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Insights into Protein Misfolding and Aggregation enabled by Solid-State NMR Spectroscopy

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Abstract

The aggregation of proteins and peptides into a variety of insoluble, and often non-native, aggregated states plays a central role in many devastating diseases. Analogous processes undermine the efficacy of polypeptide-based biological pharmaceuticals, but are also being leveraged in the design of biologically inspired self-assembling materials. This *Trends* article surveys the essential contributions made by recent solid-state NMR (ssNMR) studies to our understanding of the structural features of polypeptide aggregates, and how such findings are informing our thinking about the molecular mechanisms of misfolding and aggregation. A central focus is on disease-related amyloid fibrils and oligomers involved in neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's disease. SSNMR-enabled structural and dynamics-based findings are surveyed, along with a number of resulting emerging themes that appear common to different amyloidogenic proteins, such as their compact alternating short- β -strand/ β -arc amyloid core architecture. Concepts, methods, future prospects and challenges are discussed.

Graphical Abstract

Keywords

Amyloid; Neurodegenerative disease; Magic-angle spinning; Protein Structure; Protein Dynamics; Protein misfolding; Structural Biology; Prions

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Introduction

Biochemical, biological and structural studies traditionally focus on the characterization of proteins in their soluble state. Such a solution-centric focus risks undervaluing the high relevance and importance of a variety of insoluble protein aggregates. Aggregated proteins play a central role in protein deposition diseases, they compromise the stability and effectiveness of protein-based therapeutics, and are increasingly used as self-assembling (hybrid) bio-materials [1–6]. Solid-state NMR (ssNMR) offers unique and essential capabilities to study the aggregated state of polypeptides, through its ability to elucidate both structure and dynamics independent of macroscopic order. This *Trends* article reviews the progress made in the application of ssNMR to this important field of research in the last five years. Due to constraints of space rather than reflecting relative importance, the primary focus will be on contributions of ssNMR to studies of structural and mechanistic aspects of disease-associated protein aggregation. A growing list of diseases are associated with the aggregation of proteins, either within cells or extracellularly [1]. The most famous examples of protein deposition diseases are those in which the affected proteins form amyloid or amyloid-like fibrils. Note that a traditional definition of “amyloid” is that these are patient-derived extracellular fibrils, but increasingly the term amyloid is employed more generously to polypeptide assemblies with a characteristic cross- β β -sheet-based architecture (see below) [7]. Examples of amyloid diseases include Alzheimer’s, Parkinson’s and Huntington’s diseases. Characteristically these diseases are age-dependent, even in those cases where the disease-causing amyloidogenic protein is a mutant variant that is present from birth. Another common feature of these devastating and lethal diseases is that they lack effective treatments that target the molecular causes of disease (i.e. the misfolding and aggregation), rendering them as-yet incurable, unpreventable and ultimately fatal. In absence of a cure, affected patients require extensive and costly day-to-day care during the unstoppable progression of the disease. Efforts to design treatments that target the causative protein misfolding and aggregation events are hindered by a lack of atomic-level understanding of mechanism by which the aggregates form or how they may help cause the disease-related cellular toxicity.

Structural biology of protein aggregates

One longstanding hurdle in our understanding of protein aggregation mechanisms is the lack of atomic structures for non-crystalline protein assemblies that populate these pathways. Mature aggregates lack the three-dimensional crystalline order required for high-resolution X-ray crystallography. Amyloid fibrils can still be studied using X-rays, in the form of aligned (or unaligned) fibril diffraction experiments. Indeed, X-ray diffraction arguably provides the gold-standard test of the “amyloid” nature of aggregates, as amyloids share a highly characteristic cross- β diffraction pattern (Fig. 1a). This cross- β pattern reflects the fact that the common “core” of these assemblies is made up of extensively intermolecular β -sheets running along the length of the fibers (*vide infra*). Unfortunately, these diffraction patterns lack the resolution to extract atomic-level amyloid structures. This is exemplified by polyglutamine amyloids for which qualitatively different fiber structures were proposed based on the same experimental X-ray data [8–10]. While a subset of amyloidogenic proteins can be made to form sufficiently large crystals that are suitable for high-resolution

X-ray crystallography, the resulting structures reflect a non-amyloid state that is devoid of the structural hallmarks of the fibrillar amyloid. Most obviously this is true for proteins that are crystallized in their native fold but also form aggregates when they misfold into a distinct non-native structure. Certain *short* fragments of amyloidogenic proteins are capable of forming amyloid-like fibrils and elongated β -sheet-based nano- or microcrystals. In the 2000s, X-ray crystallographic studies of the latter crystalline amyloid mimics revealed a number of structural motifs that were subsequently shown to be common in true amyloid fibril structures [11, 12]. The interfaces between β -sheets featured “steric zippers”: a tight intermeshing of side chains, without (space for) water molecules (Fig. 1b,c). These amyloid-like crystal structures provide a growing library of high-resolution “reference” amyloid architectures in the protein databank. An intriguing, and as-yet not fully understood, question is what differentiates the formation of those 3D crystal lattices from the formation of “true” amyloid fibril structures, given that they are in fact different structures even though they form under very similar conditions [13]. Single molecule based methods such as atomic force microscopy (AFM) and electron microscopy (EM) have also been essential for the study of protein aggregates and their formation. Although these tools traditionally have been unable to gain sub-nm resolution data, it has been especially the EM field that has made significant progress toward ever-higher resolution amyloid fibril studies (Fig. 1d) [14–17]. In the few years, it has however been especially the use of advanced solid-state NMR spectroscopy (ssNMR) that has made a lot of exciting progress in detailing atomic-level structures and structural data. Mature aggregates and even the “soluble” oligomers may tumble too slowly for direct high-resolution solution NMR characterization, but they have proved quite amenable to the ever more capable tools of magic-angle-spinning (MAS) ssNMR. After an introductory review of applicable concepts in the next section, we will discuss how recent MAS ssNMR studies have revealed the structure and dynamics of amyloid fibrils, non-fibrillar protein deposits and pre-fibrillar assemblies, in unprecedented Å-resolution detail.

Concepts and mechanisms

Amyloid: definition of a generic architecture

Amyloids are characterized by an elongated, fairly narrow (5–15 nm wide) fibrillar structure that is largely or entirely made up of β -sheet structures. The most robust hallmark of amyloid is the presence of a **cross- β pattern** in X-ray diffraction studies (Fig. 1a). The cross- β pattern reveals the fundamental architecture, in which a 4.7–4.8 Å repeat distance reflects the hydrogen-bonding distance between β -strands. The β -strands from individual monomers stack atop each other to form long intermolecular β -sheets. Close contacts between opposing sheets manifest themselves in the cross- β pattern as (perpendicular) repeat distances of ~ 8–10 Å. These inter-sheet repeat distances are more variable, as they depend on the size of side chains forming the sheet-to-sheet interface [22, 23]. Fig. 1d shows an example of such an amyloid fiber structure derived from a combination of ssNMR and EM constraints [18]. This architecture is stabilized by intermolecular hydrogen bonding between the backbones of this relatively short amyloidogenic peptide. Since this structural motif is mediated by backbones, it can naturally be adopted by a wide array of unrelated proteins and peptides that all form amyloids. That said, especially for longer amyloidogenic

peptides or proteins, different types of β -sheet-based architectures can underlie an amyloid fibril assembly. Fig. 1e–h shows three basic amyloid folds predicted in a previously proposed classification [21]. Many amyloids feature in-register parallel (IP) β -sheets, whilst the extended polypeptide chain contains multiple shorter β -strand segments that end up in their own distinct β -sheet stacks (Fig. 1e). Connecting these β -strands are characteristic loops or kinks (sometimes named β -arcs; see figure). Thus, each polypeptide forms a layer of connected strands and arcs, which together are referred to as “ β -arches” that stack to form an IP amyloid architecture. IP β -sheet amyloids are characterized by a particular sequence position in one monomer being layered atop the same residue in its neighboring protein monomer (Fig. 1e; black dots). In this case, the hydrogen bonds stabilizing the β -sheets are all intermolecular. This is also the case for amyloid folds in the polypeptide also forms β -arches, but these arches are assembled supramolecularly in antiparallel (AP) rather than IP β -sheets (Fig. 1g). Notably, in the AP fold most residues in a β -strand will not face their sequence counterpart in the neighboring β -strand, which imparts particular sequence constraints that do not apply to IP amyloids. Some amyloids feature β -sheet architectures that are in part stabilized by *intramolecular* backbone-mediated hydrogen-bonding interactions. For instance, β -solenoid-based amyloids contain parallel (but not in-register) β -sheets (Fig. 1f; Fig. 2) in which two or more neighboring strands come from the same protein. Or, AP β -sheets can be formed from β -hairpins stabilized by intramolecular hydrogen bonds (Fig. 1h). The structural motif connecting the two β -strands of a β -hairpin is known as a β -turn (see figure), which is distinct from the β -arc found in β -arch architectures (see above). It is important to distinguish true β -hairpins (with their internal hydrogen-bonding) from the types of “hairpin” structures seen in e.g. certain IP A β fibrils, where the hydrogen bonding occurs between inter-molecularly rather than intra-molecularly. Note that all of these distinct amyloid folds conform to the abovementioned cross- β architecture (Fig. 1a).

Protein aggregation mechanisms and nomenclature

As Anfinsen already noted [26], a polypeptide chain's fold “*is determined by the **totality** of inter-atomic interactions and hence by the amino acid sequence, in a given environment*” (emphasis added). The same applies in protein misfolding and aggregation, where the structure of the resulting aggregate depends specifically on the exact aggregation conditions. Changes in experimental conditions, such as concentration, pH, temperature or agitation, change the nature of both intra- and inter-protein interactions, and thus enable different aggregation pathways. In this section, we will examine different types of aggregation mechanism, while also covering some of the nomenclature in use in the protein aggregation and amyloid formation literature [27, 28]. Figure 3 summarizes several different types of protein aggregation processes, with the active one dependent on the polypeptide sequence and environmental conditions.

Many disease-associated aggregation-prone proteins are *intrinsically disordered proteins* (IDPs) under physiological conditions, when they are not aggregated (Fig. 3a,b; left). These proteins (or protein domains) are present in an *ensemble* of rapidly interchanging conformations. As a precursor to full-blown aggregation, aggregation-prone IDPs often first form oligomeric, but still soluble, assemblies, usually driven by hydrophobic interactions.

These initial interactions may require a structural change (compared to the soluble monomer) that is slow or unlikely to occur, manifesting in a *lag phase* of oligomer formation. Commonly, oligomers are semi-stable at a certain defined size, but for amyloidogenic proteins oligomer formation is followed by a structural change that facilitates the formation of extendable filamentous assemblies. This amyloidogenic structural change reflects the initial creation of the amyloid architecture, based on inter-molecular β -sheets, and is commonly known as the (primary) nucleation event. A hallmark feature of amyloid structure is that it is capable of self-propagation through elongation: new monomers are recruited to fibril ends, where they hydrogen-bond to the exposed β -strands of the amyloid core. This elongation step causes the new monomer to take on the existing amyloid structure. This mechanism gives amyloids the ability to self-propagate with high structural fidelity. In-depth analyses of protein aggregation kinetics [29] reveal that fibrils do more than just elongate: fibril fragmentation and fibril-mediated secondary nucleation are essential to fully explain observed aggregation kinetics.

Not all aggregation-prone proteins implicated in disease are normally IDPs. The proteins that aggregate in transthyretin and light chain amyloidosis are globular proteins with a defined native fold [30]. The eye lens proteins that aggregate during cataract formation also normally adopt an ordered fold [31]. These kinds of non-IDP proteins form protein deposits via different types of aggregation mechanisms, with three prominent categories illustrated in Fig. 3c [32]. First, destabilization of the native fold can lead to significant unfolding that effectively opens up an amyloidogenic assembly process reminiscent of those illustrated in Fig. 3a–b. However, extensive unfolding or misfolding is not a prerequisite for aggregation. Some aggregation-prone proteins self-assemble under non-denaturing conditions into non-filamentous protein deposits that lack the hallmarks of amyloids [32]. The molecular principles of these types of aggregation pathways are less well understood. Condensation-style aggregation involves predominantly surface-surface interactions (Fig. 3c; top). Others have advocated domain swapping as a potential contributor to this type of aggregation process, noting a correlation between domain swapping propensity and aggregation propensity. To the best of our knowledge, truly domain-swapped conformations have not yet been demonstrated within amyloid fibrils, but may be part of non-amyloid aggregates or pre-amyloid oligomers [32–35].

Many aggregation processes are multistage mechanisms in which multiple structural states co-exist and inter-convert. This includes the mature fibrils and oligomers discussed above, but there are two other concepts that should be noted. Protofibrils are thinner isolated β -sheet-based fibrils, sometimes wormlike in appearance, that can be observed prior to the formation of the thicker mature fibrils, but after formation of non-fibrillar oligomers. They are conceptually different from so-called protofilaments, which are the intertwining sub-filaments that constitute amyloid fibrils (but may not be immediately visible in low to moderate resolution EM or AFM images).

Aggregate polymorphism

A predictable consequence of the diversity of accessible aggregation pathways (Fig. 3) is that they should yield differently structured aggregates. Structural information on the

aggregates is therefore useful to narrow down the set of potential aggregation mechanisms. The active mechanism depends on experimental conditions, causing a single polypeptide to form a variety of differently structured types of aggregates [36, 37]. **Polymorphism** is well known for the amyloid aggregates formed by A β , tau, and α -synuclein, and it underlies the strain phenomenon of prion proteins. A particular amyloid fibril polymorph often (but not always! [38]) propagates itself with structural fidelity. Polymorphic aggregation goes beyond the formation of different types of amyloid fibrils. This is illustrated in Fig. 3(c), in context of the condition-dependent aggregation of a cataract-related mutant eye lens protein, P23T γ D crystallin[32]. Under physiological conditions this protein forms non-amyloid “amorphous” aggregates, while denaturing conditions induce formation of amyloid fibrils with a very different internal and macroscopic structure. In other cases, relatively subtle changes in experimental conditions (e.g. concentration) differentiate the nucleation of 3D crystals from amyloid fibrils [19].

Functional properties of polymorphic assemblies

Naturally, different (polymorphic) structures translate into different functional properties. This correlation is a prominent concern for the disease-related protein aggregation diseases. The misfolding and aggregation of proteins leads to a loss of (normal) function, but also can cause a disease-causing gain of a toxic function. The exact toxic roles of different types of oligomers and fibrils remain hotly debated[39–41]. Most evidence suggests that cytotoxic effects show an inverse correlation to parameters such as aggregate size and stability, with soluble oligomers, protofibrils or small fibrillar states generally being most toxic. Sequestration of smaller species into larger assemblies may reduce the effective toxicity. This is an ongoing discussion, as there are also data supporting a contribution of mature aggregates to disease and toxicity [41].

Protein aggregation in human disease

Aggregates in Alzheimer’s disease – A β

Two types of polypeptide aggregates are associated with Alzheimer’s disease (AD). This includes on the one hand 40- and 42-residue A β peptides that form extracellular amyloid plaques. A β fibrils have been a long-standing target of MAS ssNMR studies, with several groups recently reporting 3D structures of different types of A β fibrils (Fig. 4). Most A β fibrils feature an IP β -sheet architecture, with each “layer” containing two or three peptide monomers, depending on the polymorph. One exception is the A β 1-40 Iowa mutant (D23N mutation), which forms fibrils with an AP core fold[42]. A β 40 fibrils featuring the Osaka mutation (E22) adopt the more canonical IP fold, with the conformation of the dimeric peptide building block in Fig. 4b [43]. An intriguing feature of this fibril architecture is that it achieves a dense packing of the hydrophobic core by combining steric-zipper-like interactions between short β -strand segments with intervening β -arc (ref. Fig. 1e) twists or turns. This is a recurring feature in a number of newly determined amyloid fibril structures. Indeed, an inspection of three independently determined A β 42 fibril structures shows a similar structural motif [44–46], as illustrated in Fig. 4c–e. Various ssNMR studies of A β and other amyloids have started to probe the conformation of brain-seeded fibrils [47–49]. In these studies, amyloid fibrils recovered from patient material are amplified by cyclical

seeded elongation processes performed *in vitro*. By using stable-isotope labeled monomers during the final round of amplification, labeled fibrils are obtained that are amenable to multidimensional ssNMR. For A β 1-40, this approach has yielded the three-fold-symmetric fibril structure in Fig. 4a, which is distinct from prior A β 1-40 *in vitro* fibril structures [48]. The key differences were ascribed to bends and kinks in the backbone, which were absent in prior *in vitro* fibrils that had a less complex architecture with longer straighter β -strands [48].

Recent ssNMR studies related to A β aggregation have not been constrained to the determination of high-resolution fibril structures, but have tried to provide more insights into the pathway of misfolding that leads to amyloid formation. One recurring question has been how membrane interactions can modulate the A β misfolding and aggregation process [50–52]. Others have used ssNMR to examine the conformation of pre-fibrillar A β oligomers and protofibrils, and compare them to those of the mature fibrils [53–60]. Although a high-resolution structure of these disordered assemblies has not been reported, these studies do agree that the pre-fibrillar species have β -strands that occur in regions that are also part of the amyloid core. It appears however that these β -strands must be arranged in a tertiary or quaternary fold that is different and in particular not amenable to amyloidogenic elongation into a fibril. It has been suggested that the β -rich oligomers or protofibrils formed by A β contain intramolecularly hydrogen bonded β -hairpins that need to undergo a substantial strand rearrangement to convert into the more stable fibril state [53, 55, 60–65]. At least one recent ssNMR study of A β 42 oligomers argues against β -hairpins, but favors other types of antiparallel β -sheets (lacking the *intramolecular* hydrogen bonds) [59].

Aggregates in Alzheimer's disease - Tau

AD patients also have another type of protein aggregates in the form of tau tangles. The tau protein is much larger than A β , which has made it harder to study by most structural biology methods including ssNMR. A combination of improvements in protein production protocols and in the ability of ssNMR to study smaller sample sizes have started to make effective tau ssNMR studies possible [67]. Beyond ssNMR, it is worth noting a recent breakthrough study involving the use of cryoEM to probe the conformation of patient-extracted tau fibrils [16].

Aggregates in Parkinson's disease

In Parkinson's disease it is the α -synuclein protein that undergoes aggregation into amyloid fibrils. Building on earlier ssNMR work, recent years have seen important progress reported by several ssNMR groups [66, 68–71]. This includes one study that probed the effect of phospholipid membrane binding on α -synuclein aggregation. Membrane binding modulates the α -synuclein aggregation pathway, but ssNMR shows that, perhaps somewhat surprisingly, the resulting fibril structure is relatively similar to the structure that forms in absence of membranes [68]. Moreover, a 2016 study showed that α -synuclein fibrils made *in vitro* are toxic to cells, and used extensive ssNMR experiments to delineate the atomic-resolution structure of these fibrils [66]. The obtained structure is shown in Fig. 4f, and reveals the presence of a similarly kinked backbone architecture as was discussed for some A β polymorphs above. The compact structure of alternating β -arcs and short β -strand

segments enables a dense packing of the rigid amyloid core, stabilized by a combination of backbone and side chain interactions both within and between the IP β -sheets.

Prions

Amyloid-like aggregates play a special role in prion diseases, given their role as the infectious species. The origin of this phenomenon is encoded in their capability to propagate themselves by recruiting otherwise innocuous protein monomers and subverting them into an amyloidogenic state. Differently structured aggregates made by a particular prion protein have distinct levels of infectivity and toxicity, while prions from different organisms vary in cross-species propagation. MAS ssNMR has been applied to aggregated prion proteins from multiple organisms, to probe this fascinating topic from a structural perspective[72]. Several groups are pursuing ssNMR studies of the prion strain phenomenon and its relation to toxicity and propagation, with a prominent role for correlating the ssNMR to *in-vivo* toxicity studies or seeding with *ex-vivo* aggregates [72–74]. Structurally speaking, a striking feature of the examined prion protein fibrils is that ssNMR revealed that only a small portion of the protein ends up in an ordered β -sheet amyloid core, visible in cross-polarization (CP) experiments (Fig. 5a) [73–76]. Upon freezing the hydrated sample, the disordered non-core segments become visible in the CP spectra, but are highly broadened due to static disorder and structural heterogeneity. In the unfrozen state, the dynamically disordered residues outside this core are so dynamic and flexible that they are visible in J-based ssNMR experiments (see below).

Prions are also found in lower organisms such as fungi, where they are proteins with prion behavior but without sequence similarity to the abovementioned mammalian prion protein. Although not associated with human disease, this class of amyloidogenic proteins has proven instrumental for our understanding of protein misfolding and amyloid formation. This includes a number of now-classic ssNMR studies on various fungal protein amyloids. In recent years, several groups have used ssNMR to contribute further insights on the structure, dynamics, and chaperone interactions of the Sup35p yeast prion protein (Fig. 5b) [77–80]. Structurally, most ssNMR data point to the formation of canonical IP β -sheet architectures by the mammalian as well as yeast prions [61]. In contrast, ssNMR studies of the functionally important fungal prion protein HET-s previously has provided an exciting perspective into a solenoid amyloid fibril architecture (Fig. 2a) [24]. Building on this ssNMR structure, recent studies continue to probe (by ssNMR) the dynamics, structures, NMR characteristics, and functional properties of the amyloid state [81–83]. Notably this includes a demonstration of the potential of segmental isotopic labeling in current and future ssNMR studies of larger protein assemblies [84, 85].

Aggregates in Huntington's disease

Huntington's disease (HD) is the most common example of a family of inherited polyglutamine expansion disorders[88], in which mutations in disease-specific genes result in the expansion of a normally occurring polyglutamine repeat within affected proteins. In the case of HD it is the huntingtin protein that is mutated, with its polyglutamine domain present within the first exon of the protein. The last five years have seen a number of ssNMR studies of aggregated polyglutamine, both in isolation and in context of the huntingtin exon

1 (HttEx1) [20, 87, 89–93]. HttEx1 is seen as an essential disease-relevant huntingtin N-terminal fragment, as it is observed in patients and can cause HD-like disease in model animals. A recent ssNMR study revealed that the expanded polyglutamine domain forms a long β -hairpin structure in mutant HttEx1 fibrils [20], which were subsequently shown to be toxic to neuronal cells (Fig. 5e) [87]. Like other ssNMR-studied amyloids discussed above, the HttEx1 fibrils also have a highly ordered amyloid core that is decorated with exposed non-amyloid flanking regions (with α - and PPII-helical as well as random coil regions; Fig. 5c–e) [87, 91–93]. Delineation of the fibrils' domain architecture was enabled in large part by ssNMR measurements of the domains' relative dynamics probed by ssNMR measurements of relaxation rates and dipolar order parameters (Fig. 5f–g), and the use of J-based pulse sequences (Fig. 5d) [87, 91, 92, 94].

Systemic amyloidosis

The term of amyloidosis is sometimes used to generically indicate amyloid-related diseases, but is mostly reserved for systemic amyloidosis diseases [30]. The two most well known examples are light chain amyloidosis and transthyretin amyloidosis, both of which have seen recent contributions from ssNMR studies. Both these proteins have an ordered conformation in their native state, as in Fig. 3(c), unlike IDPs such as A β or amyloidogenic proteins with low-complexity sequences. Recent progress is particularly notable in the previously poorly served (by ssNMR) area of light chain amyloidosis research with very recent work on two light chain variants [95–97]. The study of the variable domain of AL-09 included a comparison to patient-derived material, based not on the seeding of labeled protein, but rather the direct acquisition of patient material's natural abundance ^{13}C spectrum. The fibrillar and oligomeric states of the variable domain of MAK33 were very similar to each other in terms of their ssNMR spectra, whilst the fibrils were also reported not to reflect a domain swapped state [35]. Interestingly, the regions that form the amyloid core seem to differ between the two light chain variants, arguing that further ssNMR studies will be valuable to probe what (if any) commonalities are present between different amyloidogenic light chains.

Beyond studies of amyloidogenic peptides derived from TTR (Fig. 1), multiple groups are studying aggregates formed by the disease-relevant full-length protein upon long-term incubation under acidic conditions [98, 99]. No ssNMR-based structure of TTR fibrils is as yet available, but various types of structural data start to constrain our understanding of TTR's aggregate architecture, with implications for the misfolding mechanism. These data show that the aggregated protein lacks the common IP β -sheet architecture, but the reports disagree on the extent to which secondary and tertiary structures from the β -rich native fold are preserved in the fibrils.

Other amyloid-related diseases

A variety of other amyloid-related diseases have seen recent contributions from ssNMR studies of the associated protein deposits. ALS or Lou Gehrig's disease reflects a class of related diseases that are now recognized to also involve the aggregation of proteins. Affected proteins include the TDP43 and FUS proteins. Thus far, ssNMR studies on the former have been limited to fragments of the protein, which suggested the formation of a β -hairpin in the

fibril [100]. Recently, a novel ssNMR-based structure of the FUS amyloid core was reported, with similar structural features as noted above for other amyloid structures[101]. The peptide IAPP (also known as amylin) forms amyloid-like fibrils in type II diabetes, which have also been studied recently by ssNMR [102]. Human β -2-microglobulin (β 2m) is involved in dialysis-related amyloidosis. In contrast to the large flexible flanking domains in HttEx1 and prion protein fibrils, ssNMR shows the β 2m amyloid core to contain ~80% of the primary sequence (with N-terminal truncation actually extending this core structure) [103].

Functional amyloids

Amyloid-like architectures occur in nature not just in context of disease, but are also observed to have a more beneficial relevance. This category of amyloids is generally known as “*functional amyloid*” [104]. A number of bacterial proteins form functional amyloids, with relevance to biofilm formation. Most notably this includes the curli proteins, which have been studied by ssNMR [105–107]. Although no purely ssNMR-based structure exists yet, computational modeling informed by the available experimental data (including ssNMR) has been reported [25]. As illustrated in Fig. 2, the curli structure appears to be a β -helix or β -solenoid, reminiscent of the discussed fungal HET-s fibrils. This kind of architecture may be a more general feature of functional amyloids, as HET-s is itself also considered a functional amyloid. Various functional amyloids have been identified or proposed in higher organisms, some of which have been probed by ssNMR: the Pmel17 protein involved in the melanin deposition [108], memory-related CPEB and Orb2 proteins [109–111], RIP1/RIP3 kinases involved in necrosis [112], and reversible fibrils formed by β -endorphin [113].

Designer amyloids

Amyloid-like assemblies are also finding applications beyond biology, as frameworks for self-assembling hydrogels and other nanomaterials. As with their naturally occurring counterparts, structural studies of these designer amyloids benefit from valuable insights enabled by ssNMR [23, 114–118]. In most cases, these amyloid- or cross- β -based materials are based on short amyloidogenic peptides that may be modified with acyl chains or by cross-linking [23, 115, 116]. Detailed structural (and dynamic) data on these synthetic assemblies enabled by ssNMR are likely to be critical for disentangling how these designer molecules self-assemble and to find ways to rationally modify their structures and properties.

Non-amyloid protein aggregates

It is important to note that not all protein aggregation necessarily reflects misfolding into an amyloid-like state. Indeed, many studies differentiate protein aggregates (related to disease) between elongated amyloid-like fibrils and amorphous-looking assemblies [32, 119–122]. This includes for instance pre-fibrillar oligomers formed by amyloidogenic polypeptides, which are in EM and AFM studies lacking the more ordered elongated appearance of the mature fibrils. These oligomers are studied by ssNMR, but such experiments are often

complicated by the need to somehow stabilize these inherently transient assemblies (*vide supra*). Other proteins form amorphous-looking aggregates not as an intermediate form, but rather as their final mature aggregated state. It is tempting to assume these types of assemblies to be dynamically or statically disordered on the atomic level (and therefore challenging or unsuitable for in-depth ssNMR study). However, a recent ssNMR study on such amorphous looking aggregates formed by a mutant of a γ D-crystallin protein reported very high quality spectra, indicative of high rigidity and order on the atomic level [32]. Note that this protein forms canonical amyloid-like fibrils under denaturing conditions, but follows a qualitatively different assembly process at physiological pH. The remarkably high ssNMR spectral quality of the latter non-amyloid assemblies illustrates the point that EM-based morphology may not predict or reflect the atomic-level structure or the suitability for ssNMR. Clearly, MAS ssNMR is a powerful complement to EM-based structural studies, and may be invaluable for samples that feature local order, but lack the repetitive or “macroscopic” order necessary for EM image reconstruction methods.

Recurring themes

Structural features of amyloid architectures

One of the key lessons learned from ssNMR studies of amyloid fibrils is that there are a number of recurring features and canonical architectures[21]. Thus far, the most common architecture reported for amyloid fibrils is based on an IP β -sheet assembly, as illustrated in Fig. 1 and Fig. 4. These types of assemblies lead to the stacking of identical segments from individual protein monomers, stabilized by a combination of intermolecular backbone-backbone and side-chain interactions. Several ssNMR approaches have been developed for detecting the characteristic “self-to-self” interactions of *IP β -sheets*. This is most commonly achieved by intermolecular ^{13}C - ^{13}C recoupling experiments using either derivatives of the RFDR-based PITHIRDS experiment or double quantum ^{13}C - ^{13}C recoupling pulse sequences such as DQ-DRAWS [123–125]. An IP structure has been reported for A β , α -synuclein, several yeast prion proteins, and various other amyloids (Fig. 4) [13, 44, 45, 66, 75, 101, 126]. However, we also have a growing set of alternative amyloid core structures that were first predicted [21] and have now been demonstrated experimentally by ssNMR. This includes the parallel *β -sheet solenoids* as illustrated in Fig. 2, as well as antiparallel architectures with and without β -hairpins[20, 42, 100, 118].

Beyond the overall architecture classification, there is an emerging characteristic shared by many of the recent fibril structures. They tend to feature a very tightly packed core that is made possible by a collapsed supramolecular fold in which short complementary β -strands are linked by β -arc turns to form a compact alternating short-strand/arc (CASSA) architecture (e.g. Fig. 4). Typically, the short β -strands tend to be only a few residues long, yielding a striking highly kinked misfolded state that may well prove to be a typical architecture of amyloids. The short strand segments of these amyloid cores are engaged in tight zipper-like interactions, reminiscent of the structures of crystallized amyloidogenic hexapeptides[11, 12]. These peptide X-ray structures have yielded a systematic classification of amyloid-like β -sheet spine assemblies. Although it is dangerous to extrapolate the structures of the short-peptide crystals to the actual amyloid architecture of their parent

proteins [78, 79], these X-ray data are valuable for ssNMR studies as high-resolution reference structures. The peptide crystals are also ideal to experimentally validate (novel) ssNMR measurements of amyloid structure. In the case of the hexapeptide GNNQQNY (Fig. 1b), ssNMR on the (highly stable) crystalline and fibrillar states [127] indicated that the crystals do recapitulate key structural features of the amyloid state. This includes the dehydrated “steric zipper” interface between β -sheets that seems to be characteristic of various amyloids studied by ssNMR[20]. However, the hexapeptide fibrils reproducibly had a complex architecture in which both straight β -strands and bent peptides co-assembled into a “composite” core structure [13], which might in retrospect reflect a CASSA-type architecture typical of larger amyloids.

Recent ssNMR studies increasingly focus on larger proteins and protein constructs, rather than truncated fragments. These studies reveal another common feature of amyloidogenic proteins: that only relatively small portions of the protein end up in the β -sheet-based amyloid core [75, 91, 92, 111]. The non-amyloid portions can contain folded domains, isolated secondary structure elements, or be dynamically disordered (Fig. 5). The different domains within and outside the amyloid core have very different dynamics and solvent accessibility, which can be very nicely probed by ssNMR (as long as samples are sufficiently hydrated and unfrozen). The amyloid core itself tends to be highly rigid, resulting in high signal intensities in traditional dipolar-based experiments. Highly flexible disordered segments are invisible in CP-based spectra, but are effectively studied using a variety of multidimensional J-based MAS ssNMR pulse sequences. Indeed, fully scalar methods often allow for a detailed analysis of the assignments and dynamic properties of these exposed segments. Partly immobilized domains outside the amyloid core are often visible in the dipolar MAS NMR spectra, but show diminished intensity and increased relaxation. This still permits the measurements of chemical shifts and dynamic parameters, but the reduced dipolar order parameters complicate the measurements of (long range) distances or dihedral angles with precision and accuracy. Many types of protein aggregates have now been found to contain segments with dynamics that range from fully rigid to completely flexibly disordered in the same protein. As such, these samples not only benefit greatly from the wide assortment of ssNMR-based dynamics measurements, but also present a great opportunity to further develop this toolkit.

Outlook

A combination of improvements in ssNMR instrumentation, ssNMR pulse sequence development and sample preparation protocols have allowed ssNMR studies to greatly advance our understanding of amyloid formation, protein misfolding, and protein aggregation. Recent years have seen a progression from measurements of structural constraints, to the development of preliminary structural models, to increasingly well-defined atomic-resolution structures deposited in the protein databank. Thus, we have obtained the first detailed insights into the misfolded structures of true amyloid fibrils, and begun to identify their recurring characteristic architectures.

Technological and methodological advances

Going forward, there are a number of developments that are likely to further revolutionize this field of investigation. A characteristic challenge of ssNMR spectroscopy relates to the inherently low sensitivity of the method. *Dynamic nuclear polarization (DNP)* methods are under continuing development as a means to achieve dramatic increases in sensitivity. A number of published studies have demonstrated the applicability to protein aggregate studies in particular [128–132]. One interesting application is to study smaller amounts of labeled protein aggregates under dilute conditions, for instance in cellular extracts [132]. Current DNP methods are typically reliant on cryogenic temperatures, which enhance the performance of dipolar-recoupling experiments, but also limit the ability to measure highly valuable dynamics data and often result in reductions in spectral resolution [82, 133]. A complementary new development is the advent of faster MAS using ever-smaller rotor sizes, with state-of-the-art probes enabling MAS rates in excess of 100 kHz [134]. This enables ^1H detection and can greatly improve the per-mg sensitivity, which is a crucial consideration for studies of hard-to-produce protein samples [135]. Ultrafast MAS traditionally suffers the opposite challenge of cryogenic DNP conditions, in that the frictional heating is prone to overheat the sample. Fortunately, this challenge is being met by improvements in sample cooling hardware.

The field is also expected to benefit from methodological developments in terms of pulse sequence design and application. A key goal in this should be a standardization of methods for structural and dynamic analysis. At conventional MAS rates (8–20 kHz), the field is gradually settling on standard methods of measuring ssNMR chemical shifts and distance constraints, with the latter mostly based on ^{13}C - ^{13}C and ^1H - ^1H (from XHHC; with X=N/C) distance constraints. These are complemented with ^{13}C - ^{15}N distances that can be obtained via TEDOR or PAIN-CP experiments. One recurring challenge is that one needs to distinguish intra-molecular from intermolecular distance constraints, which are indistinguishable in fully labeled protein aggregates. For this purpose, ssNMR studies of amyloid structures typically include distance measurements on isotopically diluted and isotopically mixed samples. The former would reveal those inter-residue interactions that are intramolecular while the latter unambiguously identify inter-molecular contacts. Standard protocols are complemented with more tailored or specialized experiments, some of which are of particular interest in amyloid studies. For instance, as noted above, several dedicated tools are available for probing ^{13}C - ^{13}C contacts characteristic of intermolecular IP β -sheets, including DQ-DRAWS and PITHIRDS [130, 136, 137]. Other complementary structural data can be obtained via dihedral torsion angle measurements, which have been proved useful and effective in several amyloid fibril studies [20, 127]. Torsion angle experiments are effectively insensitive to intermolecular interactions and can therefore be performed without need for isotopic dilution.

When performed on hydrated and unfrozen samples [138], ssNMR studies can provide many tools to probe dynamics across a wide range of motional time scales. In the context of aggregate studies, these experiments are invaluable in efforts to identify the rigid core of the aggregated state, which commonly features the residues that drive the aggregation process. However, it is also increasingly realized that regions outside the amyloid core proper can

have important consequences for the aggregation process [87, 94]. Scalar-based or J-based methods probe highly dynamic protein segments that are distant from the rigid amyloid core. Selectivity for highly flexible residues can be accomplished by generating initial polarization using refocused INEPT featuring τ_1/τ_2 delays without any ^1H decoupling [110, 139]. Such dynamically disordered segments are observed in many types of amyloids, whether formed from IDPs [45, 77, 87, 110, 126] or by destabilization of a folded protein [32, 103]. It is important to note that not all protein segments outside the amyloid core are seen in such dynamically filtered J-based spectra, as they require a very high degree of flexibility. Proximity to the amyloid core and the presence of secondary structure can imbue non-amyloid flanking domains with intermediate dynamics resulting in short relaxation times [87, 91]. Such residues may be visible in CP spectra, permitting measurements of their dynamics via relaxation and order parameter measurements. However, intermediate motion may also render certain residues effectively invisible in both CP- and INEPT-based spectra obtained at ambient temperatures [140].

Relationship to other techniques in structural biology

The ability of ssNMR methods to provide Å-resolution structural data on protein aggregates remains a unique strength compared to other methods. However, the development of 3D structural models (or structures) requires the incorporation of long-range information. As such, ssNMR measurements are increasingly part of a multi-technique integrated structural biology approach, in which especially EM-based methods play an important role. This includes negative-stain TEM, alongside unstained STEM and dark-field methods. As the evolution of cryo-EM methods continues [16–18], we can foresee exciting opportunities for the further integration of ssNMR and cryo-EM structural data, with an early example in a recent EM/ssNMR study of A β fibrils [17]. Another notable complementary method is EPR spectroscopy, which provides both dynamic insights and long-range structural constraints. Synergy arises not only from the complementarity of obtained information, but also from the fact that EPR spin-labeled samples allow for ssNMR measurements of long-range paramagnetic effects on the nuclear shifts and relaxation properties [141].

Future challenges and (biological) questions

One of the recurring questions in ssNMR studies of disease-related aggregates is whether the aggregates made *in vitro* are truly relevant to the disease. Going forward, one can envision several approaches that address this question, some of which are already in use. The first approach relies on the characteristic feature that many amyloids show a high degree of fidelity in the propagation of strains or polymorphs. This principle has been leveraged to “amplify” small amounts of propagation-capable seeds extracted from patient materials, for instance in studies of A β and prion protein [73, 74]. Whilst limited work on unlabeled patient material has already been reported [96], new ssNMR methods based on ^1H detection and DNP-enhancement may further boost sensitivity to the point that more in-depth ssNMR measurements become feasible. Whilst obtaining large amounts of material of sufficient purity from human patients may remain a challenge, aggregates extracted from disease model animals would also be of great interest and could be more accessible. Alternatively, there are a number of reports of the successful isotopic labeling of certain types of animals, for studies of tissue by ssNMR [142].

A similarly important biological question concerns the need to connect ssNMR-enabled structural findings to the molecular origins of disease toxicity. This can be addressed in part by measuring the cytotoxicity of *in vitro* fibrils or oligomers used for ssNMR, using neuronal cell lines or animals [54, 66, 87, 90]. Integrating structural ssNMR measurements of polymorphic fibrils and oligomers with parallel toxicity measurements will provide exciting opportunities to detail the toxic mechanism behind protein misfolding diseases. An associated challenge comes from the transient nature of the pre-fibrillar species (e.g. soluble oligomers) that may be the most toxic type of misfolded protein aggregates. This feature makes their study by ssNMR challenging, due to the time requirements of (multidimensional) NMR and the destabilizing impact of densely packing the oligomers during sample preparation. Existing studies [53–55, 58] describe a variety of means to stabilize or trap oligomers, including cross-linking, freeze-drying and stabilizing antibodies. Further SSNMR studies may also directly study one of the proposed mechanisms of toxicity: the idea that oligomers disrupt the barrier function of cellular membranes [143]. This is exemplified in several existing studies that shed light on the molecular mechanism of membrane damage, by leveraging the capabilities of ssNMR to probe protein-lipid interactions and the disruption of lipid bilayer structures [52, 144, 145].

A crucial goal in current and future ssNMR-enabled studies of protein misfolding and aggregation related to disease is to facilitate the development of new treatments. On the one hand, SSNMR-enabled studies can be expected to further illuminate the mechanisms of misfolding and aggregation, which are increasingly seen as targets for developing new (and hopefully more effective) drugs [146–149]. Moreover, ssNMR will likely play a key role in studies of the ways in which inhibitors can modulate the aggregation behavior and aggregate structure [57, 150]. A related topic of substantial interest involves the use of ssNMR to learn more about players in the protein homeostasis network that is another key drug target [151]. Many chaperones are oligomeric or disordered (or both), making them challenging for traditional structural studies, but potential targets for ssNMR studies of structure and dynamics [152–154]. A natural extension of the above will be to integrate these types of projects together, with ssNMR used to probe e.g. both the chaperones and their aggregating substrates.

Extrapolating from the work covered in this review, it is clear that we can expect a further progression and expansion of investigations of the molecular mechanisms that drive the misfolding and self-assembly of polypeptides, both in disease and beyond. Seeing the crucial findings already made achieved by ssNMR, and the ongoing developments in advancing ssNMR hardware and methodology, it is clear that ssNMR will be a critical component in future interdisciplinary studies of this important research. As noted, the interest in these topics (and thus the need for ssNMR applications) is growing and spans beyond biochemistry, into other areas such as materials science and pharmaceutical research.

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Highlights

- The aggregation of peptides and proteins is a hallmark of many incurable diseases.
- Solid-state NMR site-specifically probes the conformation of protein aggregates.
- Solid-state NMR studies of dynamics and disordered regions yield unique insights.
- Recent solid-state NMR structures point to common molecular themes and mechanisms.

Fig. 1. Amyloid architecture

(a) Cross- β X-ray diffraction pattern of TTR(105–115) fibrils [18]. (Adapted from Fitzpatrick et al. 2013). (b) Steric zipper sheet-to-sheet interface of GNNQQNY amyloid-like nanocrystals (PDB 1YJP; ref. [11]; adapted with permission from Van der Wel et al., 2007 [19], Copyright 2007 American Chemical Society). (c) Steric zipper interface in polyglutamine amyloid [20]. (Adapted from Hoop et al. 2016). (d) Integrated structural model of TTR(105–115) amyloid fibrils, based on TEM, cryoEM, and ssNMR experiments [18]. (Adapted from Fitzpatrick et al. 2013) (e–h) Basic β -sheet classifications for amyloid assemblies[21]. The black dot marks the position of a particular residue number, while color-coding differentiates the peptide monomers. Note that these dots are at characteristically different distances from each other, a feature used in ssNMR experiments such as the PITHIRDS pulse sequence (see below).

Figure 2. Amyloids with parallel β -solenoid core structures

(a) HET-s fibril structure determined by ssNMR [24], with colorcoding highlighting the fact that two rungs of the solenoid come from a single protein monomer. Reproduced from Wasmer et al. 2008 [24] with permission from AAAS. (b,c) Computational predictions of left- and right-handed β -helical models of curli fibril structure. Adapted with permission from ref. [25], copyright American Chemical Society. Both proteins show extensive intra-molecular interactions that determine the structure and stability of the fibrils. This provides an element of (evolutionary) control that may be essential for these functional amyloids.

Figure 3. Molecular mechanisms of aggregation

(a) Characteristic molecular mechanism of amyloid formation, showing monomers, non-amyloid oligomers and β -rich amyloid aggregates. (b) In some cases, initial self-assembly may not be based on β -rich oligomers, but involve amphipathic α -helices. (c) Potential aggregation mechanisms accessible by non-IDP proteins, adapted from Boatz et al. 2017 [32]. (d) Schematic aggregation curve, where amyloid formation is monitored using the amyloid dye ThT. The nucleation and elongation phases of the process are indicated. The lag phase is associated with the nucleation process, while the elongation rate dictates the slope of the elongation phase.

Figure 4. Recent ssNMR-based IP amyloid structures

(a) Brain-seeded A β 1-40 fibrils, from ref. [48], which adopt the common IP architecture, like most known A β fibrils. It also shows the increasingly common feature of having a compact amyloid core featuring alternating short β -strands and β -arc kinks or bends. (b) Osaka mutant A β 1-40 fibrils also adopt the IP architecture, with twisted sets of short β -strands. Adapted from ref. [43] with permission from Wiley. (c-d) Three independent A β 1-42 structures [44–46] feature similarly alternating β -arcs and short β -strand segments. Adapted with permission from Macmillan Publishers Ltd: Nat. Struct. Mol. Biol., ref. [44], copyright 2015; from ref. [45] (Copyright American Chemical Society), and ref. [46]. (e) The amyloid architecture of α -synuclein fibrils has analogous features, featuring a structural motif that resembles an orthogonal Greek key arrangement [66]. Adapted with permission from Macmillan Publishers Ltd: Nat. Struct. Mol. Biol., ref. [66], copyright 2016. All structures represent the conformation of the immobilized amyloid core (studied by CP-based

ssNMR), with dynamic exposed residues included in the samples but not shown in these representations (see Fig. 5).

Figure 5. SSNMR of amyloid cores and non-amyloid flanking domains

(a) Variable temperature CP-based NCA spectra for human Y145Stop prion protein fibrils. Whilst at 0 °C only narrow signals from the ordered amyloid core show up (red), freezing of the solvent results in the appearance of additional broad signals from the now-immobilized non-amyloid segments of the protein (blue). Adapted from ref. [86]; (b) As illustrated for weak and strong Sup35p NM domain fibrils, J-based 2D INEPT/TOBSY spectra can be used to detect the flexible domains in the unfrozen state. Adapted from ref. [77]. (c) CP/DARR and (d) INEPT/TOBSY spectra of HttEx1 fibrils (44Q variant) detect signals of core and flanking domains, respectively. (e) Combining structural and dynamic ssNMR studies helped reveal the architecture of these HttEx1 fibrils, which feature a β -hairpin polyQ amyloid core and solvent-exposed non-amyloid flanking regions. (f–g) CP-visible signals include regions of different dynamics, as illustrated here with $^{13}\text{C}\alpha$ - $^1\text{H}\alpha$ DIPSHIFT dephasing curves for the amyloid core and its neighboring flanking regions. Panels (c–g) were adapted from ref. [87].