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Review Article

New applications of solid-state NMR in structural biology

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Various recent developments in solid-state nuclear magnetic resonance (ssNMR) spectroscopy have enabled an array of new insights regarding the structure, dynamics, and interactions of biomolecules. In the ever more integrated world of structural biology, ssNMR studies provide structural and dynamic information that is complementary to the data accessible by other means. ssNMR enables the study of samples lacking a crystalline lattice, featuring static as well as dynamic disorder, and does so independent of higher-order symmetry. The present study surveys recent applications of biomolecular ssNMR and examines how this technique is increasingly integrated with other structural biology techniques, such as (cryo) electron microscopy, solution-state NMR, and X-ray crystallography. Traditional ssNMR targets include lipid bilayer membranes and membrane proteins in a lipid bilayer environment. Another classic application has been in the area of protein misfolding and aggregation disorders, where ssNMR has provided essential structural data on oligomers and amyloid fibril aggregates. More recently, the application of ssNMR has expanded to a growing array of biological assemblies, ranging from non-amyloid protein aggregates, protein–protein complexes, viral capsids, and many others. Across these areas, multidimensional magic angle spinning (MAS) ssNMR has, in the last decade, revealed three-dimensional structures, including many that had been inaccessible by other structural biology techniques. Equally important insights in structural and molecular biology derive from the ability of MAS ssNMR to probe information beyond comprehensive protein structures, such as dynamics, solvent exposure, protein–protein interfaces, and substrate–enzyme interactions.

Introduction

Solid-state nuclear magnetic resonance (ssNMR) comprises several experimental approaches, enabled by specialized hardware, that facilitate the application of NMR experiments to various kinds of solid, semi-solid or (partly) immobilized samples. Traditional uses of NMR in structural biology have focused on the study of proteins (and other biomolecules) in solution. Solution NMR methods excel at probing the structure, dynamics, and interactions of *soluble* protein monomers and multimers. They are dependent on the fast tumbling of molecules to suppress or reduce anisotropic and inter-atomic NMR interactions, to get narrow spectral lines and enable multidimensional spectroscopy. Dedicated experiments are then used to measure weak residual interactions that encode inter-atomic distances and orientational constraints used for structure determination. The requirement for rapid tumbling places an upper limit on the size of target proteins. Larger proteins, vesicle-bound proteins, oligomeric complexes, large assemblies, and aggregates tumble too sluggishly or lack the tumbling. It is these samples, whose short-lived coherence lifetimes prohibit in-depth characterization by solution NMR, that are targeted by modern biomolecular ssNMR.

One classic approach for NMR studies of such biological 'solids' involves the alignment of the sample to enable unique orientation-dependent structural and dynamic ssNMR measurements (reviewed in [1,2]). However, most recent progress is in the area of magic angle spinning (MAS)

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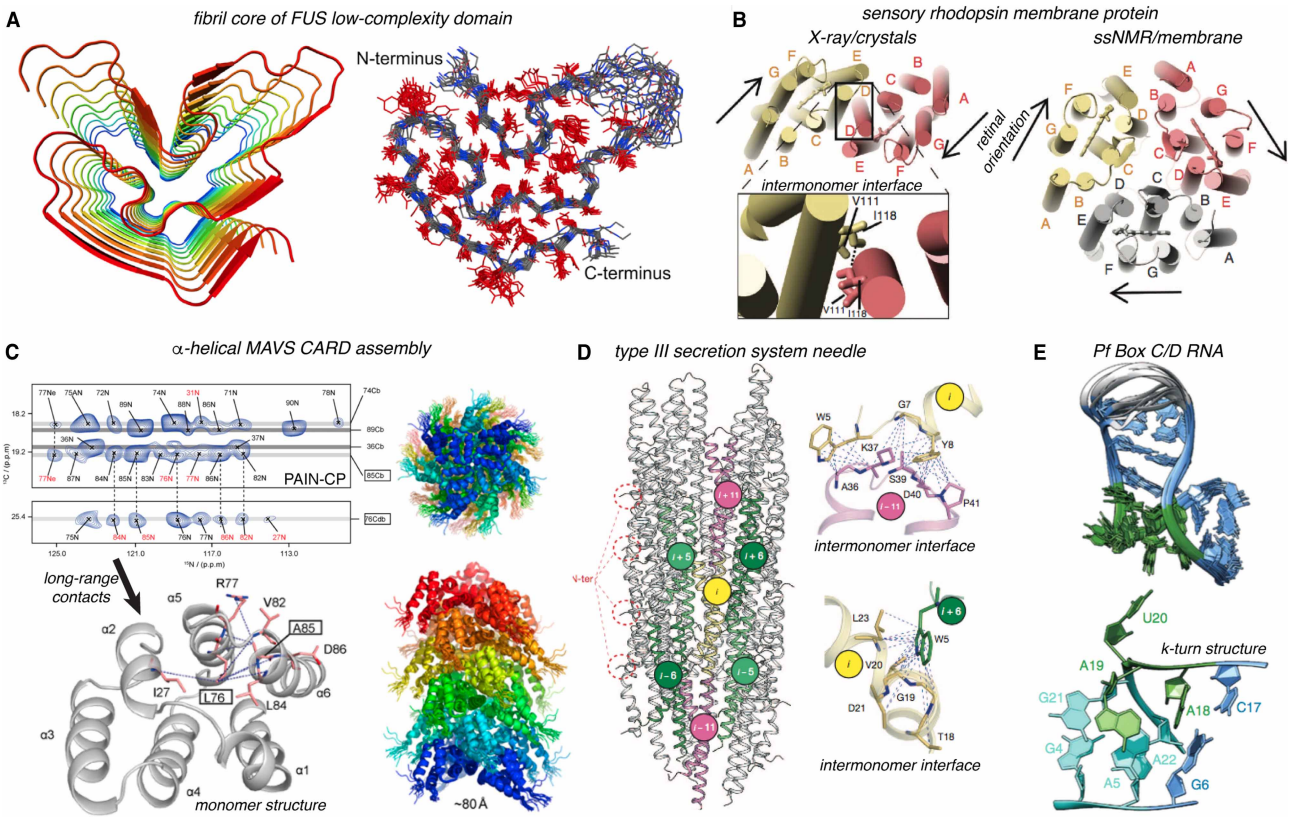

Figure 1. Selected ssNMR structures for diverse biomolecules.

(**A**) Amyloid-like fibrils formed by the FUS protein. Reprinted from ref. [102] with permission from Elsevier. (**B**) Membrane protein *Anabaena* sensory rhodopsin, comparing the 3D crystal structure (left) to the ssNMR-determined conformation in lipid membrane (right). Reprinted by permission from Macmillan Publishers Ltd: ref. [103], copyright 2013. (**C**) α-helical assemblies of the N-terminal caspase recruitment domain (CARD) of the MAVS protein, showing the assembly (right) and the monomeric structure (left), with long-range distance constraints indicated; reprinted with permission from ref. [104]. (**D**) Atomic model of the α-helical type III secretion system needle. Reprinted by permission from Macmillan Publishers Ltd: ref. [105], copyright 2012. (**E**) The 26-mer Box C/D RNA from *Pyrococcus furiosus*, reprinted from ref. [106].

ssNMR, which will be the focus of this article. In MAS NMR, samples are submitted to very rapid uniaxial rotation at an angle of 54.7° (i.e. the 'magic angle') relative to the imposed magnetic field. The whole-sample rotation mimics the isotropic tumbling of molecules in solution, thus suppressing the inter-nucleus and orientation-dependent NMR interactions that otherwise excessively broaden the ssNMR spectra. The MAS ssNMR approach is applicable to many interesting and important biomolecular samples: nano- and microcrystalline proteins and peptides, amyloid-like fibrils, non-amyloid aggregates, membrane-bound polypeptides, and various other biological assemblies (e.g. Figure 1). It is important to note that these samples are commonly not 'dry solids', but are rather studied in a fully hydrated state [3]. Moreover, our samples often contain components that are semi-solid or undergoing extensive dynamics, such as flexible protein segments or fluid lipid bilayers [4–7].

These types of samples may also be studied by other biophysical or structural methods, but ssNMR contributes many unique capabilities. It is amenable to carefully crystallized samples, but can similarly be applied to insoluble or immobilized samples that lack the supramolecular crystalline order required for X-ray crystallography or micro-electron diffraction [8]. ssNMR also has a unique ability to probe dynamics on the global and local level, and do so across a range of timescales and sample temperatures and conditions. For sufficiently rigid molecules, ssNMR provides structural information on the Å (even to sub-Å) scale, in the form of distance and geometric (angular) constraints [9,10]. Static and MAS ssNMR also provide orientational information in the form of anisotropic dynamics and (tilt) angles of proteins or peptides relative to aligned as well as non-oriented lipid membranes [11–14], reviewed in [1,2,6].

Expanding horizons in biomolecular ssNMR

The last decade has seen many critical developments that make ssNMR more powerful and more practical as a tool of structural biology, since it was originally shown capable of producing 3D structures of proteins and peptides in the early 2000s [15,16]. This is reflected in the fact that over 85% of the ssNMR structures currently in the protein structure databank were deposited in the last 10 years, with a few examples shown in Figure 1. This section briefly reviews some of the ssNMR improvements that make this possible. On the one hand, new ssNMR instrumentation developments have significantly enhanced the effective sensitivity and productivity of biomolecular ssNMR. This is due, in part, to increased access to high-field NMR instruments that increase resolution and sensitivity (reviewed in [17]). Other critical improvements affect the ssNMR probes that feature the coil used for the detection and application of radio frequency (RF) pulses, and the 'stator' assembly that enables MAS sample rotation. Notable modifications in coil designs enhance the performance of advanced ssNMR experiments (due to improvements in, for example, RF homogeneity), while also preventing RF-induced (over)heating of hydrated samples [18–20], as recently reviewed [21]. Developments in MAS hardware design and engineering enable ever-higher MAS rates using ever-smaller sample (rotor) sizes: increasing MAS rates that now exceed 100 kHz facilitate the use of ^{1}H detection on sub-mg protein samples, reflecting an enhanced sensitivity over traditional ^{13}C detection methods [22,23]. Depending on the experimental goals [22–24], ^{1}H detection at such MAS rates may allow one to avoid or reduce the need for ^{13}C and/or ^{2}H-labeling. This can be useful in cases where the latter may be expensive or difficult, such as for hard-to-express proteins or proteins obtained from, for example, mammalian cells. Access to new MAS rate regimes (and higher field strengths) is also enabling types of experiments that were not possible with more traditional ssNMR hardware. This includes measurements of dynamics, where the more effective averaging of inter-atomic interactions by faster MAS makes it easier to measure some relaxation parameters that otherwise may require non-uniform sample labeling strategies [25,26]. New types of pulse sequences continue to extend the toolkit of ssNMR experiments, in part, to take advantage of higher fields and faster MAS rates. These experiments cannot be adequately reviewed here, but they include improved approaches for residue-specific assignments, long-range distance measurements, order parameters, chemical shift tensors as well as angular constraint measurements [22,26–30].

The last decade has seen a dramatic expansion of the use of dynamic nuclear polarization (DNP) to enhance biological ssNMR. DNP uses the inherently much higher polarization of electron spins to boost the sensitivity of ssNMR by orders of magnitude (see recent review [31]). DNP enhancement has enabled remarkable measurements of structure and interactions under dilute sample conditions that are unsuitable for traditional ssNMR experiments, as illustrated with select examples in Figure 2 [32–34], and the abovementioned review [31]. Current DNP methods typically require cryogenic sample temperatures (usually 90–100 K) similar to those used in cryo-EM and X-ray crystallography. At these low temperatures, which are well below the protein glass transition [35], motions that commonly characterize functional biomolecules are largely frozen out. This includes both solvent-coupled dynamics and certain motions inherent to the polypeptide side chains. Therefore, ssNMR studies of functionally relevant dynamics are mostly done without DNP enhancement. Whenever cryogenic conditions trap dynamic molecules in a range of distinct structural states, this results in increased line broadening beyond that seen at higher temperatures [35–39]. Recent work has shown that improved linewidths under cryogenic temperatures can be achieved for suitably ordered biological samples studied by high-field DNP instrumentation [39]. Efforts are underway to enable DNP at temperatures above 100 K, by exploring optimization of polarizing agents and other experimental and sample conditions [36,40,41]. In summary, modern DNP-enhanced ssNMR is an increasingly powerful complement to more conventional ssNMR approaches.

Years of trial and error across the global ssNMR community have broadened our understanding of what types of biological samples are suitable for, or amenable to, ssNMR. Like in solution NMR, the judicious application of tailored labeling strategies is essential. Completely uniform isotopic labeling with both ^{13}C and ^{15}N continuous to be common, at times complemented with deuteration for ^{1}H-detected experiments [23,24]. At the same time, uniform ^{13}C labeling is sometimes avoided to facilitate the detection of longer distance interactions that may otherwise be masked by dipolar truncation phenomena [42] or to suppress undesired interactions that otherwise compromise dynamics measurements [25]. A recurring strategy is to selectively probe intermolecular interfaces or interactions in supramolecular assemblies by mixing differently labeled proteins or peptides, with examples in Figure 2 and references [43,44]. A notable labeling strategy that is likely to expand in the future is the increasing use of 'segmental labeling' where differently labeled sections of larger proteins are ligated together [45–47], as recently reviewed [48].

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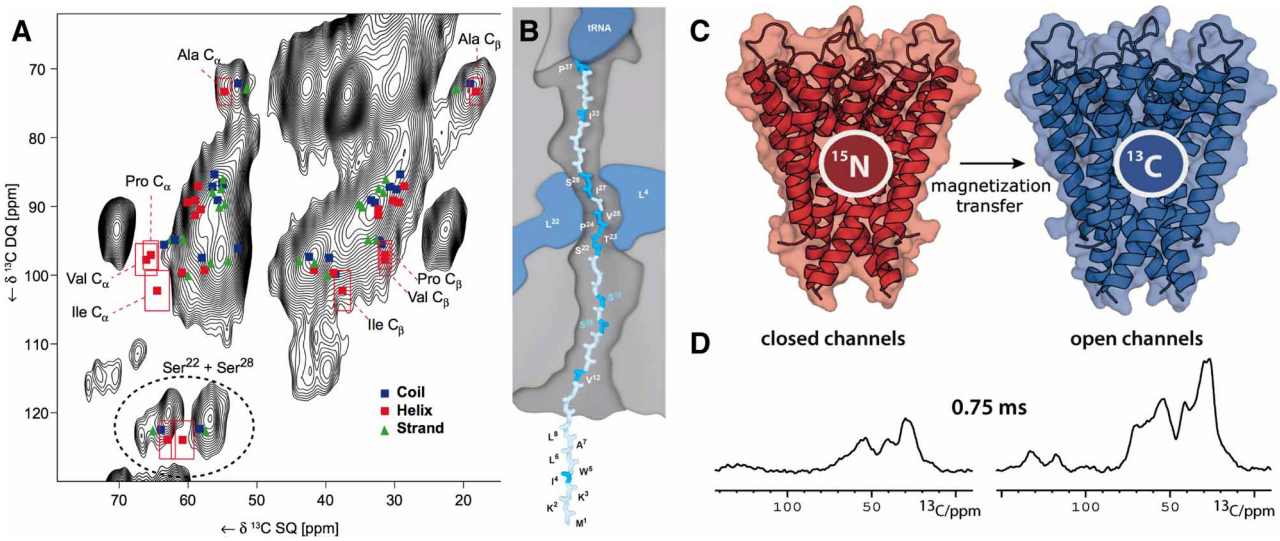

Figure 2. Examples of DNP-enhanced ssNMR studies.

(**A**) 2D ssNMR spectrum of (labeled) signal peptide in (unlabeled) ribosome, designed to probe the presence or absence of secondary structure in the labeled peptide, illustrated in (**B**). Reprinted from ref. [32]. (**C** and **D**) Long-range polarization transfer between ^{15}N- and ^{13}C-labeled ion channels (top) in membranes can be detected by DNP/ssNMR, which finds that protein clustering depends on the functional state of the protein (bottom). Reprinted from ref. [33].

Until recently, most ssNMR studies focused on large insoluble biological assemblies such as liposome-bound proteins, amyloid fibrils, or microcrystalline proteins and peptides. However, it has been shown that even moderately sized biomolecular complexes, which would generally be considered 'soluble', are productively studied by ssNMR. This stems from the realization that such complexes (or large proteins) can be sedimented under ultracentrifugal conditions, thus immobilizing them for MAS ssNMR characterization [49]. The approach of preparing ssNMR samples via 'sedimentation' of the studied macromolecules or macromolecular assemblies by ultracentrifugation is now widely used for all sorts of samples [3,50], including oligomeric chaperones, vesicle-bound proteins and 'soluble' oligomers formed by amyloidogenic polypeptides [4,51–54]. Another development worth noting is that MAS NMR studies of whole cells, cellular membranes, or cell extracts have been shown to be feasible, providing a unique and effective approach to examine proteins in their native milieu [55–57]. Interestingly, parameters that improve biological relevance (such as proper sample hydration) have, in some cases, been found to also lead to improvements in ssNMR spectral quality [3].

Recent protein and peptide structures obtained by ssNMR

Figure 1 illustrates a sampling of the range of biological assemblies for which ssNMR has recently yielded atomic-resolution 3D structures. As recently reviewed [58], ssNMR structures of amyloid-like fibrils associated with neurodegenerative diseases have identified common structural features, which may have implications for our understanding of the disease-causing misfolding processes. ssNMR also revealed the structures of various membrane-associated proteins [59], including those of the M2 channel of influenza A and sensory rhodopsin (Figure 1B) [60–64]. However, recent ssNMR structures go well beyond these traditional types of protein samples with an expanding repertoire of biologically functional protein assemblies, such as viral capsid proteins, motor proteins, α-helical protein assemblies (Figure 1C,D) and functional amyloids [17,65,66].

Beyond protein structures: dynamics, interactions and other insights

Crucially, ssNMR studies contribute much more than simply protein structures. Some of the most interesting applications of ssNMR focus on the detection and characterization of intermolecular interactions and interfaces. These can be interfaces between proteins in membranes (Figure 2), in amyloid fibrils or viral capsids, but can also involve 'asymmetric' interactions between proteins and other biomolecules. A classic example is the

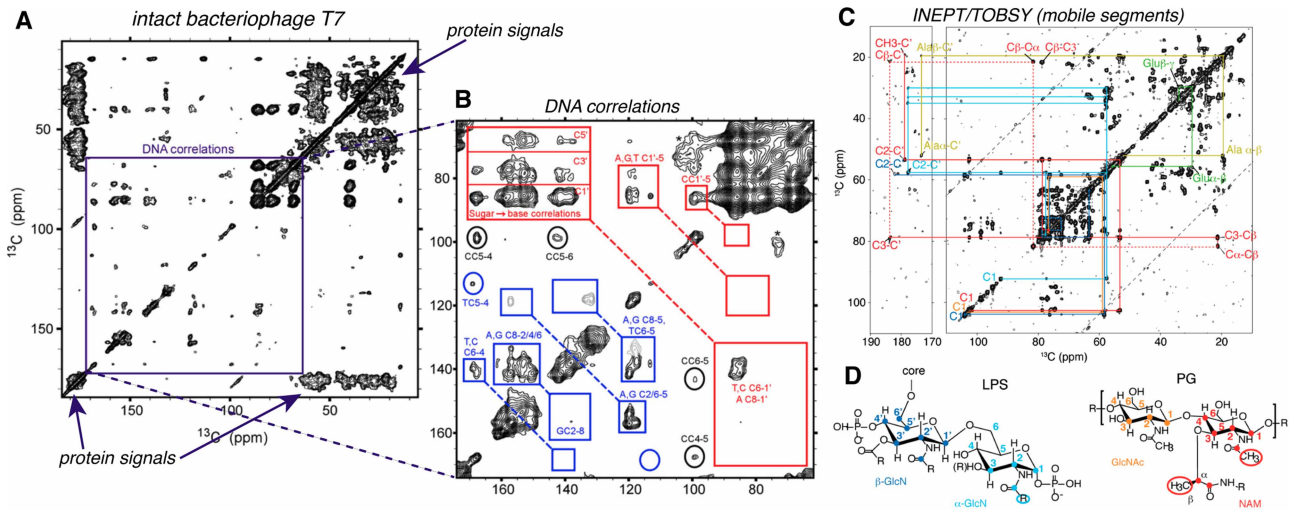

Figure 3. Distinct ssNMR spectral signatures of non-polypeptide biomolecules.
(**A**) 2D ^{13}C-^{13}C CP/DARR spectrum of labeled bacteriophage T7, showing both protein and DNA signals, with the latter enlarged in (**B**). Reprinted with permission from ref. [107]. (**C**) 2D spectrum of mobile peptidoglycan (PG) and lipopolysaccharides (LPS) acquired with INEPT/TOBSY ssNMR spectroscopy; reprinted from ref. [55] with permission from Elsevier.

case of protein–lipid interactions [6], but recent studies also show the potential of probing protein–DNA/RNA interactions (Figure 3A), substrates with receptors, amyloid-specific dyes with protein aggregates, aggregation inhibitors with oligomeric Aβ and many other examples [67–72]. One key factor in these studies is that polypeptides can be easily distinguished from the other (bio)molecules simply due to their characteristic chemical shift frequencies. This is exemplified in Figure 3, showing the well-separated ^{13}C signals of proteins and DNA or sugars. Moreover, phospholipids, DNA, and RNA contain phosphorus that is inherently NMR-active but absent from unmodified polypeptides. Even in cases where there is no innate chemical difference (e.g. for peptide-based substrates), the targeted introduction of isotopic labels in one or both binding partners can be very powerful [73]. As illustrated in Figure 2, these types of interface-mapping experiments can be ideal targets for DNP enhancement under low-temperature conditions [73–75].

ssNMR can be applied quite effectively on samples featuring static and dynamic disorder that may be problematic for other structural techniques (Figure 4). For instance, our ssNMR studies [7,10] shown in Figure 4A–D provided important structural insights into polyglutamine-containing protein aggregates that in recent cryo-EM studies were found to be too heterogeneous to allow detailed structural analysis [76,77]. Dynamics measurements by ssNMR yield exciting new insights into the dynamics in protein crystals [78,79], as well as membrane-associated proteins [4,80–82], by revealing rates, range, and directions of dynamics via NMR relaxation and order parameters. At the same time, one can make use of the sensitivity of ssNMR to dynamics to implement dynamic filtering experiments. This approach enables the measurement of simplified spectra featuring only parts of proteins with a certain type of motion. For instance, cross-polarization (CP) spectra (e.g. Figures 3A and 4C) are devoid of peaks from highly dynamic flexible residues, while solution-NMR-like INEPT spectra *only* show those residues that are highly flexible (Figures 3C and 4D). More sophisticated relaxation-filtered experiments can also be used to map solvent- and membrane-facing protein interfaces [83,84].

Integration with other methods in structural biology

The application of structural biology to the most important biological questions increasingly requires an integration of techniques that provide different perspectives on the problem at hand. In the case of ssNMR, structural measurements can provide highly localized atomic-resolution information, which is both the strength and weakness of the method. The Å-scale resolution of such measurements enables powerful structural constraints, but their sub-nm 'reach' also makes it hard to detect longer range contacts and supramolecular features such as the twisting of amyloid fibrils or the curvature of viral capsids [85,86]. As a consequence, ssNMR studies are

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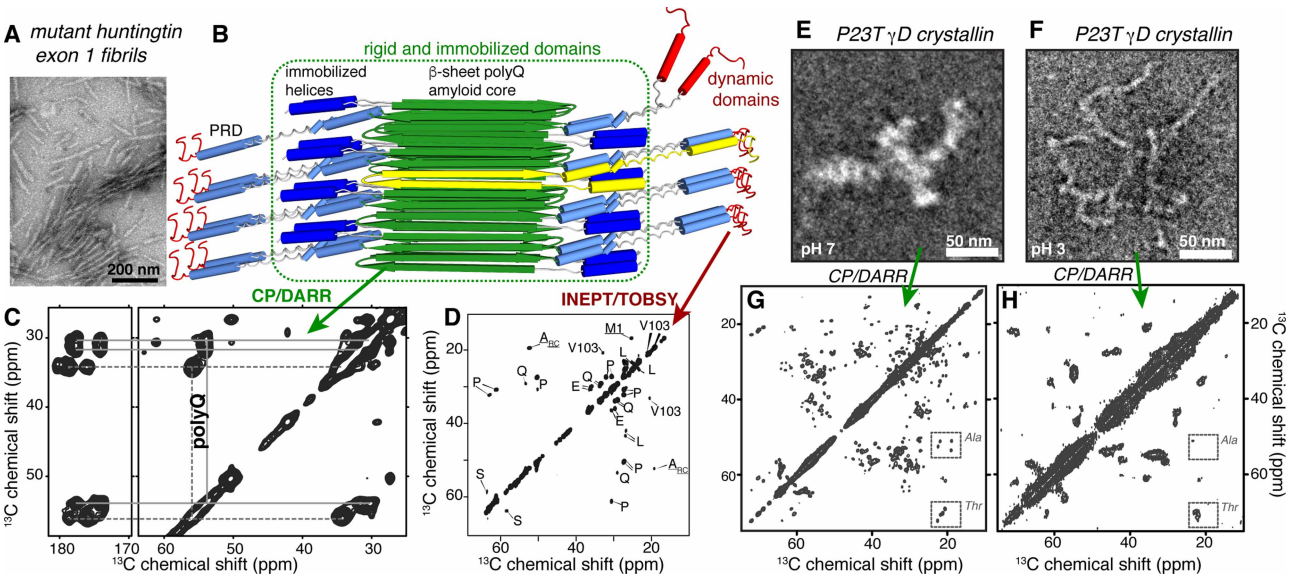

Figure 4. Samples with static and dynamic disorder.

(**A**) Negative-stain TEM of amyloid-like fibrils formed by mutant huntingtin exon 1. (**B**) SSNMR-based structural model, illustrating the rigid core (green) featuring the polyQ domain and the dynamic flanking regions. (**C**) Rigid core and immobilized parts are selectively detected in CP/DARR-based 2D spectra, while (**D**) flexible segments are detected in INEPT/TOBSY spectra. (**E** and **F**) Negative-stain TEM of aggregates formed by mutant P23T γD crystallin at pH 7 and pH 3. (**G** and **H**) 2D ssNMR spectra of both aggregates, revealing a remarkable degree of atomic order in the amorphous-looking deposits. Adapted with permission from references [7,10,96].

often combined with methods like electron microscopy (EM) for visualizing the morphology of samples. Cryo-EM methods are improving rapidly, but their achievable resolution remains dependent on the extent of molecular order or disorder [76,77]. The complementarity of ssNMR and EM methods is based, in part, on their respective resolutions, with ssNMR providing atomic-resolution local structural information [87–91]. As noted above (and visualized in Figure 4), ssNMR is also complementary by being able to yield structural data in heterogenous samples, or to directly probe dynamics or dynamic disorder. The application of ssNMR across a broad range of temperatures (from cryogenic to above ambient) also has the potential to connect biologically functional states (studied at ambient or higher temperatures) with structural data measured under cryogenic conditions [4,5,78].

ssNMR is also a valuable partner to X-ray crystallography. For instance, it can facilitate a straightforward and direct structural comparison between X-ray crystal structures and alternative conformational states such as amyloid-like fibrils, since both crystals and fibrils are amenable to ssNMR characterization [92,93]. In addition, ssNMR dynamics measurements provide unique insights into the dynamic modes present in the crystal lattice [78,79]. Another example of synergy between X-ray and ssNMR methods is found in recent MAS ssNMR studies of crystallized, but functional enzymes [94,95].

The integration of solution- and solid-state NMR methods allows one to observe structural and dynamical changes that accompany the formation of insoluble macromolecular assemblies. The spectral properties of the two methods (i.e. the measured isotropic chemical shifts) are directly comparable. Thus, structural comparisons between soluble and immobilized protein states can be performed on the level of the chemical shifts in NMR spectra. For instance, dramatic spectral changes are associated with protein aggregation via large-scale misfolding [93], which are absent when proteins become immobilized while maintaining much of their native fold [4,96]. Electron paramagnetic resonance (EPR) spectroscopy can be a useful complement to NMR studies in solution and solids, by providing long-range distance constraints and dynamic information, while having a much higher sensitivity than NMR. One promising area of integration in this regard is the use of paramagnetic compounds and spin labels to gain long-range distance information by both EPR and in ssNMR experiments [97].

Moving beyond experimental techniques, molecular dynamics (MD) simulations that are instrumental across most of structural biology are also increasingly important for biomolecular ssNMR studies [89,98]. MD

simulations are, for instance, valuable for biological samples (such as peptides within fluid lipid membranes) that feature functionally relevant dynamics with a complexity that can be challenging to capture fully with ssNMR experiments alone [98–100].

Conclusion

ssNMR has contributed a variety of novel structures or structural models for membrane proteins, biological complexes, and protein aggregates. These structures have been enabled by an increasingly standardized toolkit of structural ssNMR measurements [101], often integrated with structural data from EM, X-ray crystallography, EPR, and other complementary methods. Ongoing developments across all structural biology techniques open up exciting new research directions, in which ssNMR will continue to generate an invaluable and unique set of insights. This may take the form of 3D structures in heterogeneous non-crystalline sample conditions, but ssNMR will also provide other types of essential information. This includes supramolecular interactions, dynamics, and the mapping of condition-dependent changes in structure and dynamics. These types of information are critical for efforts to bridge the gaps between data from other methods in integrated structural biology, for instance, by relating high-resolution structures of crystalline or solubilized proteins to native or functional states that may be neither.

Summary

- Modern solid-state NMR provides high-resolution structures of protein aggregates, crystallized proteins, membrane proteins, and numerous other types of biological assemblies.
- Beyond 3D structures, solid-state NMR methods provide unique insights into the dynamics and interactions of biomolecules, including proteins, lipids, and oligonucleotides.
- Solid-state NMR can bridge the gaps between other methods of integrated biology given its applicability across a broad range of sample conditions, including the study of whole cells, cellular membranes and cell extracts.

Abbreviations

CP, cross-polarization; DNP, dynamic nuclear polarization; EM, electron microscopy; EPR, electron paramagnetic resonance; MAS, magic angle spinning; MD, molecular dynamics; NMR, nuclear magnetic resonance; RF, radio frequency; ssNMR, solid-state NMR; TEM, transmission electron microscopy.

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Competing Interests

The Author declares that there are no competing interests associated with this manuscript.

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