Direct observation of minimum-sized amyloid fibrils using solution NMR spectroscopy

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Abstract: It is challenging to investigate the structure and dynamics of amyloid fibrils at the residue and atomic resolution because of their high molecular weight and heterogeneous properties. Here, we used solution nuclear magnetic resonance (NMR) spectroscopy to characterize the conformation and flexibility of amyloid fibrils of β_2 -microglobulin (β_2 m), for which direct observation of solution NMR could not be made. Ultrasonication led to fragmentation producing a solution of minimum-sized fibrils with a molecular weight of around 6 MDa. In ^1H - ^{15}N heteronuclear single-quantum correlation measurements, five signals, derived from N-terminal residues (i.e., Ile1, Gln2, Arg3, Thr4, and Lys6), were newly detected. Signal strength decreased with the distance from the N-terminal end. Capping experiments with the unlabeled β_2 m monomer indicated that the signals originated from molecules located inside the fibrils. Ultrasonication makes the residues with moderate flexibility observable by reducing size of the fibrils. Thus, solution NMR measurements of ultrasonicated fibrils will be promising for studying the structure and dynamics of fibrils.

Keywords: β_2 -microglobulin; amyloidosis; protein misfolding; ultrasonication; HSQC spectra; capping of fibril ends

Introduction

Contrary to Anfinsen's dogma that the amino acid sequence determines the unique native structure with a free energy minimum, some proteins misfold into an alternative unique conformation known as amyloid fibrils.¹ Amyloid fibrils are physically stable

self-assemblies, the deposition of which is associated with fatal diseases including Alzheimer's disease, prion disease, human islet amyloid polypeptide, and dialysis-related amyloidosis.^{2–5} Various studies have been performed to elucidate the structural and physical properties of amyloid fibrils using microscopic images, X-ray fiber diffraction, circular dichroism, and so on. However, it is often difficult to characterize the structure and dynamics of amyloid fibrils using techniques used to analyze protein structure at the atomic level such as solution nuclear magnetic resonance (NMR) and X-ray crystallography because of their huge size and heterogeneous nature. Recently, solid-state NMR techniques have been used to determine the structure of amyloid fibrils^{6–11} and aggregation intermediates,^{12,13} and X-ray crystallography has been applied to amyloid-like microcrystals.^{14,15} Furthermore, a general method to

Abbreviations: 1D, one dimensional; β_2 m, β_2 -microglobulin; AFM, atomic force microscopy; H/D, hydrogen/deuterium; HSQC, heteronuclear single-quantum correlation; INEPT, insensitive nuclei enhanced by polarization transfer; NMR, nuclear magnetic resonance; PFG, pulsed-field gradient; ThT, thioflavin T.

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analyze the structure of amyloid fibrils using solution NMR has been developed, which combines hydrogen/deuterium (H/D) exchange of amide groups and subsequent dissolution of amyloid fibrils into a monomer by dimethyl sulfoxide, an aprotic organic solvent, to detect hydrogen-bonded amide groups.^{16–19}

In this study, we aimed to characterize the dynamics of amyloid fibrils using solution NMR. Although amyloid fibrils are too large to be observed directly in NMR experiments, highly mobile regions within the fibrils can be detected using conventional NMR experiments.^{18,20–23} However, this is not true for the amyloid fibrils of human β_2 -microglobulin (β_2m),²⁴ a light chain of the type I major histocompatibility complex and the main component of the amyloid fibrils deposited in the synovia of the carpal tunnel of patients suffering from dialysis-related amyloidosis.^{3,25} The results suggest that the absence of highly flexible regions or local mobility of β_2m fibrils makes detection by solution NMR difficult.

More recently, magic-angle spinning solid-state NMR of β_2m fibrils has been reported in which ¹³C and ¹⁵N resonance assignments for 64 residues among 99 residues, including part of the highly mobile N-terminus, have been performed.²⁶ Chemical shift analysis of the sequentially assigned residues indicated that these fibrils contain an extensive β -sheet core organized in a non-native manner. Although the solid-state NMR is a promising approach for studying the structure and dynamics of β_2m fibrils,^{26,27} it will be important to explore additional approaches.

To overcome the problem of large size, preformed fibrils were fragmented by ultrasonic waves. We previously reported that ultrasonication is useful for preparing minimum-sized and relatively monodispersed amyloid fibrils of β_2m , which is achieved by the free energy minimum under competition between ultrasonication-induced fibril production and breakdown.²⁸ The ultrasonication produced β_2m fibrils with a minimum molecular size, their structural and chemical properties remaining unchanged. With the fragmented β_2m fibrils, we succeeded in observing directly the solution NMR signals of β_2m fibrils, which were broadened beyond detection for the unfragmented fibrils.

Results

Ultrasonicated β_2m fibrils

β_2m fibrils were prepared by a seeding-dependent method²⁵ at 37°C. The formation of fibrils was monitored by measuring the fluorescence of thioflavin T (ThT).²⁵ Fragmentation of the formed fibril was performed as reported.²⁸ Atomic force microscopy (AFM) images confirmed that the fibrils were fragmented by ultrasonic waves [Fig. 1(A,B)]. We also confirmed that ultrasonic waves did not bring about large changes in the intensity of the ThT fluorescence [Fig. 1(C)]. To examine the apparent molecular

weight of the fragmented fibrils, sedimentation equilibrium measurements were conducted [Fig. 1(D)]. From the fitting, we obtained a molecular weight value of 6.2×10^6 , which corresponds to ~ 500 mer.

1D ¹H and pulsed-field gradient NMR spectra

Then, we investigated the effects of the size of β_2m fibrils on solution NMR spectra. We recorded one-dimensional (1D) ¹H spectra of the unfragmented and fragmented β_2m fibrils in solution of deuterated solvent at 37°C [Fig. 2(A)]. The NMR spectrum of the unfragmented fibrils showed few signals. Because the fibrils are hundreds of nanometers long, which corresponds to a size of several tens of MDa, they may be too large to be detected with solution NMR measurements. The results were consistent with those reported, suggesting the absence of highly flexible regions or that the local mobility of β_2m fibrils is insufficient to be detected by solution NMR.²⁴

The spectrum observed for the fragmented fibrils was distinct from that for monomeric β_2m in the acid-denatured state [Fig. 2(A)], indicating that the NMR signals observed for the fragmented fibrils are not for the dissociated monomers. The present results obtained in ²H₂O are similar to those in H₂O.²⁸ These results suggest that a reduction in the size of the fibrils by ultrasonication allows a decrease in the overall rotational correlation time, making the direct observation of moderately flexible regions possible.

To distinguish between the β_2m fibrils and monomers on the basis of molecular diffusion, pulsed-field gradient (PFG) ¹H NMR experiments were performed [Fig. 2(B)] at 37°C.^{29,30} From the fitting, we obtained effective diffusion coefficient (*D*) values of $(1.17 \pm 0.01) \times 10^{-10}$ m²/s for the acid-denatured monomers and $(6.02 \pm 0.43) \times 10^{-11}$ m²/s for the fragmented fibrils. The *D* values for 100-residue proteins in the denatured state are estimated to be 8.86×10^{-11} m²/s based on the equation reported by Wilkins *et al.*³¹ Considering the presence of a disulfide bond, the observed *D* value is likely valid for denatured β_2m . The difference in diffusion coefficient values between the β_2m monomers and fibrils also supports that NMR signals of the fragmented fibrils do not originate from monomers in equilibrium with fibrils but indeed from the fibrillar species. The *D* value obtained for the fragmented fibrils corresponds to a molecular weight of 3.3×10^5 on the basis of the Einstein–Stokes equation, using the viscosity value of 8.4×10^{-4} Pa s for deuterated solvent. This is obviously inconsistent with the fact that amyloid fibrils are a large molecular assembly of monomeric protein molecules. However, the effective diffusion coefficient values of large and rod-shaped macromolecules include rotational motion in addition to translational motion, making the

Figure 1. Effects of ultrasonication monitored by AFM, ThT assay, and analytical ultracentrifuge. (A, B) AFM images of $\beta 2m$ fibrils before (A) and after (B) ultrasonication. The black bar represents 1 μm . (C) ThT fluorescence intensity of $\beta 2m$ fibrils before (blue) and after (red) ultrasonication. Error bars indicate five independent experiments. (D) Sedimentation equilibrium measurements of fragmented $\beta 2m$ fibrils. The solid line represents a theoretically fitted curve using Eq. (1).

apparent diffusion coefficient much smaller than that expected for spherical particles.^{30,32}

HSQC spectra

We recorded 1H - ^{15}N heteronuclear single-quantum correlation (HSQC) spectra of the fragmented and unfragmented $\beta 2m$ fibrils in solution of 10% 2H_2O at 37°C. The ^{15}N -edited 1H spectrum obtained using the 1D version of 1H - ^{15}N HSQC contained no signals for unfragmented $\beta 2m$ fibrils, but several signals for fragmented fibrils [Fig. 3(A)]. The two-dimensional 1H - ^{15}N HSQC spectrum of the fragmented $\beta 2m$ fibrils included five signals for main-chain amide protons. These signals were assigned because they showed chemical shifts identical to those of the monomer [Fig. 3(B)]. All of the signals belonged to the N-terminal region (Ile1, Gln2, Arg3, Thr4, and Lys6) based on the assignment for the acid-denatured monomer, indicating that the N-terminal residues of the fragmented fibrils have enough mobility to be detected. Ile1 had the strongest signal with a gradual decrease in intensity along the sequence [Fig. 3(C)], indicative of a decrease in local mobility. Importantly, the signals from the fragmented fibrils

were weaker than those from the monomer (i.e., 0.15 for Ile1 at most) indicating that the residues in fibrils are less dynamic.

To examine the possibility that only molecules located at the edges of the fragmented $\beta 2m$ fibrils contribute to the NMR signals, the fragmented ^{15}N -labeled fibrils were capped with unlabeled $\beta 2m$ molecules. To the fragmented fibrils composed of ^{15}N -labeled $\beta 2m$, 10% (w/w) ^{14}N - $\beta 2m$ monomer was added, and the solution was incubated at 37°C overnight to elongate the fibril ends with unlabeled monomers. Assuming that a molecular weight value before capping is 6.2×10^6 , as described above, and that all the monomers are used for capping, we estimated that 50 monomers are attached to each fibril. The sedimentation pattern shifted slightly to the direction of larger molecular weight, consistent with the successful capping of the fibrils [Fig. 4(A)]. The 1H - ^{15}N HSQC spectrum of the capped fibrils did not exhibit a significant decrease in signal strength compared with the uncapped fibrils [Fig. 4(B,C)], suggesting that the observed signals for the uncapped fibrils mostly arise from the N-terminal region of the molecules located in the middle of

Figure 2. Effects of ultrasonication of fibrils on solution NMR. (A) 1D ^1H spectra of $\beta 2\text{m}$ monomers in the acid-denatured state (black), fragmented $\beta 2\text{m}$ fibrils (red), and unfragmented $\beta 2\text{m}$ fibrils (blue). (B) Normalized signal intensity decay from PFG ^1H NMR spectra of $\beta 2\text{m}$ monomers in an acid-denatured state (black) and fragmented $\beta 2\text{m}$ fibrils (red). The solid lines represent a theoretically fitted curve using Eq. (2).

fibrils. It is also noted that the intensities of the capped fibrils were slightly lower than those of the uncapped fibrils because of the longer tumbling time.

Discussion

Although no HSQC signals were obtained for the untreated $\beta 2\text{m}$ fibrils, we could see several residues of the N-terminal region after the extensive fragmentation by ultrasonication. The results indicate that the N-terminal region of $\beta 2\text{m}$ fibrils is “moderately” flexible. We previously reported that the structure of the $\beta 2\text{m}$ molecules within the fibrils is maintained after fragmentation.²⁸ The N-terminal residues of $\beta 2\text{m}$ fibrils are not so highly flexible that solution NMR signals of these residues were not detected until fragmentation of the fibrils. The capping of the edges of the fibrils with unlabeled monomers indicated that they were not derived from monomers remaining in solution. The capping

experiments also imply that the N-terminal region of $\beta 2\text{m}$ in solution may not be involved in the direct interaction in fibril formation.

There are several reports of the flexibility of the N- and C-terminals of $\beta 2\text{m}$ fibrils. H/D exchange experiments combined with the dissolution of fibrils

Figure 3. NMR spectroscopy of fragmented fibrils. (A) ^{15}N -edited 1D ^1H spectra of the $\beta 2\text{m}$ monomer in an acid-denatured state (black), unfragmented $\beta 2\text{m}$ fibrils (blue), and fragmented $\beta 2\text{m}$ fibrils (red). (B) ^1H - ^{15}N HSQC spectra of $\beta 2\text{m}$ monomers in the acid-denatured state (black) and fragmented $\beta 2\text{m}$ fibrils (red). Signals in the spectrum of the fragmented $\beta 2\text{m}$ fibrils were assigned. (C) Signal intensity of Ile1, Gln2, Arg3, Thr4, and Lys6 in the spectra of fragmented $\beta 2\text{m}$ fibrils and relative intensity (I_{rel}) calculated with the equation $I_{\text{rel}} = (I_i/256)/(I_m/16)$, in which I_i is the intensity for the fragmented $\beta 2\text{m}$ fibrils and I_m that for the monomer.

Figure 4. Capping of fibril ends with ^{14}N - $\beta 2\text{m}$. (A) Integral distribution plots of uncapped (red) and capped (green) $\beta 2\text{m}$ fibrils. (B) ^{15}N -edited 1D ^1H spectra of uncapped $\beta 2\text{m}$ fibrils [red, the same as the fragmented $\beta 2\text{m}$ fibrils shown in Fig. 3(A)] and capped $\beta 2\text{m}$ fibrils (green). (C) ^1H - ^{15}N HSQC spectra of uncapped $\beta 2\text{m}$ fibrils [left, the same as the fragmented $\beta 2\text{m}$ fibrils shown in Fig. 3(B)] and capped $\beta 2\text{m}$ fibrils (right).

at a high concentration of dimethyl sulfoxide and solution NMR measurements indicated flexible regions.^{16,17} Limited proteolysis experiments showed that $\beta 2\text{m}$ fibrils were cleaved at Val9 by pepsin, revealing a flexible N-terminal region.²⁴ A recent study with spin labeling of $\beta 2\text{m}$ fibrils³³ also confirmed the flexibility of the N-terminal and C-terminal areas. More recently, insensitive nuclei enhanced by polarization transfer (INEPT)-based solid-state NMR under magic-angle spinning condition revealed the highly mobile N-terminus.²⁶ Moreover, they indicated that these fibrils contain an extensive β -sheet

core organized in a non-native manner. However, there has been no direct solution NMR data with unfragmented $\beta 2\text{m}$ fibrils despite the flexibility of N-terminal residues.

There have been several reports that highly mobile regions within large complexes can be detected using solution NMR measurements. For example, NMR evidence of highly mobile terminal regions had been reported for the small heat shock protein Hsp25.³⁴ The C-terminal 18 amino acids showed a large flexibility with motion that is essentially independent of the domain core of the protein. A number of studies with other amyloid fibrils also have characterized the highly flexible regions using solution NMR.^{18,20–23}

Sillen *et al.*²⁰ studied the paired helical filaments of tau, a protein made of 441 residues, by NMR spectroscopy. Under conditions where the monomer and filaments coexist, with the proportion of monomer around 10%, signal intensity on the basis of the assignment of the tau monomer revealed different regions of residual mobility: the N-terminal domain was found to maintain solution-like dynamics and was followed by a large domain of decreasing mobility; finally, the core region Ser262-Thr377 was distinguished by a solid-like character. Heteronuclear-NOE data indicated that the decrease in mobility was due to both a slowing down of the rapid nanosecond movements and the introduction of slower movements that lead to exchange broadening. The results differ from our finding in that a significant proportion of the residues are highly flexible and detected even without special treatment to prepare shorter fibrils like the ultrasonication used here.

Baldwin *et al.*²¹ constructed and expressed a gene for a 294-residue protein, $(\text{SH}_3)_2\text{Cyt}$, with cytochrome fused to the C-terminus of the tandem repeat of an 86-residue SH3 domain, $(\text{SH}_3)_2$. $(\text{SH}_3)_2\text{Cyt}$ forms fibrils both in apo and heme-bound holo forms. 1D ^1H NMR spectra of apo- $(\text{SH}_3)_2\text{Cyt}$ fibrils showed sharp resonances characteristic of a mobile unfolded protein that were not observed for the holo fibrils. The results indicate that the unfolded apoprotein attached to the fibrils has extensive motional freedom but that, once heme is bound, the folded cytochrome in the fibril is motionally restricted and shows resonances too broad to be detected. PFG NMR data were then recorded for the resolved resonances.³⁰ The analysis including the effects of rotational diffusion yielded an estimate of the lengths of fibrils that is of the same order of magnitude as that measured by AFM and transmission electron microscopy.

Siemer *et al.*²² reported flexible amino acid residues of a prion fragment, HET-s(218–289), in its fibrillar state. These residues provided sharp proton resonances under high-resolution magic-angle spinning conditions, which were undetected in the past,

indicating that the averaging by local motion is almost complete.

Meehan *et al.*²³ showed that the last 12 amino acid residues of the C-terminal hydrophilic region of α B-crystallin can be observed with ^1H NMR spectroscopy, and that the region is flexible both in solution and in amyloid fibrils, where it protrudes from the fibrillar core.

Toyama *et al.*¹⁸ analyzed SupNM fibers by solution NMR. SupNM is composed of a Gln/Asn-rich N-terminal domain (N, amino-acid residues 1–123) and a highly charged middle domain (M, residues 124–253). ^1H - ^{15}N HSQC spectra from uniformly ^{15}N -labeled SupNM fibers revealed multiple robust signals. SupNM contains eight Leu residues, and seven of which are in the M domain. NMR spectra of SupNM fibers in which the leucine residues were specifically ^{15}N -labeled showed at least four robust signals with a smaller additional signal. These data together with other pieces of evidence indicated that, in solution, large regions of the M domain remain highly mobile even in the fiber.

Solution NMR has been used to characterize the $\beta 2\text{m}$ molecule in the native and acid-denatured state.^{35–37} It has been also applied for the investigations of fibril formation and folding process of $\beta 2\text{m}$.^{38–42} As for $\beta 2\text{m}$ fibrils, Platt *et al.*²⁴ carefully analyzed the ^1H - ^{15}N HSQC spectra of amyloid fibrils. Although they observed signals for the solution of fibrils, the capping of fibril ends with unlabeled monomers indicated that they were derived from the monomer remaining in solution. On the other hand, a mutant with an elongated sequence comprising six glycine and three serine residues inserted at the N-terminal of Ile1 exhibits signals for the elongated sequence independent of protein conformation and the capping, suggesting that these signals arise no longer from the monomer and that the elongated sequence is a good probe for $\beta 2\text{m}$ fibrils. Bellotti *et al.*⁴³ reported that limited proteolysis at Lys6 and Tyr10 was observed in the $\beta 2\text{m}$ molecule involved in amyloid fibrils from a patient who had been under chronic hemodialysis. The results indicate that, although the 10–20 N-terminal residues of $\beta 2\text{m}$ in the fibrillar form are susceptible to both proteolysis and hydrogen exchange, this region does not display enough mobility independent of the fibril body to be detected in HSQC spectra. It was proposed that although Val9 is exposed in the amyloid-like form of $\beta 2\text{m}$, substantial and stable interactions must exist between the ~ 20 N-terminal residues and the rest of the fibril core such that this region of the polypeptide chain does not display dynamics, at least on the nanosecond to picosecond timescale, independent of the remainder of the fibril. More recently, magic-angle spinning solid-state NMR of $\beta 2\text{m}$ fibrils has suggested that the first few N-terminal residues (MIQRTPK) of $\beta 2\text{m}$ are mobile in the fibrils.²⁶

Their results are consistent with our finding that signals were not observed for unfragmented fibrils. Fragmentation of fibrils by ultrasonication to a size of around 6,000,000 enabled the detection of N-terminal residues, although the signal was less intense than that of monomers. Thus, the mobility is less than that of highly flexible regions of other fibrils studied so far, but definitely higher than that of the tightly packed core. Other methods also indicated the high mobility of the C-terminal residues.^{16,17} Although more signals emerged on lowering the threshold of the ^1H - ^{15}N HSQC spectrum of the fragmented $\beta 2\text{m}$ fibrils, which might be the signals of C-terminal residues, they were much lower than those of N-terminal residues and therefore unable to be assigned, suggesting that C-terminal residues were less mobile than N-terminal residues.

Conclusion

It has been established that, although the overall size of amyloid fibrils exceeds the limit for solution NMR experiments, highly mobile noncore regions allow for local motion, thus yielding NMR signals. However, this also depends on a combination of the local dynamics of the region and slow overall tumbling of the entire fibrils. Although nothing was detected for the unfragmented $\beta 2\text{m}$ fibrils, the N-terminal residues were detected with fragmented fibrils. This suggests that fragmentation of fibrils decreases the molecular tumbling time, thus making the observation of the moderately flexible N-terminal residues possible. This approach with ultrasonication-dependent fragmentation can be applied to various amyloid fibrils addressing further the structure and dynamics of regions with moderate dynamics. Moreover, combined with specialized spectroscopic techniques such as transverse relaxation-optimized spectroscopy,^{44,45} which can achieve improved sensitivity and resolution for perdeuterated large proteins, direct observation of rigid core regions of amyloid fibrils might be possible.

Materials and Methods

Ultrasonicated $\beta 2\text{m}$ fibrils

Recombinant human $\beta 2\text{m}$ protein was expressed in *Escherichia coli* as previously reported.⁴⁶ It should be noted that an additional Met0 is present at the N terminus. $\beta 2\text{m}$ fibrils were prepared by a seeding-dependent method.²⁵ The seeding was carried out at 37°C without agitation by adding 0.09 mg/mL of $\beta 2\text{m}$ seeds to a solution of the monomer at a concentration of 3 mg/mL in 3.2 mM HCl and 100 mM NaCl. The fragmentation of $\beta 2\text{m}$ fibrils was performed using a water bath-type ultrasonic transmitter with a temperature controller (ELESTEIN SPO70-PG-M, Elekon, Tokyo), on which the solution

was placed and subject to 18 cycles of ultrasonication for 1 min at 9-min intervals^{28,47} at 7°C. The frequency of the ultrasonic waves was 17–20 kHz, and the power of output was 700 W.

AFM images

The sample solution was diluted 100-fold with water and 10 μ L was spotted onto freshly cleaved mica. After 1 min, the residual solution was blown off with compressed air. AFM images were obtained using a NanoScope IIIa (Digital Instruments) at room temperature. The scanning tip used was a phosphorus (n)-doped Si (Veeco). The spring constant was 40 N/m, resonance frequency was 300 kHz, and scan rate was 0.5 Hz.

ThT assay

The formation of fibrils was monitored by measuring the fluorescence of ThT.²⁵ Ten microliters of the sample was added to 2 mL of 50 mM glycine–NaOH (pH 8.5) containing 5 μ M ThT. Fluorescence intensity of ThT at 485 nm was measured at 25°C using a Hitachi fluorescence spectrophotometer F4500 with the excitation wavelength set at 445 nm and a cell with 10-mm light path.

Analytical ultracentrifugation

Sedimentation equilibrium measurements of fragmented β 2m fibrils were conducted using a Beckman Coulter Optima XL-A analytical ultracentrifuge at 37°C. The data were fitted to the following equation assuming a single size component with a molecular weight M_w :

$$A(r) = A(r_0) \exp[(\omega^2/2RT)M_w(1 - v^\circ\rho)(r^2 - r_0^2)] + A_0, \quad (1)$$

where $A(r)$ is the absorbance at the distance from the rotor center r , r_0 is the reference distance, ω is angular velocity, v° is the partial specific volume of the fibrils, ρ is the density of the solvent, calculated from the database in Ultrascan 9.9, A_0 is baseline absorbance, R is the gas constant, and T is temperature in kelvin.⁴⁸ The sample was centrifuged at 3000 rpm (3.14×10^2 rad/s), and a concentration profile was recorded by monitoring absorbance at 280 nm across the centrifugation cell with a 3-mm light path. We used the v° value of 0.697, which was obtained by Lee *et al.*⁴⁹ at 5°C. Nonlinear least-squares fitting was performed with Igor Pro software.

Sedimentation velocity measurements of β 2m fibrils were conducted using a Beckman Coulter Optima XL-A analytical ultracentrifuge at 20°C. The samples were diluted threefold before centrifugation. The diluted samples were then centrifuged at 8000 rpm (8.38×10^2 rad/s). Absorbance data at 280 nm

across the centrifugation cell with a 1.2-cm light path were collected at intervals of 5 min. The sedimentation coefficients were obtained from the data by the van Holde-Weischet method with the software Ultrascan 9.9.

1D ^1H NMR spectra

The samples for 1D ^1H were prepared in solution of deuterated solvent. 1D ^1H spectra were recorded on a Bruker DRX500 spectrometer (Bruker BioSpin, Karlsruhe, Germany) using 1-1 hard pulses for excitation and the echo⁵⁰ at 37°C. The spectral width was 10.0 kHz (20.0 ppm) with a data size of 8192. NMR data were processed using nmrPipe.⁵¹ The number of scans was 16 for monomers and 256 for fibrils.

PFG NMR spectra

The samples were prepared in deuterated solvent. PFG ^1H NMR spectra for the diffusion measurements were recorded on a Bruker DRX800 spectrometer (Bruker BioSpin, Karlsruhe, Germany) equipped with a cryoprobeTM using stimulated-echo method with sine-shaped bipolar pulse-pair gradients and a longitudinal eddy-current delay²⁹ at 37°C. Spectral width was 20.8 kHz (26.0 ppm) with a data size of 16,384. Each 1D ^1H NMR spectrum was acquired using a gradient of 6, 12, 18, 24, 30, 36, 42, 48, 54, and 60% strength, and the signal intensity (I) of each 1D ^1H NMR spectrum, integrated from 3.5 to 0.5 ppm, was fitted against the gradient strength (G) as follows:

$$I/I_0 = \exp[-\gamma^2 G^2 \delta^2 D(\Delta - \delta/3)], \quad (2)$$

where I_0 is the signal intensity in the absence of applied gradient pulses, γ is the gyromagnetic ratio of ^1H (2.675×10^8 rad/T/s), D is the effective diffusion coefficient, Δ is diffusion time, and δ is gradient duration. The diffusion time of monomers and fibrils was 350 ms and 1 s, respectively. The gradient was set to last 6 ms. NMR data were processed using nmrPipe.⁵¹ Absolute gradient strength was determined using the signal of water and determined to be 47.8 G/cm. Nonlinear least-squares fitting was performed with Igor Pro software. We used the viscosity value of 8.4×10^{-4} Pa s to calculate the molecular weight of the fragmented fibrils in solution of deuterated solvent from the D value on the basis of the Einstein–Stokes equation.

HSQC spectra

The samples were prepared in solution of 10% $^2\text{H}_2\text{O}$ solvent. ^1H – ^{15}N HSQC spectra were recorded on a Bruker DRX500 spectrometer using water flip-back and water suppression by gradient-tailored excita-

tion.⁵² Spectral widths were 12.0 kHz (24.0 ppm) and 1.01 kHz (20.0 ppm) at the ¹H and ¹⁵N axis, respectively, with a data size of 2048 (¹H) × 64 (¹⁵N). The number of scans was 16 for the monomer and 256 for the fibrils. ¹⁵N-edited 1D ¹H spectra were obtained using the 1D version of the ¹H-¹⁵N HSQC. NMR data were processed using nmrPipe.⁵¹ NMR data were analyzed with the software Sparky (Goddard and Kneller, SPARKY 3, University of California, San Francisco).

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