Supplementary Informatioin for "Structural Insight into an Alzheimer's Brain-Derived Spherical Assembly of Amyloid β by Solid-state NMR"

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Supplementary Procedures

Preparation of ASPD samples

ASPD samples were prepared as described previously with minor modifications.[1] Briefly, Aβ(1-42) was synthesized and purified as described previously using solid-phase synthesis with standard Fmoc synthesis and cleavage protocols and HPLC purification.[1,2] The synthesis was performed with an ABI 433 peptide synthesizer in the Department of Chemistry, University of Illinois at Chicago (UIC) with Fmoc protected ^{13}C- and ^{15}N-labeled amino acids at selected sites. Fmoc protection of the labeled amino acids was performed at the UIC Research Resource Center

(RRC).[3] The peptide was purified by reverse-phase HPLC with an Agilent Zorbax column at the UIC Department of Chemistry. Purity of the Aβ samples was determined based on the MALDI-TOF mass spectra collected at the UIC RRC to be approximately 90 % and 95 % before and after the HPLC purification, respectively. The lyophilized peptide after HPLC purification was completely dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (Kanto Chemical Co., Inc., Tokyo, Japan), and a solution containing ~50 nmol of the peptide was aliquoted to 1.5 mL tubes (Eppendorf, Hamburg, Germany), and subsequently lyophilized again. The lyophilized peptides were redissolved in HFIP and lyophilized again as described previously,[1] and these tubes were stored in freezer at -20°C.

For the preparation of the ASPD samples, the Aβ(1-42) peptide in each 1.5 mL tube was thoroughly dissolved in 10 μl of anhydrous dimethyl sulfoxide (276855; Sigma-Aldrich, St. Louis, MO) containing bis(2-ethylhexyl) phthalate (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) by vortexing every 10 minutes for 30 min, and then the Aβ(1-42) peptide was incubated at ~50 μM in a F12 buffer without $_{L}$-glutamine and phenol red (Kohjin Bio, Sakado, Japan) with slow rotation for 14 h at 4°C. For SSNMR measurement, ASPD samples were purified by double filtration. First, ASPD was collected as a flow through after filtration using a 0.2 μm Vivaspin filter (Vivaproducts, Inc., Littleton MA) to remove larger species such as fibrils. Next, ASPD was concentrated to ~60 μM (in a monomer Aβ equivalence) and collected as a retentate by centrifuging the sample in a 50-kDa molecular-mass-cutoff filter (Vivaspin; Sartorius AG, Göttingen, Germany) at 3×10^3 g for ~3 min to remove the low-molecular weight species such as monomers and concentrate the ASPD sample. Then, the sample in the filter was washed several times with an equal amount of phosphate buffer containing 10 mM phosphate and 5 mM NaCl

for buffer exchange in order to suppress the level of substances originated from a F12 buffer such as amino acids and sugar, which can interfere with NMR experiments. The phosphate buffer was removed by additional centrifugation of the filter for ~3 min. Cares should be taken as over-concentrating the ASPD sample may introduce irreversible aggregation. The recovered ASPD solution was quickly frozen in liquid nitrogen and lyophilized for overnight. The lyophilized sample was immediately stored in a sealed bag containing desiccants to remove any humidity. As shown in Figure 1C and Figure S2, these procedures did not alter the morphologies or the surface structure of the synthetic ASPD. From the previous study, it is indicated that ASPD is not recognized by anti-pan oligomer A11 antibody.[1]

Solid-state NMR experiments

All the SSNMR experiments were performed with a Varian Infinity-plus or Bruker Avance III SSNMR spectrometer with a homebuilt 2.5-mm triple-resonance MAS probe at 9.4 T (^{1}H frequency of 400.2 MHz) in the double- or triple-resonance configuration. The lyophilized ASPD samples (10-40mg) were packed in 2.5 mm SSNMR MAS rotors (20 μL volume) in a glove box under inert nitrogen environment with the humidity level as low as 11-13 %. The spinning speed was set to 20,000 ± 3 Hz throughout the experiments. In the 2D ^{13}C/^{13}C correlation experiments with DARR mixing[4] in Fig. 2, 1.8-3.5 mg of isotope labeled Aβ(1-42) ASPD samples were used, and the remainder of the sample amount (~80%) is originated from a F12 medium, which contains considerable amount of glucose and amino acids. The SSNMR experiments were performed following the established procedures used for amyloid intermediates of Aβ(1-40).[5]

For ^{13}CO-^{13}CO inter-strand distance measurements in Fig. S3, Aβ(1-42) samples labeled at a single ^{13}CO site in Ala-30 or Val-39 were used. Distances were measured by the fpRFDR-CT method, as previously described.[6] The total mixing period of $2\tau_R(M + 5N)$ was kept constant, while the effective dephasing period of $2\tau_R(M - N)$ was ranged, where N and M are notations in the reference. A total mixing period of 26.4 ms or 19.8 ms was used. To eliminate the contribution of the natural abundance ^{13}CO background signals, which overlapped with a signal of the labeled ^{13}CO, a constant value corresponding to the natural abundance ^{13}CO of non-glycine residues was subtracted. The resultant experimental curves were compared with simulated dephasing curves obtained using the SIMPSON program (v.4,1.1).[7] Simulation curves were obtained for a system consisting of five ^{13}C spins that are equally separated by a distance R in a linear arrangement, which corresponds to a parallel β-sheet arrangement. The dephasing curves were calculated for the middle ^{13}C spin.

Preparation of human brain extract

The brain tissues were homogenized (0.15 g/ml) in an ice-cold extraction buffer, which is a F12 buffer without $_L$-glutamine and phenol red, containing pepstatin, antipain, and complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN), using a Potter Teflon/glass tissue homogenizer at 1200 rpm on ice. The soluble fractions were collected as the supernatants after the centrifugation for 1 hr at 104,300 *g* with a Beckmann Optima MAX-XP ultracentrifuge with a TLA100.4 rotor (Beckman Coulter, Galway, Ireland) at 4°C. These manipulations were repeated 10 times, and each supernatant fraction was quickly frozen in a dryice/ethanol bath and immediately stored at -80℃. The small aliquot of each fraction was subjected to Bradford assay

and dot blotting for quantification of the amount of total protein and ASPD. To remove other assemblies (<50 kDa), soluble extracts from AD or NCI brains were concentrated using 50-kDa MWCO filters (Vivaspin; Sartorius AG, Göttingen, Germany).

Dot Blotting

Samples were diluted to the desired concentrations in PBS solution (Nacalai Tesque Inc., Kyoto, Japan), and 100 µl of solution were blotted onto nitrocellulose membrane (Protran 0.2µm; GE Healthcare, Pittsburg PA) using a vacuum-blot apparatus (Biodot, Biorad, Hercules, CA). Membranes were washed three times with PBS, dried for 30 min at 37°C, and then blocked with 5% skim milk in Tris-buffered saline containing 0.05% (v/v) Tween 20 (TBS-0.05T) for 1 hr at room temperature. Membranes were probed with rpASD1 (0.04 µg/ml) and 82E1 (0.5 µg/ml; anti-human Aβ(1-16) N-terminal mouse IgG; Immuno-Biological Laboratories Co., Ltd., Gunma, Japan) for overnight at 4°C, washed with TBS-0.05T and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (highly cross-adsorbed anti-rabbit (1:10000) or anti-mouse IgG(1:5000); Zymed Laboratories, South San Francisco, CA) for 1 hr at room temperature, and washed with TBS-0.05T. The membranes were finally washed with TBS, and reacted with SuperSignals West Femto chemiluminescent substrates (Thermo Scientific/Pierce, Rockford, IL) for 3 min at room temperature. The chemiluminescence was detected using a cooled CCD-based digital image analyzer (ImageQuant LAS4000; GE Healthcare). Dot intensities were normalized by a standard curve for synthetic ASPDs (0.03-4

pmol/dot, prepared from Aβ(1-42) for each membrane), and the average data (n=3) was used for the calculation of relative intensity of rpASD1/82E1.

TEM

Nano-scale morphology of the samples were observed by TEM using JEM-1010 (JEOL, Tokyo, Japan) operated at 100 kV and magnification of 80,000. For the grid preparation, elastic carbon-supported film grids (Okenshoji Co., Ltd, Tokyo, Japan) were glow discharged for 1 min at 4-6 mA using ion-sputtering device (PIB-20; Vacuum Device Inc, Ibaraki, Japan). Samples were diluted to the approximately 5 μM of Aβ concentration in ice-cold PBS solution, and 5 μl of diluted sample was immediately loaded on the grid, and subsequently left for 1 min; then the excess solution was removed by wicking the edge of the grid by a piece of filter paper. The samples were negatively stained with 4% (w/v) uranyl acetate solution. The grids were gently dried and stored in a desiccating chamber.

The average diameters of ASPD were measured by using the ImageJ software (http://imagej.nih.gov/ij/). The species with diameters less than 8.2 nm or circularity less than 0.5 were rejected as artifacts or smaller aggregates other than ASPD. This protocol captured most of the intact spherical species recognized as ASPD by "eye-ball" fitting. The diameter d was estimated from the area of the species detected by ImageJ assuming that the area was given by $\pi(d/2)^2$. The diameters are averaged over 65, 30, 54 spheres for synthetic ASPD, native ASPD, and rehydrated ASPD, respectively.

Supplementary Data

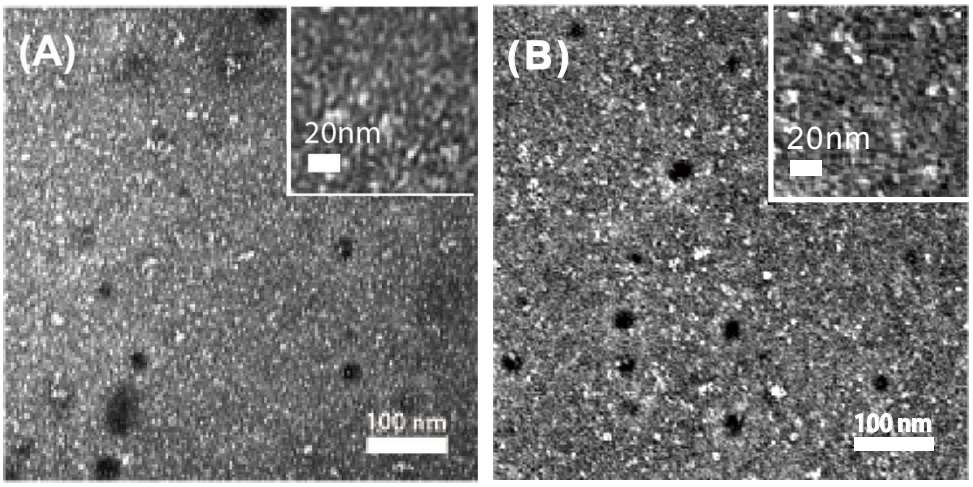

Figure S1. (A) A control TEM image for the sample collected right after the incubation of Aβ(1-42). No spherical species were identified. (B) A control TEM image for the sample collected by mouse IgG from a brain extract in place of haASD used in Fig. 1B. The TEM analysis does not show any spherical species that were found in Fig. 1B.

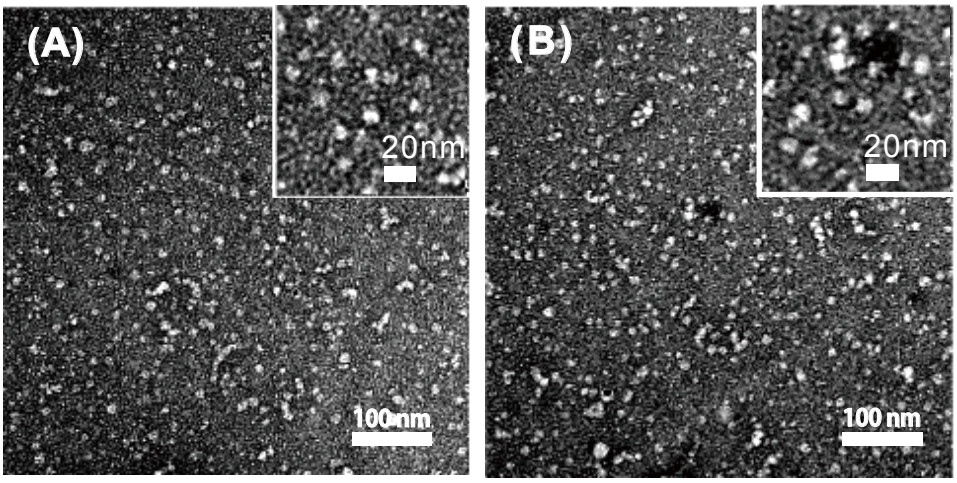

Figure S2. (A) A TEM image of the ASPD sample that was treated by a buffer exchange in 10 mM phosphate and 5 mM NaCl buffer for the NMR analysis and (B) a TEM image of rehydrated sample after lyophilization. Little morphological changes were observed. The average diameter of the ASPD after rehydration is 11.0 ± 2.0 nm, which agrees well with the corresponding diameters observed for synthetic ASPD (11.0 ± 2.1 nm) and native ASPD (10.9 ± 1.7 nm; n=54) in Fig. 1.

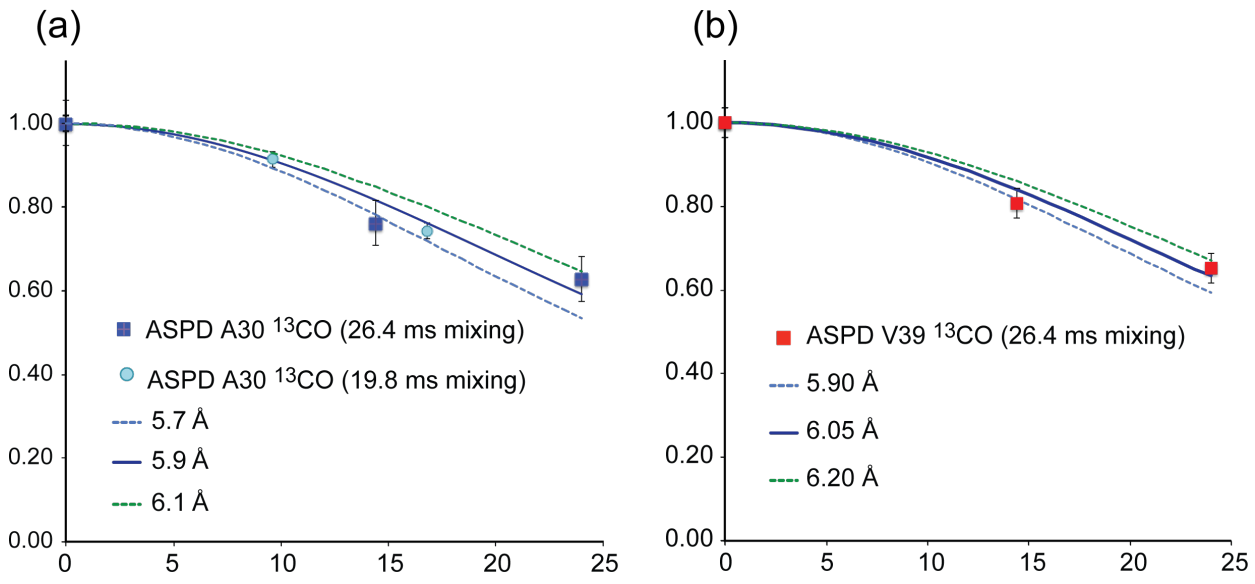

Figure S3. The ^{13}CO-^{13}CO interstrand distance measurement (solid circle or square) for (a) Ala-30 and (b) Val-39 by CT-fpRFDR measurements[8,9] with simulated dephasing curves for varied distances as indicated in the inset. The total constant-time mixing period was 26.4 ms (square) and 19.8 ms (circle). The best-fit curves were obtained for (a) 5.9 Å and (b) 6.05 Å. For Ala-30, the ^{13}CO-^{13}CO distances were determined to be 5.9 ± 0.2 Å and 5.85 ± 0.10 Å from the data collected with total mixing periods of 26.4 ms and 19.8 ms, respectively. A similar ^{13}CO-^{13}CO distance of 6.05 ± 0.15 Å was obtained for Val-39.

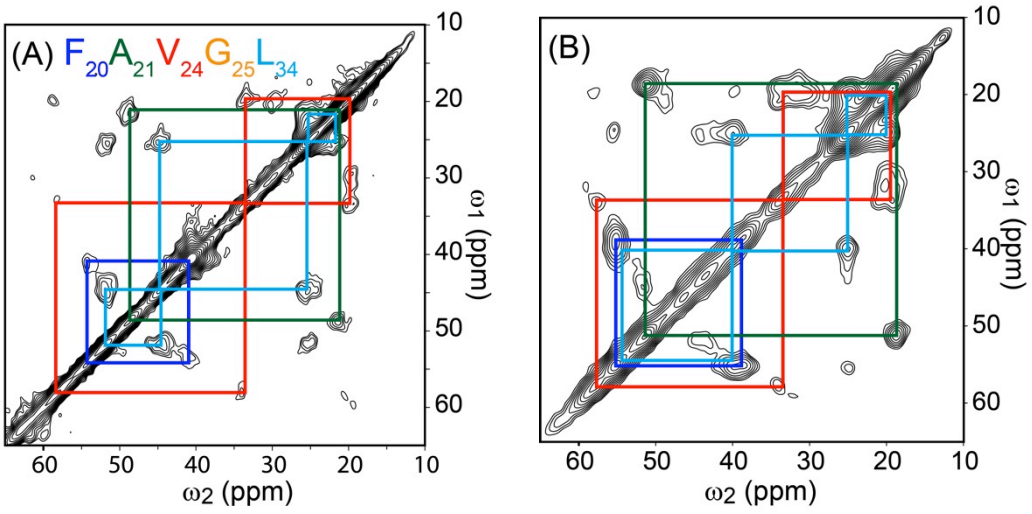

Figure S4. Comparison of 2D $^{13}C/^{13}C$ correlation SSNMR for Aβ(1-42) in the (a) ASPD and (b) amyloid fibril samples. For both experiments, the Aβ(1-42) samples were labeled with uniformly 13C- and ^{15}N-labeled amino acids at Phe-20, Ala-21, Val-24, Gly-25, Leu-34. The amyloid fibril sample was prepared by incubating a solution of Aβ(1-42) at 50 μM in a 10 mM phosphate buffer at a room temperature. The data suggest that the observed ^{13}C chemical shifts for ASPD and amyloid fibril are notably different. The details of the sample preparation and structural analysis of the amyloid fibril sample will be discussed in a separate work.

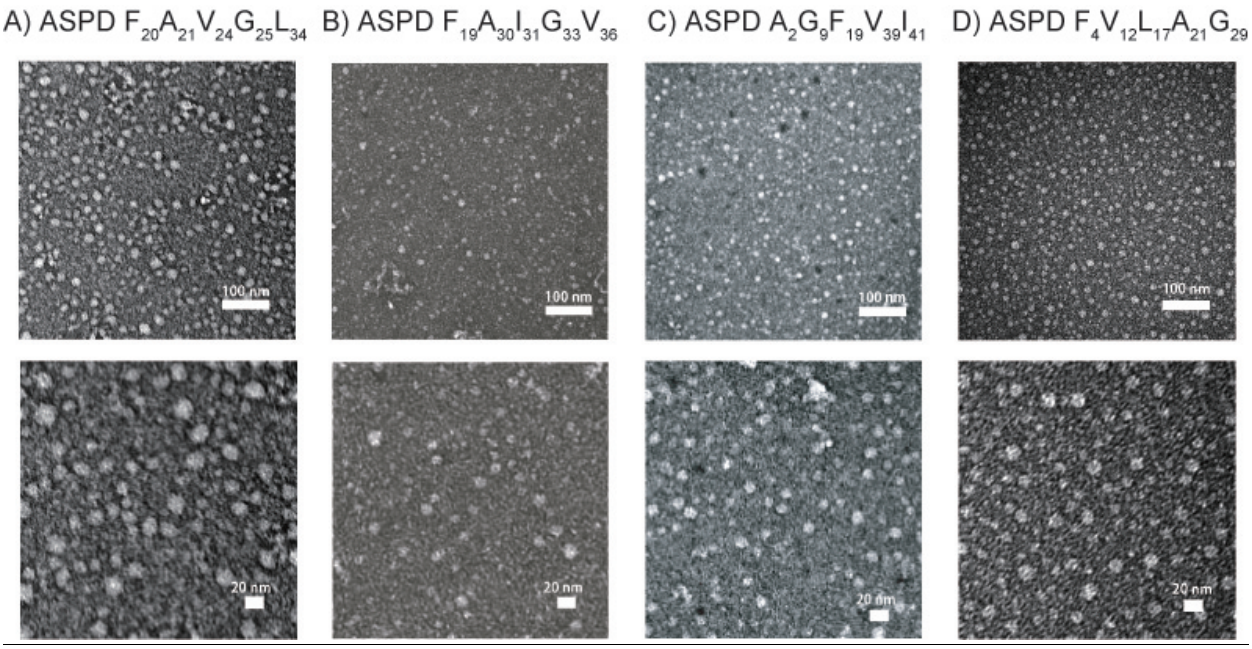

Figure S5. Transmission electron micrograph for the entire ASPD sample used for the NMR analysis (Figure 2). The images show spherical morphologies of ASPD having diameter of 10-18nm morphologies.

Samples analyzed using SSNMR

The ASPD samples (10-40mg) as received were packed in a 2.5 mm 20 µL volume SSNMR rotor in a glove box under inert nitrogen environment with the humidity level as low as 11-13 %. We have analyzed 5 ASPD samples by SSNMR spectroscopy so far.

Table S1. A list of the ASPD samples used for this study

Sample in Figure 2	Uniformly ^{13}C ^{15}N labeled sites/residues	Total sample in the rotor (mg)	Peptide amount by amino-acid analysis (mg)
A	F20, A21, V24, G25, L34	38	2.7
B	F19, A30, I31, G33, V36	24.9	3.5
C	A2, G9, F19, V39, I41	30	2.4
D	F4, V12, L17, A21, G29	11	1.8

Table S2. ^{13}C chemical shifts assigned for the ASPD samples with predicted torsion angles

Residues	**Chemical shift (ppm)[1)]**							**Torsion angle (degrees)**	
	CO	**C_α**	**C_β**	**C_γ**	**$C_{\gamma 1}$**	**$C_{\gamma 2}$**	**C_δ**	**Ψ**	**ϕ**
Ala-2		50.5	16.6						
Phe-4			38.0[2)]				129.2[2)]		
Gly-9	170.7[3)]	42.6[3)]							
Val-12	172.5	58.8[4)]	31.8		20.1			129(±11)	-120(±11)
Leu-17	173.1	51.7 53.3*	44.3 42.4*	25.6				134(±14)	-124(±16)
Phe-19	171.3	53.9	40.9	137.0			128.9	137(±23)	-129(±16)
Phe-20		53.4	41.2	136.5			129.6	136(±11)	-130(±9)
Ala-21	172.9	48.8	21.4					146(±10)	-142(±12)
Val-24	172.3	58.3 59.0*	34.0 29.9*		19.9 19.6*			151(±16)	-133(±13)
Gly-25	169.9[3)]	43.2[3)]							
Gly-29	170.0[3)]	43.0[3)]							
Ala-30	173.2	49.2	21.4					146(±10)	-130(±21)
Ile-31	173.2	57.6	38.6 35.9* 41.1*		26.5 26.0* 25.2*	14.7	12.8	132(±12)	-122(±10)
Gly-33	169[3)]	43.2[3)]							
Leu-34	172.5	51.8	44.8	25.4				139(±16)	-133(±14)
Val-36	172.5	57.7	33.4		19.4			153(±16)	-123(±13)
Val-39		57.7	33.9		19.3			149(±12)	-138(±6)
Ile-41		58.8	37.4		15.1	25.9	12		

1) Referenced to TMS, which is off from the DSS reference by 2.01 ppm.[10,11] 2) Assigned from the side chain correlation between $^{13}C_\beta$ and $^{13}C_\gamma$. 3) Assigned from the correlation between $^{13}C_\alpha$ and ^{13}CO. 4) Assigned from the correlations between $^{13}C_\alpha$ and $^{13}C_\gamma$ and $^{13}C_\alpha$ and ^{13}CO. * represent minor species, which were not used for the TALOS and secondary chemical shift analysis.

References:

(1) Noguchi, A.; Matsumura, S.; Dezawa, M.; Tada, M.; Yanazawa, M.; Ito, A.; Akioka, M.; Kikuchi, S.; Sato, M.; Ideno, S.; Noda, M.; Fukunari, A.; Muramatsu, S.; Itokazu, Y.; Sato, K.; Takahashi, H.; Teplow, D. B.; Nabeshima, Y.; Kakita, A.; Imahori, K.; Hoshi, M. *J. Biol. Chem.* **2009**, *284*, 32895.

(2) Hoshi, M.; Sato, M.; Matsumoto, S.; Noguchi, A.; Yasutake, K.; Yoshida, N.; Sato, K. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 6370.

(3) Chimon, S.; Ishii, Y. *J. Am. Chem. Soc.* **2005**, *127*, 13472.

(4) Takegoshi, K.; Nakamura, S.; Terao, T. *J. Chem. Phys.* **2003**, *118*, 2325.

(5) Chimon, S.; Shaibat, M. A.; Jones, C. R.; Calero, D. C.; Aizezi, B.; Ishii, Y. *Nat. Struct. Mol. Biol.* **2007**, *14*, 1157.

(6) Petkova, A. T.; Leapman, R. D.; Guo, Z. H.; Yau, W. M.; Mattson, M. P.; Tycko, R. *Science* **2005**, *307*, 262.

(7) Bak, M.; Rasmussen, J. T.; Nielsen, N. C. *J. Magn. Reson.* **2000**, *147*, 296.

(8) Ishii, Y. *J. Chem. Phys.* **2001**, *114*, 8473.

(9) Ishii, Y.; Balbach, J. J.; Tycko, R. *Chem. Phys.* **2001**, *266*, 231.

(10) Morcombe, C. R.; Zilm, K. W. *J. Magn. Reson.* **2003**, *162*, 479.

(11) Harris, R. K.; Becker, E. D.; De Menezes, S. M. C.; Granger, P.; Hoffman, R. E.; Zilm, K. W. *Pure Appl. Chem.* **2008**, *80*, 59.