent flexibility in many systems can challenge structure determination techniques, and, for these cases, in particular, solution Nuclear Magnetic Resonance (NMR) spectroscopy is a powerful method for 'filling in' important gaps in cryo-EM and X-ray generated structures that are often necessary to understand function. With continued developments in strategies for isotopic labeling of biomolecules and further improvements in using spin-physics to enhance experimental sensitivity, along with increased magnetic field strengths and improved probe technologies, solution NMR studies of molecular machines with aggregate masses on the order of 1 MDa are now possible. Moreover, these developments have fueled studies of biomolecules in condensed phases of phase-separated systems, where atomic resolution insights into the structural dynamics of the condensate components are now available. In this review we highlight recent solution NMR studies contributing to these two important areas. For additional insights into the roles of NMR in structural biology, including studies of molecular machines, the reader is referred to a 2021 review and references therein [3].

Breaking down limitations through methods development: molecular machines are no longer too big for solution NMR

Development of more sensitive NMR experiments: the Delayed Decoupling (DD) approach

Molecular assemblies are ubiquitous throughout the cell, performing numerous critical biological functions.

Investigation of these large biomolecules, such as multi-subunit proteins and (deoxy)ribonucleoprotein complexes, however, presents considerable challenges for solution NMR, primarily due to their slow tumbling in solution. Given that the intensity of NMR signals is inversely correlated with a molecule's tumbling time, the development of more sensitive NMR techniques is essential for such applications. The sensitivity of an NMR experiment is dictated by i) the initial amount of magnetization, that, in turn is related to sample concentration and magnetic field strength, ii) the rate at which this magnetization decays during the course of an experiment (relaxation), and iii) the extent to which the initial magnetization can be transformed into detectable signal by exploiting interactions between NMR spins that are either coupled through bonding or via spatial proximity. Improvements in these general areas have enabled the characterization of progressively higher molecular-weight particles. Methyl groups have often been the probe of choice for studies of large biomolecular assemblies as they have three equivalent protons that contribute to the initial magnetization, and their inherent flexibility results in unusually slow (favorable) relaxation properties that enhance the sensitivity of the resulting spectra.

Although methodological developments in solution NMR have been ongoing for many decades, remarkably, improvements are still forthcoming, in some cases involving repurposing experiments that were developed for a different reason many years before. An NMR experiment can be likened to a set of building blocks during which manipulations of spins or recording of signals occur. Such manipulations come at a cost because they take time, during which signals decay, and because they involve the application of pulses which are imperfect, further signal attenuation occurs. Bax and Sarkar developed a shortened experiment for correlating one-bond coupled ^{13}C and ^{1}H nuclei in which several pulses were eliminated, an important consideration in an era where probe limitations led to more severe pulse imperfections than with current generation probes [4]. However, the same approach, exploiting a phenomenon referred to as delayed decoupling (DD, in what follows), can be used to decrease the length of specific classes of experiments, and hence significantly improve sensitivity in certain applications (Figure 1a and b). The Gossert group first applied a DD approach to enhance the sensitivity of spectra recorded on fully protonated large complexes, reducing the need for deuteration [5]. On average, when compared to an experiment lacking the delayed decoupling element, and focusing on methyl probes, the so-called XL-ALSOFAST-HMQC experiment was approximately 3-fold higher in sensitivity in applications to a number of systems in the 200 kDa molecular weight range (Figure 1c). Our laboratory has modified the classic HMQC experiment which is, by-itself, optimized for studies of highly deuterated, methyl-labeled samples, and referred to as methyl-TROSY in what follows [6], to include a DD element [7]. Sensitivity gains of 2-fold, on average, and significantly higher for some peaks, were quantified in studies of a 1 MDa homo-oligomeric protein assembly. Recently, a 'gradient-enhanced' version was introduced to overcome dynamic range issues in condensed phase samples [8], as discussed in Mechanistic studies of molecular machines. These developments pave the way for methyl-TROSY studies of even larger biomolecular assemblies as well as applications to a wider range of biomolecular systems. Lineshape distortions introduced by DD, and currently corrected using time-domain deconvolution procedures [5,7], are likely to be remedied more efficiently using machine-learning (ML) approaches. Applications of ML in NMR are presented elsewhere[9–12].

Improvements in heterologous expression of isotopically-labeled molecular machines in mammalian cells

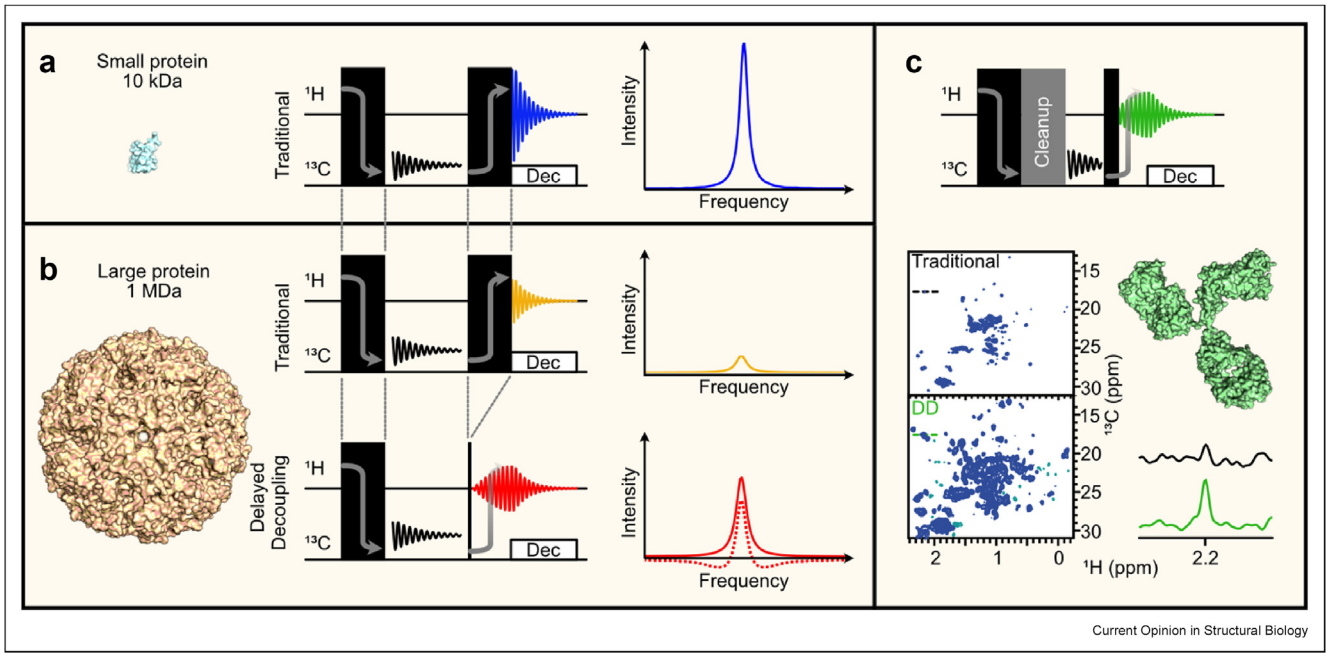
Advances in NMR pulse sequence design have been paralleled by significant improvements in protein expression systems for producing large biomolecular assemblies. The use of mammalian expression systems for isotope labeling of biological macromolecules provides opportunities to study systems whose production is incompatible with bacterial expression hosts. A major restriction for protein production in mammalian cells, however, is the large number of essential amino acids that must be added to growths and a limited ability to make use of precursors. Thus, prohibitively expensive labeled amino acids are required for expression. Notably, new protocols and strategies for the economic production of isotope labeled proteins in HEK293 cells grown in suspension have recently been published [13–15], so that uniform or amino acid-specific labeled samples can be generated. With the barrier for protein expression in a mammalian host reduced, an increase in NMR studies focused on difficult-to-produce or post-translationally modified targets is anticipated in the future.

Mechanistic studies of molecular machines

Interprotomer cooperativity regulates HtrA2 function

Large multi-chain protein complexes are commonplace in the cell, with their oligomeric state closely tied to their function. The activities of individual subunits in these complexes are often interdependent via a phenomenon referred to as cooperativity. Solution NMR experiments employing asymmetric labeling of protomers can help to reveal the underlying cooperativity in numerous oligomeric assemblies, such as in p97 [16] and in the proteasome [17]. Recent work by Toyama et al. involves the application of asymmetric labeling to elucidate cooperativity in the human high-temperature requirement A2 (HtrA2) mitochondrial protease [18], an important component in mitochondrial proteostasis. In the absence of the N-terminal membrane anchoring

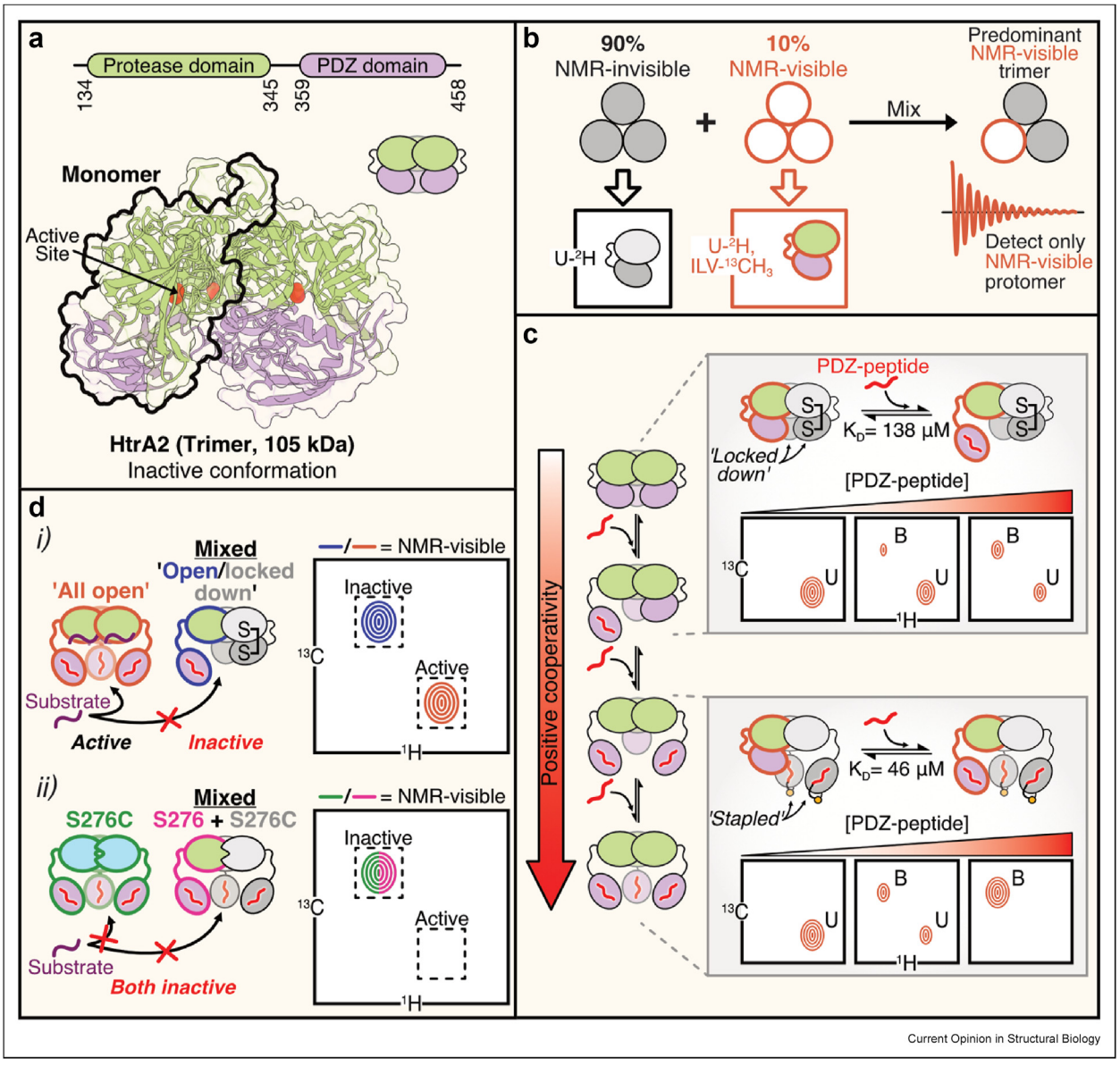
Figure 1



Powerful 'short' NMR experiments for studies of big proteins. Schematics of ^{13}C–^{1}H correlation experiments for studies of molecular machines. Boxes indicate elements that include pulses and delays, and arrows represent the flow of magnetization throughout the experiment. **(a)** Acquiring methyl spectra of a small protein (e.g., ubiquitin, PDB ID: 1UBQ) with a traditional pulse sequence consisting of complete transfer elements results in spectra with sharp, high intensity signals **(b)** Top: Spectra of large biomolecular assemblies (e.g., lumazine synthase, PDB ID: 1HQK) contain broad, low intensity peaks due to signal losses from relaxation. Bottom: Minimizing the second transfer element leads to an increase in signal intensity [5,7]. A direct Fourier transform of the resulting signal will give rise to distorted peaks (dotted red line), however, applying a correcting time-domain function removes these artifacts (red solid line) and leads to significant signal enhancements for applications involving MDa-sized complexes. Studies typically involve highly deuterated, methyl protonated samples. **(c)** Applications to protonated and excipient-rich samples require additional elements to 'cleanup' (gray box) the signal [5,8]. The XL-ALSOFAST-HMQC was designed for fully protonated samples and can be used for validation of the structures of therapeutic monoclonal antibodies (PDB ID: 1IGT) at natural isotope abundance. Traditional approaches (top spectrum, HSQC) are outperformed by the XL-ALSOFAST-HMQC experiment (bottom spectrum) by factors of 2–3. Slices originate from the indicated region in the 2D spectra. Spectra were adapted from Ref. [5].

domain HtrA2 is a 105 kDa homotrimer, with each subunit composed of an N-terminal protease domain whose active site (red sphere highlights the catalytic serine) is blocked by a C-terminal PDZ domain in the closed, inactive conformation (Figure 2a) [19]. Prior studies have found that binding of a hydrophobic activator peptide (PDZ-peptide), a mimic for the C-terminus of cognate substrates, to the PDZ domain causes it to 'open', exposing the active site [20]. To explore the underlying cooperativity of this process, hybrid molecules were prepared by mixing NMR-visible ($^{13}CH_3$ labeled) and NMR-invisible trimers in prescribed ratios, followed by unfolding and subsequently refolding the protomers as shown in Figure 2b [18]. Individual binding events in the trimer could then be studied by using NMR-invisible protomers that are locked in distinct conformations (either open or closed) via the formation of disulfides. For example, the first PDZ-peptide binding event was quantified by performing an NMR titration on a mixed sample containing NMR-visible unmodified protomers and NMR-invisible 'locked down' protomers wherein the PDZ domain is covalently linked to the protease domain by a cysteine disulfide linkage in two of the three protomers, on average, preventing opening and, therefore, ligand binding (Figure 2c, *top inset*). Subsequently, the third PDZ-peptide binding event could be probed using molecules where, on average, a single NMR-visible unmodified protomer is coupled with a pair of invisible 'stapled' protomers in which a PDZ-peptide is covalently linked to the PDZ domain, keeping it in an open, bound conformation (Figure 2c, *bottom inset*). The factor of three difference in affinity for the first and third events highlights the positive cooperativity of this binding process (Figure 2c, *left*). This is established directly, through simple fits involving single ligand binding events in this case, in agreement with results from more traditional methods where titration curves derived from native molecules were analyzed with a model which includes cooperative association, with cooperativity assessed based on a goodness-of-fit criteria.

Figure 2



HtrA2 cooperativity probed by NMR spectroscopy. (a) Domain organization of a single HtrA2 chain (top) and corresponding crystal structure of the trimeric complex in the inactive conformation (bottom, PDB ID: 1LCY [19]), along with a cartoon representation to the right. The active site S306 residue is indicated in red; in studies illustrated schematically here a mutant is used so that substrate cannot be cleaved. **(b)** Schematic of a protomer mixing experiment producing asymmetrically-labeled HtrA2 trimers comprising NMR-invisible protomers (perdeuterated, grey-filled circles) and NMR-visible protomers (perdeuterated with $^{13}CH_3$-methyl groups, orange circles). **(c)** Experiments probing the cooperativity of sequential binding of a hydrophobic activator peptide (PDZ-peptide, red curved line) to the PDZ domain. The first binding event was studied with trimers comprising one NMR-visible unmodified (orange), and two NMR-invisible 'locked down' (binding incompetent; grey) protomers, on average (top inset). Similarly, the third binding event was probed using trimers containing one NMR-visible unmodified (orange), and two NMR-invisible 'stapled' (open) HtrA2 protomers (grey, bottom inset). Peaks in spectra recorded as a function of addition of PDZ-peptide can be quantified to obtain dissociation constants. U and B denote the unbound and bound states, respectively. **(d)** Mixing experiments can also be used to query cooperativity of the catalytically inactive to active transition upon addition of substrate (purple curved line). (i) Spectra from mixed HtrA2 trimers with one NMR-visible unmodified protomer ('Mixed', blue outline & contours) and two NMR-invisible 'locked down' protomers (grey) were compared with corresponding spectra recorded of an HtrA2 trimer consisting of only NMR-visible unmodified protomers in the open conformation ('All open', orange outline & contours). Only in the case where all protomers of the trimer were 'open' could an active state of the enzyme be formed, leading to chemical shift changes in spectra. (ii) The impact of the S276C disease associated mutation on HtrA2 activation was studied by recording spectra of trimers with one or more disease protomers. Spectra of a homogeneous S276C trimer (green outline & contours) and of a mixed protomer with one subunit containing the wild-type serine at position 276 (pink outline & contours), on average, indicate that an active conformation of HtrA2 cannot be formed upon addition of substrate. Figure was adapted from Ref. [18].

As described above, binding of the PDZ-peptide to the PDZ domain leads to an open protomer structure in which the active site is exposed. Is this open conformation catalytically active, independent of the conformations of the remaining protomers of the trimer? To answer this question, a hybrid HtrA2 trimer with NMR-visible unmodified and NMR-invisible, 'locked down' protomers was prepared, saturated with PDZ-peptide to expose the active sites in the subunits that are not 'locked down' (Figure 2d*i, 'Mixed'*, blue protomer is NMR-visible). A second set of trimers was also prepared where all three protomers were 'open' and NMR-visible (Figure 2d*i*, orange protomers). NMR studies revealed that binding of a second peptide that serves as a mimic for the region of the substrate that is cleaved (referred to as substrate) results in a cooperative conformational change to an active state for the homogeneous HtrA2 trimers, with concomitant large changes to NMR spectra. In contrast, trimers with one or more 'locked down' subunits were unable to transition to an active conformation (no changes in spectra), indicating that this transition must involve all three subunits simultaneously. Similar experiments were recorded on PDZ-peptide loaded HtrA2 trimers containing one or more protomers with a disease-associated mutation, S276C, which causes Parkinson symptoms in mice models. In this case, addition of excess substrate peptide could not trigger a catalytically active conformation (Figure 2d*ii*; green and pink protomers are NMR-visible), even though all the protomers are in the open conformation, because the S276C mutation eliminates the coupling between them.

Dynamic regions are indispensable for the function and regulation of biomolecular assemblies

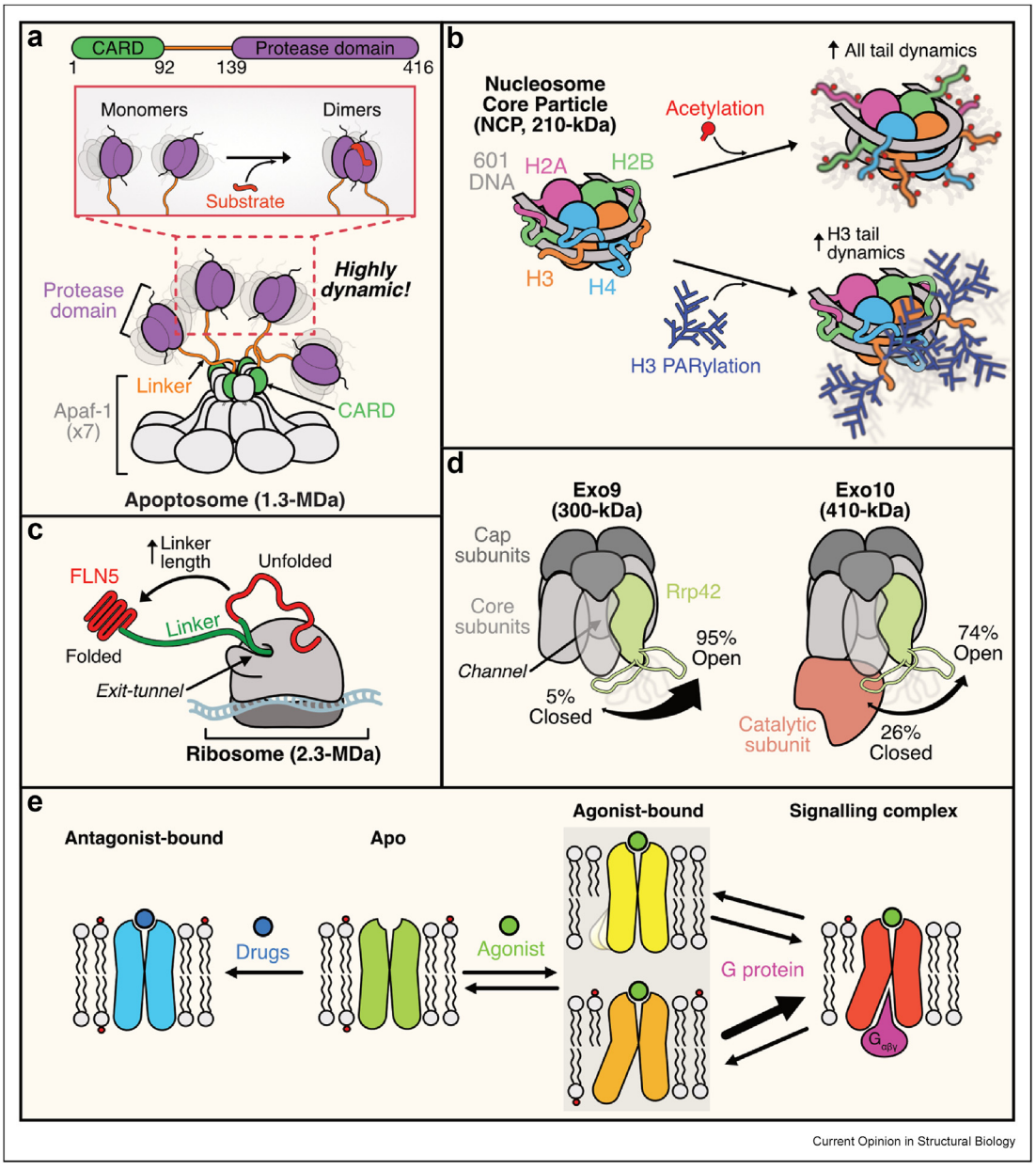
As mentioned in the Introduction, nearly 60 % of all human proteins contain folded domains connected by long flexible linkers [2]. When these folded domains are not fixed by interdomain contacts, structure determination of the complete complex by X-ray or cryo-EM methods becomes extremely difficult or impossible, as the flexible portion is rendered invisible to these techniques. A recent study by our laboratory highlights how NMR can be a powerful tool to study protein assemblies with this 'beads-on-a-string' architecture. Here, methyl-TROSY NMR was used to elucidate the interactions between protease domains of caspase-9 (Casp9) molecules (Figure 3a, top) in the 1.3 MDa apoptosome scaffold [21] (Figure 3a, *bottom*), a complex that is involved in the intrinsic apoptosis pathway. Casp9 consists of an N-terminal Caspase Activation and Recruitment Domain (CARD), which is responsible for interacting with CARD domains of the apoptosome, followed by a ~45 residue linker connecting the CARD and protease domains. Previous studies established that the protease domains must dimerize for catalytic activity, but it remained unclear as to whether dimerization occurs simply by the increase in effective concentration associated with localization of multiple domains to the surface of the apoptosome [22]. Methyl-TROSY NMR experiments indicate that Casp9 protease domains remain monomeric (inactive state) on the scaffold until substrate is added, promoting homodimerization (active state) (Figure 3a, *inset*) [21].

Solution NMR can alternatively be employed to study disordered regions of an otherwise structured complex. While static structures of macromolecular assemblies can provide insights into the folded regions of these complexes, such structures often lack information regarding functionally relevant disordered elements. Simple ^{15}N-directed experiments of backbone amides in the context of inherently flexible protein segments are powerful for characterizing conformational ensembles, even in complexes exceeding 100 kDa, as demonstrated in studies of the histone tails of the Nucleosome Core Particle (NCP) - the basic structural unit of chromatin. Each NCP is an octamer of four different histones - H2A, H2B, H3, and H4, around which ~150 base pairs of DNA is wrapped to form a 210 kDa protein:DNA complex (Figure 3b, *left*) [23]. Each histone has a positively-charged, disordered tail that interacts with the highly negative DNA and although these tails are unresolved in structural models their importance in the regulation of chromatin structure cannot be understated [24]. In one recent study, Sun et al. utilized paramagnetic relaxation enhancement (PRE)-tags attached to histone H3 to study the localization of ^{15}N-labeled histone H4 tails. By comparing experimentally determined PRE effects with distances derived from molecular dynamics simulations a conformational ensemble of the histone H4 tail could be elucidated, suggesting that these tails engage nucleosome DNA through fuzzy interactions [25].

Histone tails undergo frequent post-translational modifications which affect the surrounding chromatin structure and dictate function. Using ^{15}N-labeled nucleosomes our laboratory has studied how acetylation and PARylation perturb histone tail dynamics [26,27]. Lysine acetylation removes a positive charge, and ^{15}N spin-relaxation experiments performed on a series of NCPs where each histone tail is acetylated in turn, demonstrated an increase in picosecond-nanosecond timescale dynamics in each histone tail that is most simply explained as arising from a partial dissociation from the DNA (Figure 3b, *top right*) [26]. Alternatively, adding negatively charged poly ADP-ribose (PAR) chains to Ser10 and Ser28 on H3 enhanced the ps-ns dynamics of the H3 tails, contributing to increased accessibility of DNA repair enzymes (Figure 3b, *bottom right*) [27].

The ribosome is a 2.3 MDa protein:RNA complex responsible for the synthesis and folding of nascent polypeptide chains. ^{15}N NMR experiments, again

Figure 3



NMR studies probing structural dynamics in macromolecular assemblies. Cartoon representations of various dynamic complexes are shown. **(a)** Apoptosome activation of Casp9 during the apoptotic signalling cascade. Casp9 binding to the homoheptameric apoptosome scaffold (light grey, 7 Apaf1 copies) via CARD domain (green) interactions brings multiple protease domains (purple) into proximity – the domains remain dynamic due to a long, disordered linker connecting each to the scaffold (orange). Proximity-induced dimerization of protease domains, essential for Casp9 activity, only occurs when substrate is present (inset) [21]. **(b)** Positively charged histone tails interact with the negatively charged DNA in the nucleosome core particle. Post-translational modifications such as acetylation (top) and PARylation (bottom) decrease histone tail charges and weaken tail:DNA interactions [26,27], leading to an increase in ps-ns dynamics of the modified tails. **(c)** The nascent FLN5 polypeptide undergoes co-translational folding on the ribosome, with the ribosome surface (grey) promoting an unfolded state (red line). As more translation occurs, the linker (green) is extended and the FLN5 domain is further removed from the ribosome surface, allowing it to adopt a folded conformation [28]. **(d)** The RNA exosome degrades RNA substrates in a vectorial manner with substrates entering the hexameric core channel at the top (via the Cap) and degraded nucleotides exiting via the distal side. Loop dynamics help maintain the directional substrate – product flow via an open/close loop equilibrium at the base of the exosome that is modulated during the catalytic cycle [31]. **(e)** GPCRs are in an equilibrium between multiple conformational states. Binding of antagonists to the apo protein (green) locks it into an inactive closed conformation that is not competent for signalling (blue). Addition of an agonist causes the GPCR to populate (pre)active conformations (yellow, light orange). Notably, the membrane composition can have a significant influence on this equilibrium, with anionic lipids (red spheres) drastically enhancing the population of active state conformations (dark orange), thereby promoting interactions with G proteins (purple).

exploiting backbone amide probes, have provided insights into the mechanism of co-translational folding of polypeptide chains through studies of interactions between the highly dynamic nascent chain and the charged surface of the ribosome. Focusing on the immunoglobulin-like FLN5 filamin domain attached to a stalled *E. coli* ribosome by a variable length linker, and using a mutagenesis approach, residues in the unfolded conformation which strongly interact with the ribosome surface were identified by the Christodoulou group [28]. Mutation of these residues increased the extent of folding, as did lengthening of the linker, suggesting that nascent chain:ribosome interactions reduce the stability of the folded state (Figure 3c). Further studies using methyl-TROSY experiments have identified interactions between the folded FLN5 domain attached to the ribosome and the ribosome surface [29]. Relatedly, ^{19}F NMR studies of FLN5 using a single trifluoromethyl-L-phenylalanine probe established folding-intermediates unique to the ribosome bound domain, which could not be observed using NMR experiments focusing on other nuclei [30].

A preprint by Sprangers and co-workers further highlights the benefits of using multiple labeling-schemes in NMR studies of molecular machines and illustrates that studies need not be limited to homo-oligomeric particles. In this report a combination of ^{19}F and methyl-TROSY NMR was used to study the eukaryotic RNA exosome, an essential complex involved in ribonucleostasis through the degradation of RNA substrates [31]. The overall architecture of the ~400 kDa complex involves ten distinct subunits, with six of these assembling to form a core hexamer with a central channel, and three cap-subunits forming a substrate entrance pore on-top of the core hexamer (Figure 3d). This nonameric complex (Exo9) recruits a catalytic subunit which attaches to the bottom of the core hexamer (Exo10). While structures of eukaryotic RNA exosomes have been solved by X-ray and cryo-EM approaches, there are several disordered, functionally relevant regions which cannot be observed in the structures. One of these is a loop in a core subunit of the hexamer (Rrp42). By attaching a PRE-tag to this loop and measuring changes in peak intensities in methyl TROSY spectra (related to the inverse 6th power of the distance between the unpaired electron in the PRE-tag and the methyl protons in question) this group determined that the loop is localized to the bottom of the hexameric core. ^{19}F spin relaxation experiments further established that the Rrp42 loop interconverts between open and closed positions. This equilibrium plays a role in controlling entry and release of RNA substrates and products, respectively, as well as the direction by which RNA enters the complex, as closure of the loop prevents substrates from entering the machine from the bottom of the barrel-like structure. For example, although in the absence of the catalytic subunit the loop is 95 % open (Exo9, Figure 3d), when it is bound the extent of loop closure increases five-fold, biasing substrate entry to the top of the barrel (Exo10, Figure 3d). Finally, when RNA is in the exosome channel, the loop switches to a completely open conformation facilitating release of the degraded ribonucleotides.

G protein-coupled receptor conformational equilibria studied in membrane-like environments

G protein-coupled receptors (GPCRs) are a large class of membrane proteins with a prominent role in human health and disease. In general, GPCRs are composed of 7 transmembrane helices, connected by 3 extra- and 3 intracellular loops. GPCRs sense a vast array of environmental stimuli through, for example, the binding of extracellular ligands, with the accompanying structural rearrangements leading to their activation. The signal is propagated in the cell by the binding of heterotrimeric G proteins to the activated receptor (Figure 3e), with outcomes that depend on the activated subfamily of G proteins. GPCRs are highly dynamic, constantly interconverting between multiple conformations, and the exquisite sensitivity of NMR spectroscopy to molecular dynamics spanning a range of over ten orders of magnitude makes it a valuable tool to gain insights into structure-dynamics-function relationships in this important class of biomolecules. The first step in any NMR study involves spectral assignment, whereby each peak is 'assigned' to a specific site in the molecule from where it originates. Canonical assignment strategies are often difficult to apply to GPCRs, but a recent method using GPCR-bound nanobodies containing metal ions that shift peaks in GPCR spectra in quantifiable ways provides an exciting strategy to overcome the assignment problem [32].

The surrounding membrane can drastically affect the structural dynamics of GPCRs and the impact of different membrane compositions on GPCR conformational equilibria can be studied in detail using solution NMR. In an ^{19}F NMR study observing the GPCR directly, Prosser and coworkers found that an increase in membrane cholesterol led to an elevated active state population of the receptor [33]. Subsequently, by using a fluorinated cholesterol derivative, this group was able to demonstrate that the shift in equilibrium was not due to a direct interaction between the cholesterol and the receptor, but, rather, arises solely from physical changes of the membrane itself. A similar ^{19}F study in which the amount of anionic phospholipid in the membrane was titrated, established that increasing the anionic lipid concentration led to higher populations of the active state of the human A_{2A} adenosine receptor (A2AR) [34]. As NMR studies frequently use reconstituted lipid membranes, it is paramount that these accurately mimic properties of a true biological system. Recent studies by the Wüthrich group emphasize the importance of selecting the proper membrane environment for *in-vitro* studies of GPCRs [35].

GPCR signal transmission arises largely from interactions with specific G protein sub-families. Given the similarity between different families, how do these G proteins selectively-bind the correct GPCR? Using a combination of $^{13}CH_3$-methionine and ^{19}F-labeling, Jones et al. found that the β_1 adrenergic receptor G protein subtype selectivity was not due to structural features of the formed ternary complexes involving G proteins from different subtypes, as these were all similar [36]. Rather, they hypothesized that favorable interactions of the active state of an agonist-bound GPCR with its canonical G protein subfamily enhances the rate of formation of the preferred ternary complex, with G proteins from other subfamilies outcompeted during complex formation. Remarkably, NMR studies of a ternary complex comprising the A2AR, a heterotrimeric G protein, and an agonist, showed extensive dynamics in the GPCR core, suggesting a possible mechanism for an allosteric connection linking the agonist and the G protein, components that are bound to opposite sides of the GPCR [37]. Modulating the formation of these ternary complexes is an important aspect in the design of drugs. In this regard, Baumann et al. [38] have characterized methyl-group dynamics in the α_{1B}-adrenergic receptor in the presence of both orthosteric and allosteric inverse agonists. While the dynamics are similar on a global scale for all tested agonists, there were some distinct changes observed close to a motif that regulates GPCR helix orientation when an allosteric inverse agonist was present, implying differences between allosteric and orthosteric GPCR inactivation.

Insights into GPCR:G protein interactions, focusing on the G protein, can also be obtained from NMR studies. By using ^{19}F labeling on selected sites of the Gα subunit of the human stimulatory G protein, Prosser and coworkers were able to observe different states of the molecule [39]. Two distinct G protein states were reported by all probed sites, corresponding to the two-domain structure of the G protein in either closed or open conformations. Although the closed conformation is preferred in the nucleotide-bound form, the open/closed equilibrium can be biased towards the open state in the presence of a GPCR (A2AR), other G protein subunits (G$\beta\gamma$), or by proximity to membranes or membrane-like environments.

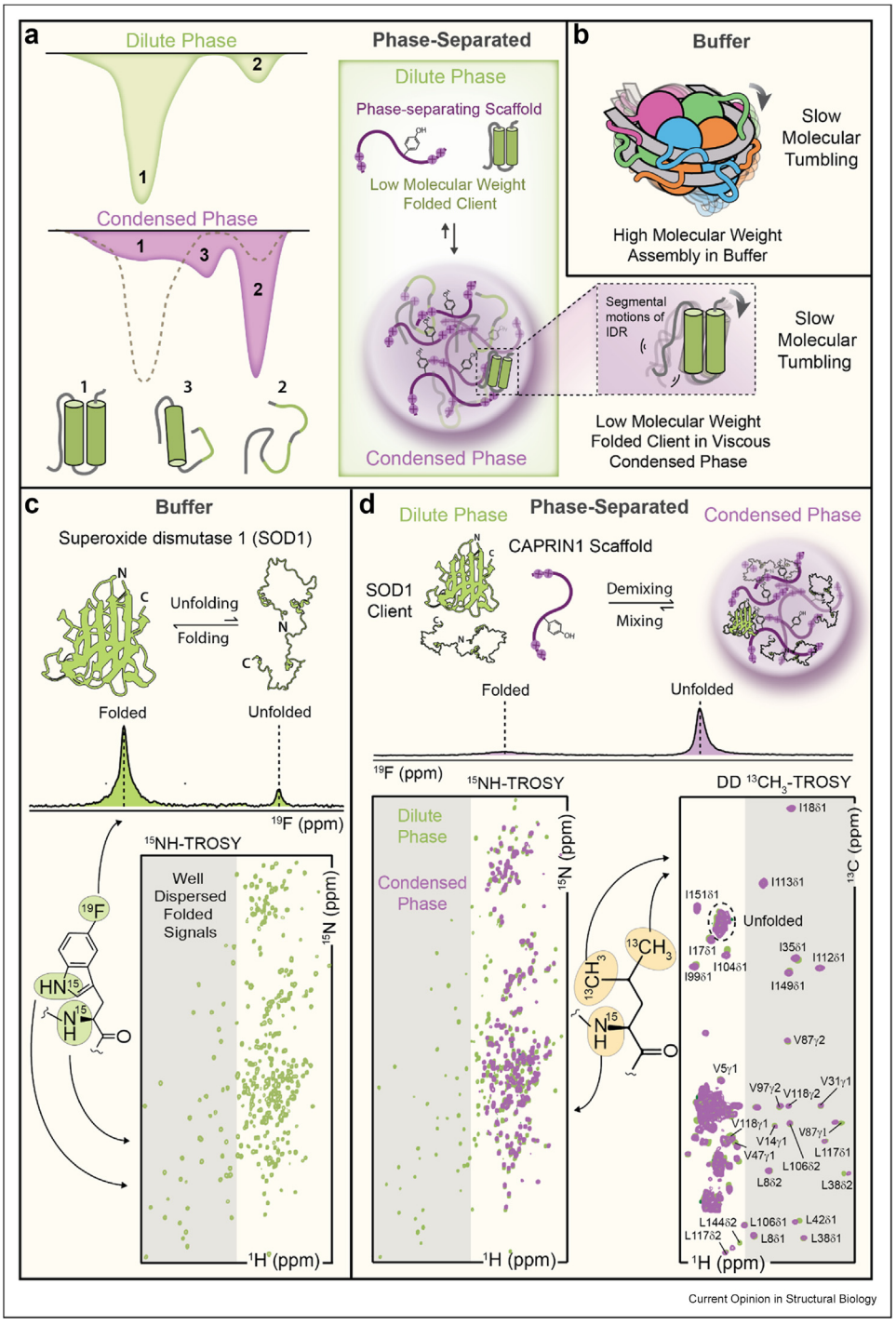
Approaches developed for NMR studies of molecular machines can be exploited in atomic resolution studies of biomolecular condensates

It is estimated that at least 18 % of the human proteome is organized into non-membrane-bound compartments called condensates [40], with 80 % of the proteome components residing in condensates during some stage of their life cycles [41]. Condensates typically contain hundreds of molecules, including "scaffolds" that are essential to their structural integrity and "clients" that partition into them but are not necessary for their formation. The concentrations of scaffold proteins inside condensed phases can be extremely high (>400 g/L) [42,43], with these proteins contributing, therefore, significantly to the solvation of clients, as does water. These unique condensate environments can remodel the free energy landscape of client proteins, enabling access to otherwise unattainable conformations with, potentially, distinct, or at least modulated, functions (Figure 4a, *left*). Characterization of client structural dynamics in condensates is, therefore, important for understanding how molecular function is regulated in these complex biomolecule-enriched environments.

Atomistic studies of condensate components are challenging, however. In the context of phase separation, the dynamic interactions between participating molecules and the requirement for studies at physiological temperatures and under conditions that do not support crystalline samples or sample freezing severely hinder applications involving cryo-EM and X-ray crystallography. In contrast, solution NMR offers a unique opportunity to probe dynamic interactions over a broad spectrum of timescales in such liquid-like environments. However, the high viscosity of the resulting condensed phase samples [42,43] challenges applications, as even folded proteins of less than 100 amino acids tumble as slowly as molecular machines in a buffer solution (Figure 4a, *right*, Figure 4b). In studies of IDRs or intrinsically disordered proteins (IDPs) signal intensities can be recovered via local segmental motions that compensate for the reduction in global tumbling rate (Figure 4a, *right*). Accordingly, amide-based solution NMR has been the dominant approach for providing detailed descriptions of the IDP/IDR components in condensates, as reviewed previously [44]. In contrast, corresponding motions are absent in folded domains, precluding the observation of structured proteins in viscous condensed phases using $^{15}N-^{1}H$ correlation spectroscopy.

Notably, the approaches developed for the characterization of high-molecular-weight proteins in buffer (as described in previous sections) are of utility for studies of folded domains in condensates. Indeed, ^{19}F NMR has been successfully exploited to study client proteins in condensed phases. ^{19}F has a natural abundance of 100 % and is, in general, not found naturally in biomolecules. Additionally, it can be site-specifically incorporated into the sidechains of client proteins, while highly concentrated scaffold proteins, lacking ^{19}F, are rendered NMR-invisible, effectively eliminating the dynamic range issue that results from the concentration discrepancy between client (low) and scaffold (high). As the ^{19}F chemical shift is exquisitely sensitive to the local

Figure 4



NMR applications focused on atomic resolution studies of folded protein conformational changes. (a) Hypothetical free energy landscapes for a client protein in condensed (purple) *vs.* dilute (green) phases of a phase-separated scaffold system. The solvation of a client by scaffolding proteins in the condensed phase can alter the populations of thermally accessible states already present in the dilute phase (states 1 and 2) but also enable access to previously inaccessible conformations (state 3). **(b)** Schematic indicating that the tumbling of a low molecular-weight folded client protein in a viscous condensed phase can be similar to that of a high molecular-weight assembly in buffer (in this case the NCP), presenting similar challenges for NMR applications in both cases. Unlike disordered protein regions, which exhibit local segmental motions that can compensate for the reduction in global tumbling, corresponding motions are absent in the folded domains. **(c)**–**(d)** Solution NMR experiments are ideally suited to probe protein structural dynamics in buffer **(c)** and condensed phases of phase-separated systems **(d)**. Specifically, ^{19}F NMR is useful for gaining global insights into the populations of various conformers, while ^{15}NH-TROSY and DD $^{13}CH_3$-TROSY experiments provide atomic-resolution information on the interactions and dynamics of the disordered/unfolded and folded protein segments, respectively. Spectra were adapted from Ref. [45].

environment, resolution is often sufficient in spectra recorded in a one-dimensional fashion, minimizing signal decay that normally occurs during magnetization transfer steps required for multidimensional NMR experiments. For example, Figure 4c, *top* shows a ^{19}F spectrum of a buffer sample of the protein superoxide dismutase 1 (SOD1) where a single ^{19}F-Trp probe has replaced the endogenous Trp in the molecule [45]. A pair of peaks results, one from each of the folded and unfolded conformers, from which the folding equilibrium can be quantified. A high quality ^{15}N−^{1}H correlation map can also be recorded of an ^{15}N-labeled sample under these conditions, with many more probes available (Figure 4c, *bottom*). By recording the corresponding ^{19}F spectrum in a condensed phase comprised of a CAPRIN1 (stress granule protein) scaffold it is readily shown that the equilibrium is shifted to the unfolded state [45] (Figure 4d, *top*). Using a similar approach the folding−unfolding equilibrium of the N-terminal SH3 domain of the *Drosophila* adapter protein Drk was shown to unfold in a condensed phase environment comprised of the germ-cell specific protein DDX4 [46].

^{19}F probes, however, are disadvantaged by the fact that only a few sites can be labeled. By recording DD methyl-TROSY based experiments with modifications that efficiently suppress signals from unlabeled scaffold molecules in condensed phases [8] it has become possible to study the structural dynamics of clients in the condensed phase via methyl group reporters. DD methyl-TROSY spectra of SOD1 in CAPRIN1 condensates establish little change to the conformation of folded SOD1, as spectra in dilute and condensed phases are nearly superimposable [45] (Figure 4d, *bottom right*). Yet, consistent with the ^{19}F data, a comparison of peak intensities indicates a dramatic shift in the equilibrium to the unfolded state. Notably, under these conditions, only signals from the flexible disordered and unfolded regions of SOD1 could be observed in TROSY-based ^{15}N−^{1}H spectra (Figure 4d, *bottom left*). DD HMQC-based studies of RNA duplexes dissolved in CAPRIN1 condensates show that base pairing is destabilized in this condensate environment, and that the on-rate for the hybridization reaction is slowed relative to buffer solution by 10-fold more than what is expected based on viscosity effects, with a concomitant increase in the melting rate of 15-fold [47]. A mechanism based on the solvent nature of CAPRIN1 can explain these changes in rates.

In summary, NMR studies are helping to define the conformational landscapes of the protein and RNA components of condensates, providing atomic level details that are not available through other methods. While the focus here has been on studies of folded protein clients, solution NMR has also been the method of choice for atomistic studies of proteinaceous, unfolded scaffolds as well [8,46−52].

Future perspectives

Solution NMR spectroscopy is an invaluable tool for atomic-resolution characterization of the mechanistic aspects of biomolecular function. Advances in NMR spectroscopy, such as the development of spectrometers operating at higher magnetic fields, the emergence of more sensitive experiments and more robust data analysis tools, including AI-based approaches, will enable studies of bigger and more complex systems. Concurrent improvements in the production of isotopically-labeled protein systems, such as provided through novel mammalian expression hosts, are further expanding the scope of NMR studies. Notably, efforts are being made to increase magnetic field strengths from the current commercially available 28 T (corresponding to 1.2 GHz ^{1}H frequency), offering the exciting possibility of improving resolution in spectra of biomolecules still further, especially in studies involving IDPs or other classes of molecules where overlap is limiting. Although, in principle, spectral signal-to-noise (S/N) ratios scale with the magnetic field strength to the power of 1.5, in practice there are other mitigating factors that could limit the impact of ultra-high fields on sensitivity. However, approaches such as Dynamic Nuclear Polarization (DNP) offer a promising solution for boosting S/N. DNP takes advantage of the much larger magnetic moment of the electron (650 times that of the proton) to transfer a high degree of polarization from electrons to nuclear spins in the biomolecule of interest. Significant gains have already been demonstrated in solid state bio-NMR applications at low temperatures [53]. More recently, the development of dissolution DNP, involving the transfer of 'signal' from highly polarized water to biomolecules at ambient temperatures [54], opens exciting possibilities for reducing the sensitivity bottleneck associated with the sorts of studies involving large biomolecular systems outlined in this review.

Declaration of competing interest

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Data availability

No data was used for the research described in the article.

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