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Perspective

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In Search of Aggregation Pathways of IAPP and Other Amyloidogenic Proteins: Finding Answers through NMR Spectroscopy

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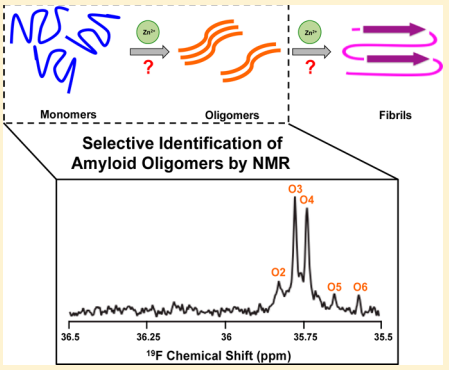
ABSTRACT: The deposition of aggregates of human islet amyloid peptide (hIAPP) has been correlated with the death of insulin-producing beta (β) cells in type II diabetes mellitus. The actual molecular mechanism of cell death remains unknown; however, it has been postulated that the process of aggregation and amyloid fibril growth from monomeric hIAPP is closely involved. Intermediate IAPP aggregates are highly toxic to islet cells, but lack of structural knowledge of these oligomers and complications in applying biophysical techniques to their study have been the main obstacles in designing structure-based therapeutics. Furthermore, the involvement of metal ions (Cu^{2+} and Zn^{2+}) associated with hIAPP has demonstrated an effect on the aggregation pathway. In the absence of well-defined targets, research attempting to attenuate amyloid-linked toxicity has been substantially slowed. Therefore, obtaining high-resolution structural insights on these intermediates through NMR techniques can provide information on preventing IAPP aggregation. In this Perspective, a review of avenues to obtain fundamental new insights into the aggregation pathway of IAPP and other amyloidogenic proteins through NMR and other techniques is presented.



The formation of insoluble protein aggregates by the assembly of initially monomeric protein into highly ordered β-sheet-rich fibers known as amyloids[1,2] has been associated with a growing family of degenerative systemic and neurological diseases. Amyloids are characterized by a cross-β sheet quaternary structure, in which individual β-strands are thought to be oriented orthogonal to the long fiber axis.[3] Human islet amyloid polypeptide (hIAPP), commonly known as amylin, is an amyloidogenic hormone containing 37 amino acids associated with the pathogenesis of type II diabetes.[4] hIAPP is coproduced and cosecreted along with insulin within β-cells, which are found in the pancreas. These cells have been shown to be damaged by the aggregation and fibril formation of IAPP. Islet amyloid deposits are found in more than 90% of patients with diabetes mellitus type 2 (DM2), which strongly suggests that islet amyloidosis are a common pathogenic feature in this multifactorial disease.[5] Other diseases, such as Alzheimer's and Parkinson's, have also been linked with the formation of other misfolded amyloid proteins (amyloid-β (Aβ) and α-synuclein, respectively); however, there appears to be little or no sequence homology between the peptides involved in the various amyloid diseases.[6] A growing body of evidence suggests that fibril formation may follow similar pathways; a nucleation process, in which the formation of numerous small oligomeric assemblies, also known as prefibrillar aggregates, of proteins is believed to precede the formation of mature fibrils.[7−10]

It is critical to study and understand hIAPP oligomers, and the early stages of fibril assembly and biophysical techniques such as NMR may provide the necessary platform to do so.

IAPP Misfolding Remains Uncharacterized. Fibrillization of hIAPP can be affected by many factors. While the exact mechanism by which hIAPP kills β-cells is unknown, disruption of the integrity of the cell membrane may play an important role,[11,12] although many others factors are likely involved.[13] Specifically, oligomeric structures within the membrane can act as largely nonselective ion channels and disrupt membrane permeability and misregulate metal ion homeostasis.[14−16] However, other studies have shown the direct disruption of the membrane by the uptake of lipid molecules during fibrillogenesis into protofibril units.[17,18] Membranes can therefore indirectly contribute to the cytotoxicity of IAPP by accelerating the formation of these toxic species (Figure 1). Although there is much evidence for mechanisms by which hIAPP aggregates cause cytotoxicity, it is becoming crucial to

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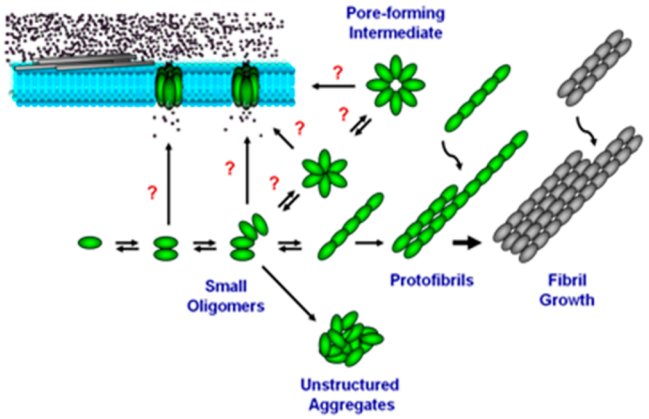

Figure 1. Potential routes toward the formation of toxic oligomers and amyloid fibers from the monomeric state via multiple intermediate states.

identify the (toxic) intermediates along the amyloid pathway.[19,20] These intermediates are structurally diverse, and the role of these species in fibrillation process has been the subject of much debate.[10,21−23] Therefore, it is critical to study and understand hIAPP oligomers and the early stages of fibril assembly and biophysical techniques such as NMR may provide the necessary platform to do so.

How Are IAPP Aggregates Detected? Experimentally characterizing the structures of the individual species involved in the fibrillation process is still extremely difficult,[22,24,25] and simulations continue to play an important role.[26,27] Currently, most of the experimental approaches to analyze the misfolding of IAPP are based on the Aβ protein, which is a more extensively studied amyloidogenic protein than hIAPP. A common method to investigate the process of fibril formation has been the thioflavin T (ThT)-based fluorescence assay as it provides indirect evidence of how individual monomers convert into oligomeric species and eventually to the growth of fibrillar IAPP.[28] Although this method is usually sensitive and reliable for most amyloidogenic proteins in the absence of other cofactors such as small-molecule inhibitors, in some cases, it cannot differentiate between oligomeric intermediates.[29] Furthermore, the ThT fluorescence is dependent on the surface charge of the fiber and therefore can give skewed results with alterations of pH[30] or when highly charged amyloidogenic proteins are considered.[31] A more serious complication occurs when small-molecule inhibitors are introduced as these often either have an intrinsic fluorescence that interferes with ThT detection[32] or bind to a similar site on the amyloid fiber, resulting in a false positive from competitive inhibition.[33,34]

Another method to follow the conformational transitions involved in IAPP amyloid formation is circular dichroism (CD).[35] Changes in secondary structure content can be followed through CD during the fibrillation process. While CD can effectively distinguish between monomers and aggregates, it does not distinguish specifically between amyloid fibers and other β-sheet-containing aggregates and therefore needs to rely on other techniques for quantitative prediction in this respect. CD is also problematic in inhibition studies when compounds that absorb in the near-UV range are considered. Time evolution of the size of aggregates can be tracked by dynamic light scattering (DLS), but the heterogeneous nature of the aggregates poses tremendous challenges to the application of DLS experiments. Furthermore, the accuracy of light scattering experiments on small complexes is severely affected by the presence of even a small percentage of high-molecular-weight species such as amyloid fibers.[36] This can be partially eliminated by running the solution through a size exclusion column (SEC) prior to acquisition.[36] However, in general, separation-based techniques can suffer from serious artifacts when applied to weakly associating aggregates like early intermediates. A careful study showed that SDS gels, a common method for detecting nonfibrillar aggregates,[36] can underestimate the size of oligomers by more than a factor of 20 by fragmenting larger oligomers.[37] Similar, although less severe, results have been obtained with SEC in the absence of detergents.[38] Several other techniques such as atomic force microscopy (AFM) and electron microscopy (EM) can detect the morphology of aggregates. Both transmission and scanning EM have been used comprehensively for low-resolution characterization and visualization of mature protein fibrils.[24] In particular, these techniques have proved invaluable for investigating membrane-bound oligomers,[39] which are inaccessible to most other techniques. However, these methods suffer from extensive surface contact between the amyloid and sample support[24] and have been shown to show a bias toward certain oligomers with a high affinity toward the surface.[37]

The ideal method for biophysical characterization would therefore (1) be sensitive enough to pick up intermediate species that likely occur at low population; (2) not suffer from interference effects when used with small-molecule inhibitors; (3) be nonperturbative; (4) have a high distinguishing capability between different aggregates; (5) be applicable to a broad range of aggregates in terms of size and structure; (6) be capable of providing an unbiased view of the total aggregates present, or at least an unbiased view of a subset that can be defined in a precise way; and (7) yield atomic-level structural details. While no method currently meets all of these requirements, along these lines, atomic ultracentrifugation (AUC) has also been employed to study the aggregation pathway of IAPP under equilibrium conditions.[40] Sedimentation has isolated monomeric and fibrillar species; however, the long experiment duration has been a drawback to utilize this technique for studying other intermediates. Recently, X-ray fiber diffraction and small-angle X-ray scattering (SAXS) have been used to study amyloid structure with similar goals.[41] Fiber diffraction provides spatial information on frequently occurring distances in the amyloid structures and provides structural fingerprints to identify the cross-β sheet pattern of fibers while SAXS has a similar objective in the solution state;[42] however, the relatively low sensitivity and limited time frame of acquisition due to radiation damage limit its application in time-resolved studies.[43] Two-dimensional infrared spectroscopy (2D-IR) has also been utilized to understand the secondary and tertiary structures of IAPP aggregates during aggregation.[44] By specifically labeling residues and measuring frequency shifts, 2D-IR has given information regarding residues in the β-sheet versus turn region of the peptide, which has provided insights into the fiber structure. This technique can also be used for rapid amyloid formation kinetic measurements.[45] Finally, EPR spectroscopy can provide long-range constraints difficult to obtain by other means, which in combination with molecular modeling are useful in establishing contacts between protofilaments within the amyloid fiber[46] and refining amyloid fiber structures.[47] Overall, various techniques have been utilized to tackle the need in the characterization of oligomeric species. Nonetheless, progress in this area has been hindered by the

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inability to track amyloid formation at atomic-level resolution in real time.

Through ^{19}F NMR and saturation transfer difference experiments (specific for binding to the fiber and other large aggregates), it could be confirmed that the inhibitor EGCG (epigallocatechin-3-gallate) has weak to nonexistent binding to the monomeric state, is a competitive inhibitor for ThT on the fiber surface at likely the same binding site, and forms nonfibrillar oligomers but does not dissociate preformed fibers.

Tracking the Misfolding Pathway through NMR. Real time NMR is another promising alternative that fulfills most of the seven requirements listed above. NMR is moderately sensitive in modern spectrometers (spectra of 1 μM Aβ_{1-40} can be recorded),[48] is nonperturbative, can easily track aggregation in the presence of inhibitors using labeled protein samples, detects a broad range of oligomers within certain size limits, and can yield rapid atomic-level structural details. While most of the spectra obtained from common solution NMR experiments are only observable for smaller oligomers, larger oligomers (up to 50 nm)[49] give rise to a characteristic spectra that is easily identified by pulse field gradient experiments.[50−52] A particular advantage of real time NMR is the large amount of data encoded even in a 1D experiment and its ability to track the individual components of a heterogeneous sample (for example, monomers, small oligomers, amyloid fibers) separately, at equilibrium, and nonperturbatively through differences in their chemical shift frequency or diffusion constants.[49,52] In this respect, real time NMR can therefore provide a clearer understanding of the early stages of amyloid assembly and other amyloidogenic proteins. However, traditional NMR experiments on amyloids suffer from low sensitivity and low time resolution, severely limiting their applications to investigate a class of proteins that inherently have low solubility and display time-dependent aggregation. Despite these challenges, NMR has been able to track the conformational changes that occur during aggregation in real time for a limited number of amyloidogenic proteins.[53−55] In particular, the aggregation of hIAPP at pH 6 and 4 °C is slow enough that changes in ^{1}H or ^{15}N chemical shift values have provided residue-specific information on the amyloid nucleation process.[54] This study showed that the peaks for individual residues do not disappear uniformly. Instead, the N-terminal cross peaks disappear before the C-terminal cross peaks, implying that the formation of large aggregates that are invisible to NMR begins with the formation of N-terminal contacts.[54]

However, the time resolution of NMR is currently limited by the time requirements to acquire multidimensional spectra using conventional radio frequency pulse sequences. To track the transient formation of small oligomers of hIAPP, accelerated versions of the traditional HMQC and TROSY techniques (SOFAST-HMQC and BEST-TROSY)[56,57] can be used to acquire real time 2D NMR spectra in ∼5 min compared to >60 min for a conventional spectrum (Figure 2a). In an earlier study, diffusion-based NMR experiments were used to show that IAPP does not form a stable population of small oligomers;[49] however, the limited time resolution of these experiments prevented a direct detection of transient oligomers. Using the SOFAST-HMQC technique, transient oligomer formation is readily detectable during the lag phase of aggregation (approximately the first 200 min in Figure 2b).

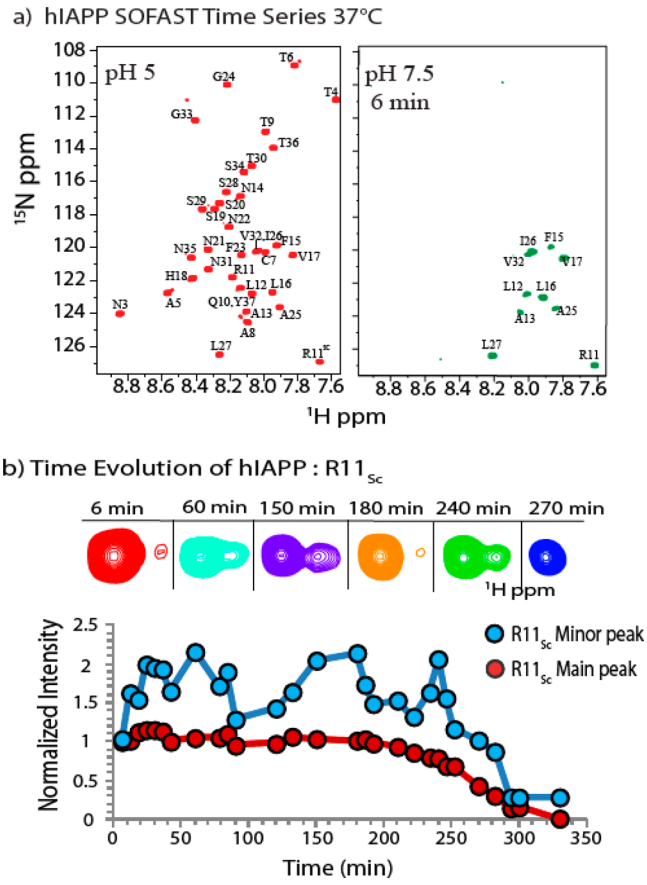

Figure 2. Real time, residue-specific kinetics of aggregation using SOFAST-HMQC NMR. (a) Representative SOFAST spectra of IAPP acquired at 37 °C. (b) Time evolution of the R11 side-chain resonance at 37 °C showing the emergence of intermediate conformations during the lag phase of aggregation (Unpublished data, Manuscript in preparation).

A recent study also has shown that alternative pathways of hIAPP aggregation can be successfully distinguished by using ^{19}F NMR by substituting Phe23 with 4-trifluoromethylphenylalanine (tfmF).[33] ^{19}F NMR provides a direct, sensitive, and real time measurement of amyloid formation by monitoring the consumption of monomeric protein. Due to the large chemical shift dispersion of the ^{19}F nucleus, small changes in structure and aggregation state can be directly observed by ^{19}F NMR for the monomeric species and small aggregates (<100 kDa). From the coincidence of the ThT, CD, and ^{19}F kinetics (Figure 3a) and the absence of new ^{19}F peaks (Figure 3b), it is apparent that IAPP fibrillizes without an appreciable buildup of small, nonfibrillar intermediates aggregates, in agreement with previous sedimentation equilibrium[40] and pulsed field gradient NMR experiments[49] at low time resolution. This is very

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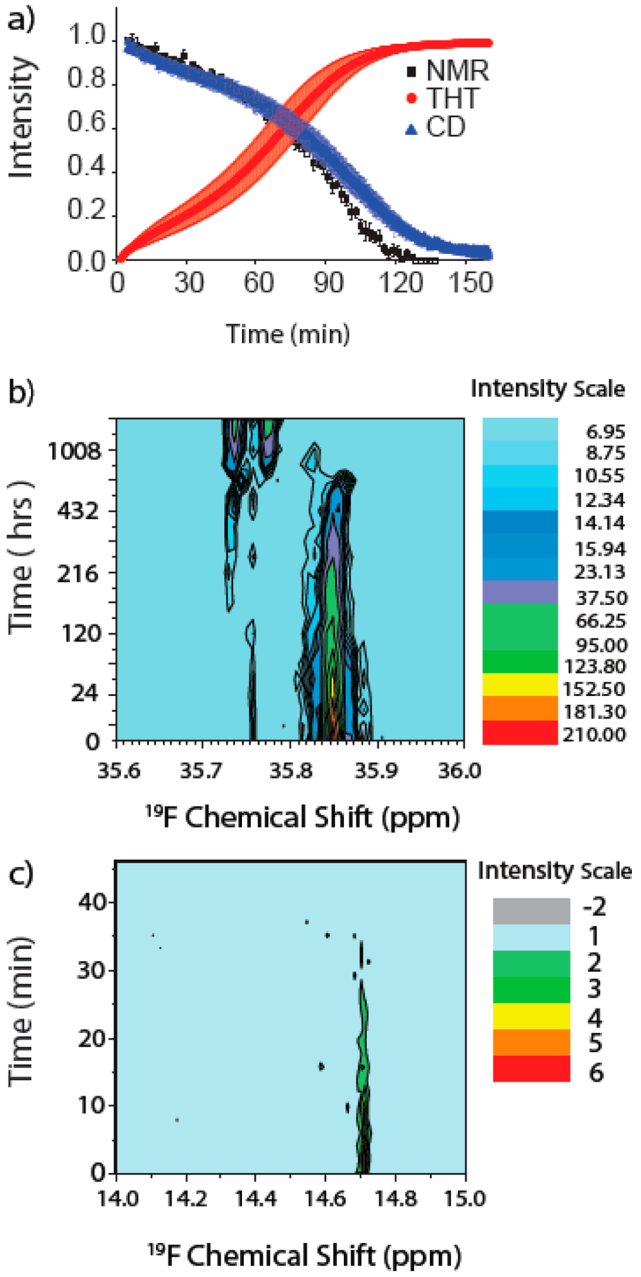

Figure 3. Comparison of the rates of monomer consumption and fiber formation. (a) Overlay of kinetic traces obtained via ^{19}F NMR (black), ThT fluorescence (red), and CD (blue).[33] Contour plots showing changes in the ^{19}F spectrum of amyloid as a function of time. (b) Aβ1-40-tfM$_{35}$ (182 μM)[58] and (c) IAPP-tfmF$_{35}$ (85 μM) at 37 °C showing the absence of new peaks from small species that may be formed during the aggregation[33] (Reprinted with permission, Copyright 2012, American Chemical Society).

different from Aβ_{1-40}, which is commonly used as model for amyloid formation from unstructured proteins and peptides. Similar experiments with ^{19}F-labeled Aβ_{1-40} showed the existence of six small oligomeric species during aggregation, indicating a fundamentally different aggregation mechanism than IAPP (Figure 3c).[58] Interestingly, some of these small aggregates persisted well after fiber formation appeared to be complete.[58] Finally, ^{19}F NMR shows that the inhibitor EGCG (epigallocatechin-3-gallate) has weak to nonexistent binding to the monomeric state, is a competitive inhibitor for ThT on the fiber surface at likely the same binding site as ThT, and forms nonfibrillar oligomers but does not dissociate preformed fibers of IAPP.[33] In a similar manner, using saturation transfer difference NMR experiments specific for probing the binding of small molecules to the fiber and other large aggregates, it is possible to identify the functional groups within a small-molecule inhibitor responsible for binding. Such experiments have been used to successfully validate the docked conformation of another small-molecule inhibitor to amyloid fibers.[59] NMR can therefore give substantial information on inhibitor binding beyond what is available from simple kinetic studies.

Real time 2D NMR spectroscopy has also been used to study the involvement of zinc in IAPP aggregation. Metal ions have been implicated to be involved in the aggregation and toxicity of IAPP. Studies have investigated the direct involvement of metal ions, specifically zinc (Zn(II)) and copper (Cu(II)); however, the physiological roles of metals have not been clearly revealed.[60−64] High levels of Zn(II) have been found in β-cells, which is also where IAPP is produced and stored.[60,65−67] Binding has been suggested to occur in a 6:1 peptide/Zn(II) molar ratio with an octahedral geometry and a low micromolar binding affinity, suggesting strong interactions between the peptide and metal.[64] Fibril formation of IAPP is significantly suppressed at concentrations of Zn(II) similar to those found in the extracellular matrix after secretion (10−25 μM). The fiber binds Zn(II) with a weaker affinity, and the metal ion is displaced upon fibrillation due to an unfavorable coordination environment in the fibril structure.[60] EXAFS experiments suggest that oxygen or nitrogen donor atoms are involved in coordination, consistent with imidazole nitrogens from multiple peptides.[61] Experimental evidence also suggests His18 as the primary residue to be involved in Zn(II) coordination. The simplest explanation, therefore, is that Zn(II) binds a hexamer of IAPP, shifting the equilibrium away from the fiber state and possibly preventing the addition of IAPP hexamers to other aggregates.

Analysis by real time NMR and other biophysical techniques reveals a considerably more complicated picture. Such a Zn-bound hexamer should be detectable by ^{1}H NMR and should show large chemical shift deviations upon zinc binding such as those found for Aβ.[68] Indeed, such changes can be found in organic solvents when IAPP is forced to adopt a helical structure and prevented from aggregating.[60] However, under more physiological conditions, the imidazole chemical shift is unchanged (Figure 4).[61] Instead, large changes are seen in the aliphatic regions implicated in peptide−peptide interactions for IAPP (Figure 4).[61] This finding suggests that Zn is binding to a large, NMR-invisible oligomer, a finding supported by mass spectrometry experiments, indicating that only a fraction of the available IAPP is binding Zn. Consistent with zinc-catalyzed oligomer formation, real time NMR shows a steady, nearly uniform drop in intensity during the lag phase not seen in zinc-free samples, which is in agreement with time-lapse AFM images showing dense aggregates in the presence of Zn(II) at short incubation times compared to the metal-free samples. A possible explanation for the effect of micromolar Zn(II) on IAPP aggregation is the diversion of the aggregation pathway to non-ThT binding aggregates, whose cellular toxicity remains undetermined. However, the actual pathway may be even more complicated. At higher Zn(II) concentrations reflective of the free zinc concentration in the secretory granule where zinc is stored (1 mM and above), the lag phase is decreased, and the

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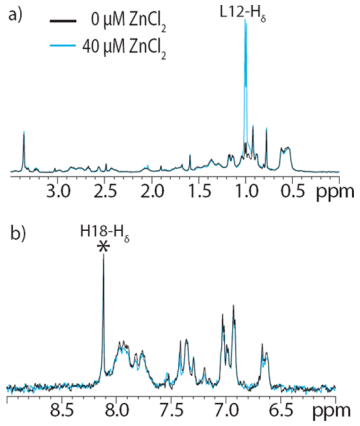

Figure 4. (a) Aliphatic and (b) amide regions of the ^{1}H spectra of 78 μM hIAPP$_{1-19}$ with (blue) and without (black) 40 μM $ZnCl_2$. The imidazole resonance is marked with an asterisk (*)[61] (Reprinted with permission, Copyright 2013, Royal Society of Chemistry).

fibril growth rate is accelerated with the total amount of amyloid fibril reduced at equilibrium.[60,64] The change in kinetics at high zinc concentrations may either reflect a coordination event at a secondary metal binding site or binding to a low-affinity species such as the amyloid fiber. Though preliminary studies to investigate the metal coordination and effects of metal on IAPP aggregation have progressed, high-resolution insights into metal-bound hIAPP species such as those obtained for Cu-bound Aβ[69] have yet to be revealed.

The use of dipolar recoupling magic angle spinning (MAS) solid-state NMR techniques, in combination with dynamic nuclear polarization (DNP) and ultrafast MAS, can provide piercing insights into intermolecular interactions that drive the aggregation process.

The various studies mentioned here clearly demonstrate the advantage of using NMR, in combination with other biophysical techniques, to follow fibrillogenesis in real time. Two areas can be identified as focuses for the further development of the technique, (1) computational techniques to identify the intermediate species directly from a series of multidimensional NMR data[70] and (2) solid-state NMR techniques to monitor large aggregates and membrane-bound species. In this respect, the use of dipolar recoupling magic angle spinning (MAS) solid-state NMR techniques, in combination with dynamic nuclear polarization (DNP) and ultrafast MAS, can provide piercing insights into intermolecular interactions that drive the aggregation process. Internuclear distances measured from these experiments can render high-resolution structures of intermediates that are difficult to obtain by other techniques.

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Notes

The authors declare no competing financial interest.

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