Antiparallel Triple-strand Architecture for Prefibrillar Aβ42 Oligomers*

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Background: Soluble Aβ42 oligomers, rather than insoluble amyloid fibrils, are toxic species in Alzheimer’s disease.

Results: We obtained structural restraints at all 42 residue positions in Aβ42 oligomers and performed structural modeling.

Conclusion: In oligomers, each Aβ42 protein forms a single β-sheet with three antiparallel β-strands.

Significance: Our novel structural model provides new structural framework for understanding oligomer-fibril interconversion and designing oligomer-targeted therapeutics.

Aβ42 oligomers play key roles in the pathogenesis of Alzheimer disease, but their structures remain elusive partly due to their transient nature. Here, we show that Aβ42 in a fusion construct can be trapped in a stable oligomer state, which recapitulates characteristics of prefibrillar Aβ42 oligomers and enables us to establish their detailed structures. Site-directed spin labeling and electron paramagnetic resonance studies provide structural restraints in terms of side chain mobility and intermolecular distances at all 42 residue positions. Using these restraints and other biophysical data, we present a novel atomic-level oligomer model. In our model, each Aβ42 protein forms a single β-sheet with three β-strands in an antiparallel arrangement. Each β-sheet consists of four Aβ42 molecules in a head-to-tail arrangement. Four β-sheets are packed together in a face-to-back fashion. The stacking of identical segments between different β-sheets within an oligomer suggests that prefibrillar oligomers may interconvert with fibrils via strand rotation, wherein β-strands undergo an ~90° rotation along the strand direction. This work provides insights into rational design of therapeutics targeting the process of interconversion between toxic oligomers and non-toxic fibrils.

Alzheimer disease is a fatal neurodegenerative disorder and the most common form of dementia (1). Deposition of amyloid β (Aβ)3 peptide in the form of amyloid plaques is a hallmark of Alzheimer pathology. Amyloids refer to fibrillar protein aggregates with common tissuerelated and structural characteristics and are involved in a range of neurodegenerative, localized, and systemic disorders including Parkinson and Huntington diseases, and type II diabetes (2). Soluble Aβ oligomers have been increasingly recognized as primary neurotoxins in Alzheimer disease (3–5). Several Aβ oligomers have been identified in vivo (6, 7), including dimers, trimers, and Aβ56. Different in vitro protocols have been used to prepare oligomers such as Aβ-derived diffusible ligands (8), globulomers (9), prefibrillar oligomers (10), and amylospheroids (11). Because the molecular structures of these oligomers are unknown, it is impossible to know how many unique structures exist in these Aβ oligomers. Currently, structural classification of these oligomers is largely restricted to the use of conformation-specific antibodies (12). Based on immunoreactivity to the oligomer-specific polyclonal antibody A11, Aβ oligomers can be classified into A11-positive prefibrillar oligomers and A11-negative fibrillar oligomers (12).

One challenge in the structural studies of Aβ oligomers is related to their transient and heterogeneous nature. Aβ oligomers represent a series of intermediate assemblies on or off the pathway to fibril formation. Oligomers prepared using different protocols have been shown to be structurally diverse (13). Some Aβ oligomers have been shown to have similar parallel in-register β structures as amyloid fibrils (14), and other oligomers adopt distinct structures (15–19). Heterogeneity can also occur within the same oligomer sample (20, 21). Structural heterogeneity has been a major obstacle in obtaining high-resolution structural data.

Site-directed spin labeling (SDSL) in combination with electron paramagnetic resonance (EPR) spectroscopy has emerged as a powerful approach to characterize the structures of amyloid fibrils (22). The general strategy of SDSL includes substitution of a selected residue with cysteine and subsequent modification of the cysteine residue to produce a spin label side chain. The EPR sample can be in solutions, aggregates, or membrane environments, and of any size. As shown previously in the studies of Aβ and yeast prion protein Ure2p, EPR can resolve structural heterogeneity and separate different structural states (23–26). Distance measurements with continuous-wave and
pulsed EPR can cover a wide range of distances from 5 to 70 Å (27, 28). These advantages make SDS-L EPR a promising technique to obtain detailed structural information of the inherently heterogeneous \( \alpha \beta \) oligomers.

In this work, we performed a comprehensive structural study on A\(_{42} \) oligomers prepared using a fusion protein, GroES-ubiquitin-A\(_{42} \) (GU-A\(_{42} \)). This fusion protein construct forms highly ordered oligomers without further assembling into fibrils, and enables us to obtain detailed structural information of these A\(_{42} \) oligomers. The fusion protein system is similar to yeast prion proteins such as Sup35p and Ure2p, which contain both a prion domain and a globular domain, and the globular domain does not participate in the amyloid formation of these yeast prion proteins (29). The fusion protein approach also provides for some other unique applications. For example, a split luciferase-\( \alpha \beta \) system allows high sensitivity detection of oligomer formation in mammalian cells (30). Fusion protein approaches also enable studies of mutational effects at specific residue positions in yeast (31) and \textit{Escherichia coli} (32) cells and \textit{in vivo} high throughput screening of small molecule inhibitor libraries (33). Fusion proteins also facilitate structural characterization of \( \alpha \beta \) fragments using x-ray crystallography (34).

These GU-A\(_{42} \) oligomers recapitulate the characteristics of prefibrillar oligomers, such as immunoreactivity to oligomer-specific antibody A11 (12). For structural studies with EPR, spin labels are introduced, one at a time, at all 42 residue positions of A\(_{42} \) sequence. Residue-specific mobility analysis using EPR reveals three ordered segments at residues 1–10, 13–23, and 28–42. Distance measurements show two major intermolecular distance distributions at each of the 42 residue positions: 9–10 Å and 15–17 Å. These results allow us to suggest a triple-strand antiparallel model for the A\(_{42} \) prefibrillar oligomers. Our model for prefibrillar oligomers points to a mechanism of oligomer-fibril interconversion wherein rotation of \( \beta \)-strands reorganizes the \( \beta \)-sheets of the oligomers into new fibril \( \beta \)-sheets that run \textit{i.e.} have \( \beta \)-hydrogen bonding) approximately perpendicular to the original \( \beta \)-sheets of the oligomers. We term this mechanism of nucleated conformational conversion (35) (conversion of an oligomer from one conformation to another without adding or losing material) to be “strand rotation.”

**EXPERIMENTAL PROCEDURES**

\textit{Preparation of A\(_{42} \) Fusion Proteins and Full-length A\(_{42} \)}—The DNA construct of GroES-ubiquitin-A\(_{42} \) (36) and the deubiquitylating enzyme Usp2cc (37) were kindly provided by Dr. Rohan T. Baker at Australian National University (Australi) and Dr. Il-Seon Park at Chosun University (South Korea).

Single cysteine mutations at various sites were introduced into A\(_{42} \) sequence using QuickChange kit (Agilent Technologies). Mutations were confirmed with DNA sequencing. Expression of GroES-ubiquitin-A\(_{42} \) in \textit{E. coli} and their purification were performed as previously described in Ngo and Guo (38). Expression of Usp2cc and removal of fusion protein GU to prepare full-length A\(_{42} \) was performed as previously described in Gu et al. (39). The purity of A\(_{42} \) fusion proteins was checked with SDS-PAGE. The fusion protein GU without A\(_{42} \) was prepared using the same protocol as GU-A\(_{42} \).

\textit{Spin Labeling of A\(_{42} \) Fusion Proteins}—Dithiothreitol was added to purified A\(_{42} \) fusion proteins to a final concentration of 10 mM and was allowed to incubate for 20 min at room temperature to break any disulfide bonds. Then A\(_{42} \) fusion proteins were buffer exchanged into the labeling buffer (7 mM guanidine hydrochloride, 50 mM NaCl, 20 mM MOPS, pH 6.8) using a 5-ml Hitrap desalting column (GE Healthcare). The spin labeling reagent MTSSL (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl methanethiosulfonate, Enzo Life Sciences) was at 10-mers molar excess and then incubated for 1 h at room temperature. Spin-labeled A\(_{42} \) fusion proteins were then precipitated with methanol, air dried, and then stored at \(-80^\circ \text{C}.\)

\textit{Preparation of A\(_{42} \) Fusion Protein Oligomers}—Methanol precipitated A\(_{42} \) fusion proteins were resuspended in PU buffer (50 mM phosphate, 8 mM urea, pH 10.0) to a final concentration of 1 mM. Then A\(_{42} \) fusion proteins were diluted 20-fold in PBS buffer (50 mM phosphate, 140 mM NaCl, pH 7.4) to 50 \( \mu \)M. After incubation at room temperature for 24 h, oligomers form loosely associated precipitates. As a control, the fusion protein GU without A\(_{42} \) was also precipitated with methanol, resuspended in PU buffer, and followed by 20-fold dilution to PBS buffer and incubation at room temperature for 24 h.

\textit{Transmission Electron Microscopy}—GU-A\(_{42} \) oligomer samples were diluted with water to 5 \( \mu \)M and then applied onto glow discharged copper grids covered with 400 mesh formvar/carbon film (Ted Pella) and negatively stained with 1% uranyl acetate. Samples were examined under a JEOL JEM-1200EX transmission electron microscope with an accelerating voltage of 80 kV.

\textit{X-ray Powder Diffraction}—GU-A\(_{42} \) oligomers were collected by centrifugation at 20,000 \( \times \) g for 20 min, and washed with water. The GU sample was buffer exchanged to water using a HiTrap desalting column (GE Healthcare). The lyophilized powders for both GU and GU-A\(_{42} \) were mounted on the tip of a broken glass rod. Then, the specimen was placed on the goniometer of an in-house x-ray machine and shot using a Rigaku-FR-D x-ray generator equipped with a Rigaku HTC imaging plate detector.

\textit{Thioflavin T Fluorescence Assay}—Thioflavin T (Sigma) was dissolved in PBS and filtered with a 0.22-\( \mu \)m filter. GU and GU-A\(_{42} \) samples were diluted to a final concentration of 5 \( \mu \)M in PBS containing 50 \( \mu \)M ThT. Fluorescence was measured immediately on a Jasco FP-6200 spectrofluorometer. Excitation was at 440 nm (3-nm slit width), and emission was at 485 nm (5-nm slit width).

\textit{Dot Blot Assay with A11 Antibody}—GU sample and GU-A\(_{42} \) oligomers were diluted with PBS to 25 \( \mu \)M. Then 5 \( \mu \)l of GU and GU-A\(_{42} \) samples were spotted on a nitrocellulose membrane (Bio-Rad). The membrane was blocked in 10% fat free milk in Tris-buffered saline (TBST) (50 mM Tris, 150 mM NaCl, 0.05% Tween20) at room temperature for 1 h, followed by incubation with the rabbit polyclonal A11 antibody at 2.4 \( \mu \)g/ml in 5% fat free milk, TBST at 4 \( ^\circ \text{C} \) overnight. TBST was used to wash the membranes for three times. Then, the membrane was incubated with anti-rabbit HRP-conjugated second-
ary antibodies (1:5000 in 5% fat free milk, TBST) (Jackson ImmunoResearch) for 1 h at room temperature, followed by further washing in TBST buffer for three times. The blots were developed using the Super Signal West Pico Chemiluminescent Substrate kit (Pierce).

**SDS-PAGE**—SDS-PAGE was performed on the 24-h samples of GU and GU-Aβ42 using Mini-PROTEAN tetra system (Bio-Rad). 4–20% gradient Tris-glycine gels (Bio-Rad) were used. Samples were mixed at 1:1 volume ratio with SDS loading buffer (4% SDS, 0.5 M β-mercaptoethanol, 125 mM Tris, 20% glycerol (v/v), 0.2 mg/ml bromphenol blue, pH 6.8) without boiling (unless specified otherwise).

**Circular Dichroism Spectroscopy**—Secondary structures of GU sample were analyzed by CD spectroscopy. The GU sample (200 μl), 24 h after dilution from PU to PBS, was placed in a 0.1-cm path length quartz cell (Starna). A Jasco J-715 CD spectrometer was employed. The measurement was carried out in a wavelength range of 190–260 nm at a rate of 20 nm min⁻¹ with a step resolution of 0.5 nm, a time constant of 4 s and a bandwidth of 1 nm. The CD spectra were obtained by averaging 6 scans. The temperature was set at 25 °C. The spectra were corrected by subtracting the buffer background. The FT voltages were above 800 for the wavelength range of 190–203.5 nm, so only the CD data in the wavelength range of 204–260 nm were reported. Because of the formation of insoluble oligomers, the GU-Aβ42 samples were not studied with CD spectroscopy. The CD results are reported as mean residue ellipticity in Fig. 1F.

**Aggregation Kinetics of Aβ42**—Purified full-length Aβ42, without fusion protein partner, was buffer exchanged to 30 mM ammonium acetate, pH 10.0, then lyophilized and stored at −80 °C. For aggregation experiments, lyophilized Aβ42 powder was dissolved in CG buffer [20 mM CAPS, 7 M guanidine hydrochloride, pH 11], filtered through 0.2-μm syringe filter (Corning 431212), and then buffer exchanged to PBS using a 5-ml HiTrap desalting column (GE Healthcare). The sample was then filtered through a 0.2-μm syringe filter (Corning 431212), and concentration was determined using an extinction coefficient of 1.28 mm⁻¹ at 280 nm. The Aβ42 was diluted to 50 μM with PBS, supplemented with 20 μM ThT, either in the presence or absence of 2.5 μM GU or GU-Aβ42 samples. After mixing all components, 50 μl of each mixture was transferred to the 384 well black polystyrene plate with clear bottom and PEG coating (Corning 3655). The plate was then sealed with a plastic film (Corning 3095). All these steps were performed on ice if possible. The aggregation was initiated by placing the plate in a Victor 3V plate reader (Perkin Elmer). The plate is incubated at 37 °C with orbital shaking (1 mm shaking diameter, normal shaking speed). The thioflavin T fluorescence was measured through the bottom of the plate at every 5 min (with excitation filter of 450 nm and emission filter of 490 nm). Each sample was prepared in duplicates.

**EPR Spectroscopy**—EPR measurements were performed at X-band frequency on a Bruker EMX spectrometer equipped with the ER4102ST cavity at room temperature using a microwave power of 20 milliwatt. A modulation frequency of 100 KHz was used. Modulation amplitude was optimized to each individual spectrum. Scan width is 200 G. For each sample, 20 μl of oligomer sample was loaded into glass capillaries (VitroCom) sealed at one end. EPR spectra in each figure panel were normalized to the same number of spins.

**EPR Distance Analysis**—Distance analysis was performed using the program ShortDistances, developed by Dr. Christian Altenbach at UCLA. The detailed fitting procedure to obtain distances has been previously described (40). The 20% labeled spectra were used as the spectra without dipolar interactions. To avoid over fitting of the experimental data, we emphasized on using minimum number of variable parameters. The width of the distance distribution was fixed at 2 Å. The distance, percentage of the spin labels at the fitted distance, and the percentage of non-interacting spin labels were allowed to vary. The fitted spectra, distances, and their relative populations are plotted in Fig. 8.

**Modeling of Aβ42 Oligomers**—EPR distance information was used to create harmonic distance constraints for backbone hydrogen bonding for Aβ42 plus four residues of ubiquitin (Lys-Arg-Gly-Gly) at the N terminus of Aβ42. Models were varied in number of oligomers in the directions of β-hydrogen bonding (direction of sheets), and in number of sheets. Models were also varied in β-twist, which changes the overall twist along the β-sheet direction. Models were energy-minimized by molecular dynamics with harmonic distance constraints using the CNS program (41). The best fit model satisfied the distance constraints from EPR and also fit well to the x-ray powder diffraction, using the Rg goodness of fit metric as previously described (18).

## RESULTS

**Aβ42 Fusion Protein Forms A11-positive Prefibrillar Oligomers**—We studied the oligomer formation of Aβ42 using a fusion protein containing GroES-ubiquitin at the N terminus of Aβ42 sequence (36). Here this construct is termed GU-Aβ42. Oligomers are formed by a 20-fold dilution from a denaturing buffer containing 8 M urea to phosphate-buffered saline (PBS) and incubated at room temperature for 24 h without agitation. The final GU-Aβ42 concentration is 50 μM. Shortly after dilution from urea to PBS, GU-Aβ42 forms visible precipitates. Twenty-four hours after aggregation began, most of the GU-Aβ42 was in precipitate, and soluble GU-Aβ42 was below the detection limit by absorbance at 280 nm. We attribute the ability to form visible aggregates to the presence of GroES as a fusion protein partner. GroES is known to form oligomers (42, 43). When we performed the same aggregation assay using full-length Aβ42 without the fusion protein, we did not observe any visible aggregates. At the same time, Aβ42 quickly aggregates into amyloid fibrils in the absence of fusion protein partners. We conclude that the GroES fusion partner promotes the trapping of GU-Aβ42 into oligomers and prevents fibril formation, allowing us to further characterize its structure below. One disadvantage, however, is that formation of precipitates prohibits us from characterizing the properties of GU-Aβ42 oligomers using solution-based methods such as size exclusion chromatography, light scattering, and sedimentation.

Transmission electron microscopy (TEM) shows that the GU-Aβ42 sample consists of globular oligomers (Fig. 1A). Most
of the GU-\(\beta\)42 oligomers have a diameter of 10–12 nm. No fibrils or any elongated protofibrils were observed by electron microscopy, even after more than 2 weeks of incubation at room temperature.

To establish the relationship between the GU-\(\beta\)42 oligomers and other \(\beta\)42 oligomers, we performed dot blot analysis using the oligomer-specific A11 antibody (10). Fig. 1B shows that the GU-\(\beta\)42 oligomers stained strongly with A11 antibody. This result suggests that the GU-\(\beta\)42 oligomers should be classified as “prefibrillar” oligomers (12), which are distinct from fibrillar oligomers that share the same epitope as amyloid fibrils.

X-ray powder diffraction of GU-\(\beta\)42 oligomers shows two sharp reflections at 4.6 and 10.0 Å (Fig. 1C), which have been previously suggested as characteristics of cross-\(\beta\) structure (44). The 4.6-Å reflection corresponds to the interstrand spacing within the same \(\beta\)-sheet, and the 10.0-Å reflection corresponds to the sheet to sheet spacing in the oligomers. The sharp nature of reflections at both 4.6 and 10.0 Å suggests that GU-\(\beta\)42 oligomers contain highly ordered \(\beta\)-sheet structure.

Thioflavin T (ThT) binding assay shows that GU-\(\beta\)42 has weak ThT binding (~2.5-fold change in fluorescence intensity compared with ThT alone), and GU alone has only a marginally greater ThT signal (Fig. 1D). This is consistent with previous studies on full-length \(\beta\)42 oligomers, which also show much weaker binding to ThT than mature fibrils (45).

On SDS gel, the GU-\(\beta\)42 sample contains two major species of SDS-resistant oligomers (Fig. 1E). The apparent sizes for these two oligomers are 86 and 128 kDa according to the calibration with molecular weight standards. The molecular mass for GU-\(\beta\)42 monomer is 25 kDa. The commercial molecular weight standards consist of denatured single polypeptide chains and thus adopt extended structures. SDS-resistant GU-\(\beta\)42 oligomers, however, must adopt some compact structures to stay oligomeric. Therefore, GU-\(\beta\)42 oligomers would migrate faster on the gel than the molecular weight standards of comparable size. For these reasons, we conclude that the SDS-resistant GU-\(\beta\)42 oligomers are tetramers and hexamers. This finding is similar to our previous study showing that GU-\(\beta\)42 oligomers also form tetramers and hexamers in the presence of 8 M urea (38). Here the oligomer sample contains only 0.4 M urea. Even with such low urea concentration, no other oligomeric species were observed within the detection limit of Coomassie staining on the gel. Tetramers and hexamers are also the major oligomer forms for \(\beta\)42 without fusion protein partners, as revealed by ion mobility mass spectrometry (46), suggesting GU-\(\beta\)42 oligomerizes similarly as \(\beta\)42.

We also checked if the fusion protein GU alone is refolded under the condition of our oligomer preparation. As shown in Fig. 1F, the CD spectrum of the GU sample is qualitatively similar to previously published CD spectra of GroES (47) and ubiquitin (48), suggesting that the protein is refolded to the native structure under our oligomerization condition.

These results show that GU-\(\beta\)42 forms globular oligomers. Lack of fibril formation suggests fusion protein partners effectively trap GU-\(\beta\)42 in a stable oligomeric state. These GU-\(\beta\)42 oligomers recapitulate the characteristics of full-length \(\beta\)42 prefibrillar oligomers including A11 reactivity, weak ThT binding, and presence of SDS-resistant oligomers.

Residue-specific Side Chain Mobility in \(\beta\)42 Oligomers—To study the structure of GU-\(\beta\)42 oligomers with EPR, we introduced spin labels, one at a time, at all 42 residue positions of \(\beta\)42 sequence. A commonly used spin labeling reagent (see “Experimental Procedures”) was used to generate the spin label side chain named R1 (Fig. 2A).

TEM studies show that the spin-labeled GU-\(\beta\)42 proteins form globular oligomers (Fig. 2B). The morphology of these oligomers is indistinguishable from the oligomers of wild-type GU-\(\beta\)42, suggesting that spin labeling has little effect on the formation of oligomers.
Structure of Prefibrillar Aβ42 Oligomers

Previously, we found that cysteine substitutions of hydrophobic residues (Ile-31, Ile-32, Leu-34, Val-39, Val-40, Ile-41) at the C-terminal region disrupted the formation of tetramers and hexamers (38). To check if spin labeling at these residue positions also disrupts oligomer formation, we performed SDS-PAGE analysis for the GU-Aβ42 oligomer samples labeled at C-terminal residues (Fig. 2C). We found that tetramers and hexamers are largely unaffected by spin labeling. We propose that the hydrophobic nature of the nitroxide ring makes it very tolerable in Aβ oligomers. These results also suggest that hydrophobicity, rather than size, of the amino acid side chain is critical for Aβ oligomerization.

To study the side chain mobility using SDSL EPR, we prepared GU-Aβ42 oligomers using a mixture of spin-labeled protein and wild-type protein at 1:4 molar ratio. This sample is referred to as “20% labeled.” The EPR spectra of 20% labeled samples are shown in Fig. 3. In the 20% labeled sample, intermolecular spin-spin interactions are minimized, so the EPR spectral lineshape is mainly determined by the mobility of the spin label, which reflects local structure at the labeling site. The side chain mobility is estimated using inverse center line width of the EPR spectra (49). Based on the plot of residue-specific side chain mobility (Fig. 4), Aβ42 consists of four structural segments. Segment 2 (residues 13–23) and segment 4 (residues 28–42) are the most ordered segments in the oligomers. The relatively more flexible segment 3 (residues 24–27) separates segments 2 and 4. X-ray powder diffraction studies suggest the presence of ordered β-sheet structures in GU-Aβ42 oligomers (Fig. 1C). Therefore, we conclude that both segments 2 and 4 adopt β structures, and segment 3 forms a turn connecting the two β strands.

Segment 1 (residues 1–12) has higher mobility than other segments (Fig. 4). However, the presence of two peaks at low-field resonance line (labeled M and N in Fig. 5A) suggests that the EPR spectra in this segment are composed of two components, which we termed M and N. The M component has similar mobility as the well ordered positions (Fig. 5B). Using spectral subtractions, we were able to reveal the lineshape of component N, whose relatively narrow lines indicate high mobility (Fig. 5). Therefore, even though center line width measurement, which has contributions from both components M and N, shows that residues 1–12 have higher mobility than other residues, spectral subtractions show that these residues consist of two structural states: a structured state and a locally disordered state. The structured state has mobility similar to segments 2 and 4 in Fig. 4.

GU-Aβ42 Oligomers Adopt a Distinct Structure from Fibrils—Most amyloid fibrils studied to date adopt parallel in-register β-sheets (50), in which the side chains at the same residue position stack on top of each other. When spin label is introduced at a β-strand site in fibrils, the stacking of spin labels leads to strong spin exchange interactions (23, 26). As demonstrated in Fig. 6A, strong spin exchange interactions (i.e. spin exchange frequency >100 MHz) manifest as single-line EPR spectra due to the collapse of the low-field and high-field resonance lines to the center-field line. The EPR spectra of 100% labeled GU-Aβ42 oligomers show significant broadening as indicated by lower spectral amplitude compared with 20% labeled oligomers (Fig. 7). However, the EPR spectral lineshape of GU-Aβ42 oligomers is distinct from the single-line spectrum of Aβ42 fibrils (Fig. 6B). Therefore, the EPR data suggest that the structure of GU-Aβ42 oligomers is distinct from the parallel in-register β-sheet structure of Aβ42 fibrils.

Distance Analysis and Structural Modeling of GU-Aβ42 Oligomers—To gain detailed structural information, we analyzed the intermolecular distances between spin labels in the GU-Aβ42 oligomers by spectral fitting (Figs. 7 and 8). Distances were obtained for every labeling position. All labeling positions show two intermolecular distances at ~9–10 and 15–17 Å (Fig. 8A). For the 9–10 Å distance, there are two possible structural origins. First, in a β-sheet structure, the interstrand distance is 4.75 Å, thus the distance between alternate β-strands is 9.5 Å. Second, the distance between adjacent β-sheets is ~10 Å (Fig. 1C). A face-to-back packing between β-sheets would also give rise to inter-residue distance of ~10 Å for all residue positions. For the 15–17 Å distance, a likely origin is the spacing between

FIGURE 2. Characterization of the spin-labeled GU-Aβ42 oligomers. A, a stick model of spin label R1 (PDB ID: 2Q9E). B, transmission electron microscopy images of representative spin-labeled GU-Aβ42 oligomers show globular structures with similar diameters as the wild-type oligomers. Arrows point to several of the oligomers. C, SDS-PAGE analysis of spin-labeled GU-Aβ42 oligomers. Note that overall spin labeling did not disrupt the formation of tetramers and hexamers.
every third β-strands within the same β-sheet. A structural model consistent with both distances is presented below.

Distance analysis also shows the populations for spin labels at each measured distance. The population of spin labels at 9–10 Å increases from ~20% for N-terminal regions to ~40% for C-terminal regions (Fig. 8B), suggesting that the repeat giving rise to the 9–10 Å distances has differing structural orders between N- and C-terminal regions. The population of spin labels at 15–17 Å account for ~30–40% of total spins, and it remains relatively unchanged from N terminus to C terminus (Fig. 8B). There is also a significant population of spin labels that are further apart (>20 Å) and do not contribute to the broadening of the continuous-wave EPR spectra. The different populations of spin labels reveal structural heterogeneity in GU-Aβ42 oligomers. The N-terminal region is least ordered, and 50% of spin labels at each labeling site are ~20 Å apart. For C-terminal regions, only 20–30% of spin labels are >20 Å apart. A less ordered N-terminal region and more ordered

FIGURE 3. EPR spectra of spin-labeled GU-Aβ42 oligomers. The oligomers were prepared with spin-labeled and WT proteins at 1:4 molar ratio to minimize the effect of intermolecular spin-spin interactions on EPR lineshape. The sample preparation is referred to as “20% labeled” in the text. Scan width is 200 G.

FIGURE 4. Residue-specific side chain mobility in GU-Aβ42 oligomers. The inverse center line width (δ⁻¹) is determined using the EPR spectra of the 20% labeled GU-Aβ42 oligomers, in which spin-labeled and wild-type GU-Aβ42 are mixed at 1:4 molar ratio. The inset shows how the center line width was measured from the EPR spectrum.
C-terminal region are consistent with the two-component analysis for the N-terminal residues in Fig. 5. In structural studies of Aβ fibrils (51, 52), the N-terminal region is also less ordered than C-terminal region, suggesting that some common mechanisms may underlie the assembly of both oligomers and fibrils.

The x-ray powder diffraction and near atomic-scale EPR distance information provide several structural restraints for prefibrillar Aβ oligomers: (i) β-rich structure; (ii) sheet repeats of three strands; (iii) head-to-tail packing of Aβ subunits within the same β-sheet; (iv) intramolecular antiparallel β-sheets; and (v) face-to-back packing of sheets. From these restraints, we developed an atomic model of prefibrillar Aβ oligomers (Fig. 9). This model fits the powder diffraction data well, with a goodness of fit of 0.220 using the previously described R metric (18), which has a scale of 0 (perfect fit) to 1 (worst possible fit). The best-fit model of prefibrillar Aβ oligomers is composed of 16 Aβ molecules arranged into four face-to-back packed β-sheets (Fig. 9A). Although the architecture of these oligomers is fibril-like, their size (~60 x 60 x 35 Å in the absence of fusion protein partners) suggests that they are expected to have a globular appearance by transmission electron microscopy (Fig. 1A). A schematic of the model of prefibrillar Aβ oligomers is shown in Fig. 9B to illustrate the measured distances from EPR. The model predicts another spin-spin distance at ~20 Å, which was not observed from the distance measurements. This is likely due to the fact that the upper limit of distance measurements with continuous-wave EPR is ~20 Å. Our analysis already revealed two interspin dis-
distances at 9–10 Å and 15–17 Å. Spin-spin interactions at ~20 Å produce very weak broadening in the EPR spectral lineshape. We attempted to include a third distance, which did not significantly improve the fitting. Therefore, we limited our analysis to two interspin distances. This structural model is also capable of accommodating the fusion protein partners, as illustrated in Fig. 9C. The fibril-like β-sheets in our oligomer model suggest that oligomers may be able to convert to fibrils through a mechanism of β-stand rotation (Fig. 9D).

GU-Aβ42 Oligomers Promote the Fibrillization of Aβ42—To further explore the relationship between the GU-Aβ42 oligomers and full-length Aβ42 oligomers (without the fusion protein partner), we studied the aggregation kinetics of Aβ42 in the presence and absence of GU-Aβ42 oligomers. As shown in Fig. 10, GU-Aβ42 oligomers shortened the lag phase of the Aβ42 aggregation. This suggests that GU-Aβ42 oligomers likely adopt a structure that is on pathway to amyloid formation and support the possibility of oligomer-to-fibril conversion as proposed in Fig. 9D. In contrast, GU alone dramatically lengthened the lag phase of Aβ42 fibrillization. This is likely due to the chaperone activity of GroES, consistent with reports in the literature that molecular chaperones generally have anti-amyloid activities (53).

DISCUSSION

Oligomeric assemblies of Aβ, particularly Aβ42, have been widely hypothesized as the primary neurotoxins that cause the pathology in Alzheimer’s disease. Structural knowledge of Aβ oligomers is critical for understanding the structural basis of toxicity and the mechanism of Alzheimer pathogenesis. An accumulating body of evidence supports the notion that Aβ oligomers adopt a different structure from the parallel in-register β-sheet structure of Aβ fibrils (15–19). Particularly, Fourier transform infrared spectroscopy (FTIR) studies on Aβ oligomers prepared using various protocols (15–19) show a common 1695-cm⁻¹ peak, which is absent in fibril samples, suggesting that antiparallel β structure may be a common feature for Aβ oligomers (54). However, concerns have been raised...
regarding whether the 1695-cm⁻¹ peak can distinguish parallel from antiparallel β-sheets (55, 56). For example, Khurana and Fink (57) show that β-helix proteins give rise to an FTIR peak at ~1690 cm⁻¹. Ahmed et al. (16) interpreted the high frequency FTIR peak at 1675–1695 cm⁻¹ as side chain vibrations of arginine, asparagine, and glutamine in their Aβ42 oligomers. Detailed structural information such as inter-residue distances is very limited (16, 58). Currently there are no structural models of oligomers based on extensive experimental restraints. Computational modeling studies have provided a number of structural models (59, 60), but these models still await experimental validation.

In this work, we employed site-directed spin labeling to probe the side chain mobility (Fig. 4) and measure intermolecular distances (Fig. 8) at all 42 residue positions of Aβ42 sequence in a prefibrillar oligomer preparation. Together with x-ray powder diffraction (Fig. 1C), the spin label mobility analysis reveals a turn at residues 24–27, which connects two β-strands at residues 13–23 and 28–42. This β-turn-β motif has also been observed in other Aβ oligomer preparations (61, 62) and has been proposed in Aβ42 fibril structural models (51, 52). Mobility analysis also suggests that N-terminal residues consist of both a disordered state and a structured state (Fig. 5), consistent with recent hydrogen exchange and solid-state NMR
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The essence of our structural model is the antiparallel triple-strand sheet with three β-strands in an antiparallel arrangement. Each Aβ42 molecule participates in the same β-sheet with three β-strands at 9–10 Å and 15–17 Å, but the spin label population for these critical units (63). Globulomers are shown to consist of 12 subunits (9). Prefibrillar oligomers are eluted as a 90-kDa peak using size exclusion chromatography, corresponding to 20 subunits (64). The essence of our structural model is the antiparallel triple-strand architecture. Because of the head-to-tail arrangement between Aβ subunits within the same β-sheet, and the face-to-back packing between β-sheets, our oligomer model is open-ended in both the backbone hydrogen bonding direction and the side chain direction, allowing the growth of the oligomer in size.

The fibril-like appearance of β-sheets in prefibrillar oligomers suggests that prefibrillar oligomers may interconvert with fibrils via a transition from a β-hairpin to a heterosteric zipper (Fig. 9D). The EPR distance restraints determined herein (Fig. 8) dictate that intersheet interactions are mediated by identical segments in oligomers (Fig. 9). Oligomers may convert to fibril seeds by strand rotation, wherein these identical segments rotate about 90° around their β-strand axes to make β-sheets of identical segments. The strand rotation model is illustrated in Fig. 9D. Such strand rotations would be facilitated by flexibility in the turn connecting the two β-strands. Indeed, our EPR data unambiguously reveal that this turn is flexible (Fig. 4). Furthermore, the possibility of oligomer-to-fibril conversion is supported by the result that GU-Aβ42 oligomers reduced the lag phase of Aβ42 aggregation (Fig. 10).

Nussinov et al. previously modeled various triple β-sheet structures for Aβ amyloid (65). In their model, each Aβ42 molecule consists of three β-strands with two turn regions: one at residues 25–29, and the other one at residues 9–14. Each β-strand participates in a different β-sheet in the triple β-sheet structure, which has a better correlation with the hydrogen exchange data than the double β-sheet structure (65). The triple β-sheet structure has been previously reported for Aβ40 fibrils based on solid-state NMR data (66). Hydrogen exchange studies of Aβ42 fibrils also found that N-terminal region is protected in approximately half of the population (52). These studies highlighted the structural importance of N-terminal region, which is likely structured in the amyloid fibrils of both Aβ40 and Aβ42. In the structural model of Aβ42 oligomers, we also show that each Aβ42 molecule consists of three β-strands at similar residue positions as previously found in Aβ42 fibrils (52, 65). At the tertiary structure level, however, three β-strands from the same Aβ42 molecule participate in the same β-sheet in an antiparallel fashion. Therefore, the Aβ42 structure in the oligomers can be characterized as triple-strand, single β-sheet, which distinguishes it from the Aβ42 structure in fibrils.

Structural heterogeneity or polymorphism has been observed for Aβ fibrils (67, 68). For Aβ oligomers, different oligomer preparation protocols have been reported, and these oligomers are classified largely by morphology, size, and immunoreactivity to mono- and polyclonal antibodies (12). The findings in this work allow us to assess the heterogeneity of the underlying molecular structure in Aβ42 oligomers. The EPR spectral line shape (Fig. 3) and spin label mobility profile (Fig. 4) suggest that residues 13–42 adopt a single β-turn-β conformation at secondary structure level. This β-turn-β secondary structure is very similar to those observed in Aβ42 fibrils (51, 52). This similarity can be rationalized in the general framework of hierarchical protein folding (69). Assuming the same force is driving both fibril and oligomer formation, it is not surprising to see structural similarity between oligomers and fibrils at secondary structure level. In contrast to the rest of Aβ42 molecule, the N-terminal residues 1–12 show significant amount of heterogeneity. In the N-terminal region, a locally disordered conformation co-exists with a well-ordered conformation. The population of the disordered conformation is ~10–20% of the total population (Fig. 5). In Aβ42 fibrils, a previous hydrogen exchange study shows that ~50% of the total population for the N-terminal region is structured (52). The higher percentage of the ordered population in oligomers is likely due to the presence of the fusion protein partner, which traps and stabilizes the oligomeric state. Intermolecular distance measurements reveal another layer of structural heterogeneity at quaternary structure level. We observed two major distance distributions at 9–10 Å and 15–17 Å, but the spin label population for these
two distances combined is \( \sim 50\% - 70\% \). The rest of the spin labels, at \( \sim 30\% - 50\% \) of total population, give rise to distances over the detection limit of continuous-wave EPR, which is \( \sim 20 \) Å (Fig. 8B). The spin label population with \( > 20 \) Å distances may represent other structures that are not currently modeled. The spin label mobility studies (Figs. 3 and 4) shows that these other structures are also highly ordered, at least at secondary structure level.

Our modeling explains both the distances at 9–10 Å and 15–17 Å with a unifying structure (Fig. 9). It is likely that other structures satisfying only one set of distances also exist. In Fig. 11, we modeled two such alternative structures in which only
the 9–10 Å distance is accounted for. In alternative model A (Fig. 11), each Aβ42 molecule adopts the same three β-strands, but each β-strand participates in a different β-sheet in an antiparallel fashion. Except for the N-terminal β-strand, the rest of the residues adopt a structure that is very similar to the antiparallel structure of Aβ40 D23N fibrils (70). Although conceptually similar to the triple β-sheet models of Nussinov et al. (65), this alternative model distinguishes itself in the N-terminal region, which folds back to the double β-sheet of the C-terminal region. In alternative model B (Fig. 11), the N-terminal region adopts a locally disordered conformation. This may partly explain the structural heterogeneity observed for this region in our spin label mobility analysis (Figs. 4 and 5) and distance measurements (Fig. 8).

Our structural models have implications about the toxicity of oligomers. Several different oligomer preparations have been shown to have cytotoxicity (8–11). If different oligomers exert their toxicity through similar mechanisms, these oligomers may have similar structural features underlying their toxicity. This work and previous studies (15–19, 39) suggest that one common structural feature observed in different oligomers is the antiparallel β-sheet. Oligomer-specific polyclonal antibody A11 recognizes pore-forming bacterial toxin α-hemolysin and block its toxicity (71). The membrane-spanning core of α-hemolysin is a β-barrel consisting of antiparallel β structures (72). The antiparallel structure may explain the reactivity of our GU-Aβ42 oligomer with the A11 antibody. An alternative explanation to toxicity is that toxicity is associated with properties that are related to the aggregation process and are not specific to particular oligomer species (73). One candidate for such a property is hydrophobicity. In Aβ fibrils, due to symmetric packing of protofilament, the C-terminal hydrophobic region is packed inside the fibril core (50). In the oligomer model of this work (Fig. 9), the packing between adjacent β-sheets is face to back, so more hydrophobic residues are exposed to solvent compared with Aβ fibrils.

Structural conversion from oligomers to fibrils is a critical step in Aβ aggregation. In Fig. 12 we summarize several potential mechanisms for oligomer-fibril interconversion. The mechanism of strand rotation for oligomer-fibril interconversion has been proposed for Aβ based on biochemical studies of Aβ40 protein (74, 75). Here we provide direct structural evidence for strand rotation conversion in that (i) exhaustive EPR distance restraints dictate the model for prefibrillar oligomers and (ii) EPR mobility measurements demonstrate the essential flexibility of the turn connecting the two β-strands. Conversion between toxic oligomers and non-toxic fibrils may represent an attractive point of therapeutic intervention to treat Alzheimer disease. Understanding the conversion mechanisms is essential for rational design of potential therapeutics targeting this process.

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