

Conformational Differences between Two Amyloid β Oligomers of Similar Size and Dissimilar Toxicity^{*[5]}

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Background: The Alzheimer A β peptide assembles into multiple small oligomers that are cytotoxic.

Results: Increased solvent exposure of hydrophobic residues within non-fibrillar A β oligomers of similar size increases cytotoxicity.

Conclusion: A β non-fibrillar oligomers display size-independent differences in toxicity that are strongly influenced by oligomer conformation.

Significance: Identifying the conformational determinants of A β -mediated toxicity is critical to understand and treat Alzheimer disease.

Several protein conformational disorders (Parkinson and prion diseases) are linked to aberrant folding of proteins into prefibrillar oligomers and amyloid fibrils. Although prefibrillar oligomers are more toxic than their fibrillar counterparts, it is difficult to decouple the origin of their dissimilar toxicity because oligomers and fibrils differ both in terms of structure and size. Here we report the characterization of two oligomers of the 42-residue amyloid β (A β 42) peptide associated with Alzheimer disease that possess similar size and dissimilar toxicity. We find that A β 42 spontaneously forms prefibrillar oligomers at A β concentrations below 30 μ M in the absence of agitation, whereas higher A β concentrations lead to rapid formation of fibrils. Interestingly, A β prefibrillar oligomers do not convert into fibrils under quiescent assembly conditions but instead convert into a second type of oligomer with size and morphology similar to those of A β prefibrillar oligomers. Strikingly, this alternative A β oligomer is non-toxic to mammalian cells relative to A β monomer. We find that two hydrophobic peptide segments within A β (residues 16–22 and 30–42) are more solvent-exposed in the more toxic A β oligomer. The less toxic oligomer is devoid of β -sheet structure, insoluble, and non-immunoreactive with oligomer- and fibril-specific antibodies. Moreover, the less toxic oligomer is incapable of disrupting lipid bilayers, in contrast to its more toxic oligomeric counterpart. Our results suggest that the ability of non-fibrillar A β oligomers to interact with and disrupt cellular membranes is linked to the degree of solvent exposure of their central and C-terminal hydrophobic peptide segments.

The seminal role of protein misfolding in several aggregation disorders has motivated the identification of protein aggregates that are highly toxic relative to those that are either less toxic or non-toxic. One logical approach to accomplish this aim is to classify aggregated conformers based on their size, and evaluate the relationship between size and toxicity. Extensive work has convincingly demonstrated that aggregate size is a critical determinant of toxicity (1–6). For example, small oligomers of the A β peptide (as well as other misfolded polypeptides) are generally more toxic than large oligomers and amyloid fibrils (2, 7–11).

Nevertheless, it is becoming increasingly clear that protein aggregates of the same size can have unique structures and, therefore, unique toxicities (8, 12–15). An important advance in classifying misfolded proteins in terms of structure has been the development of conformation-specific antibodies that recognize unique misfolded isoforms (12, 16–24). For example, the A11 polyclonal antibody selectively recognizes prefibrillar oligomers of several amyloidogenic proteins relative to fibrils and monomers of the same proteins (17). Conversely, multiple fibril-specific antibodies have also been developed that selectively recognize amyloid fibrils of several aggregation-prone proteins (18, 19, 23). These antibodies have revealed that proteins can form multiple oligomeric and fibrillar conformers with similar sizes and unique conformations (8, 12, 17, 18, 25).

An attractive strategy for understanding how conformational differences between misfolded proteins mediate toxicity is to evaluate the structures of aggregated conformers of similar size and dissimilar toxicity. Chiti and co-workers (13) performed an elegant analysis of two oligomers of a bacterial protein (HypF-N) of the same size that differ in their cytotoxicity. HypF-N folds into two unique oligomers at different solution conditions that are indistinguishable in terms of size and morphology, yet only one oligomer is toxic to mammalian cells. The authors performed site-specific fluorescent labeling analysis of each oligomer and found that hydrophobic residues within the more toxic oligomer were less structured (and more solvent-

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exposed) than those within the less toxic oligomer. This important study of a non-pathogenic protein provides an important basis for similar studies of disease-linked misfolded polypeptides.

A previous report suggests that A β 42 also forms two different oligomers of similar size and dissimilar toxicity (8). Klein and co-workers (8) found that A β 42 forms toxic oligomers in cell culture media at 4 °C that are recognized by an oligomer-specific antibody. Notably, A β oligomers of similar size occasionally formed that were neither toxic nor recognized by oligomer-specific antibodies, revealing that A β oligomers can also possess size-independent differences in toxicity. We recently reported a reproducible procedure for forming A β oligomers that are weakly toxic and similar in size to their more toxic counterparts (14). Here we characterize the biochemical and structural properties of each A β oligomer relative to A β fibrils and monomers to understand the origins of their size-independent differences in toxicity.

EXPERIMENTAL PROCEDURES

Preparation of A β Conformers—Human A β 42 (American Peptide) was dissolved in an aqueous, 50% acetonitrile solution (1 mg/ml), aliquoted, dried under vacuum and lyophilized, and then stored at –20 °C. A β oligomers were prepared by dissolving the peptide in 100% hexafluoroisopropanol (Fluka). The solvent was evaporated, and A β was dissolved in 50 mM NaOH (1 mg/ml A β), sonicated (30 s), and diluted in PBS (25 μ M A β). The peptide was then centrifuged (22,000 \times *g* for 30 min), and the pelleted fraction (5% of starting volume) was discarded. The supernatant was incubated at 25 °C for 0–6 days without agitation. A β fibrils were prepared via the same procedure except that monomers were mixed with preexisting fibrils (10–20 weight percent seed) without mixing for 24 h at 25 °C.

Thioflavin T (ThT)² Assay—A β (25 μ M) was diluted with ThT (44 μ M; 1:19 volumetric ratio of A β /ThT solutions). The fluorescence was measured using a Tecan Safire² plate reader (450/482-nm excitation/emission, 15-nm bandwidth). The seeding experiments were conducted with A β monomers (25 μ M) and 5% preformed A β oligomers and fibrils.

Atomic Force Microscopy (AFM)—A β samples (25 μ M) were spotted on cut mica mounted on glass slides. The samples were adsorbed (30 min), washed with water, and dried overnight. Images were taken using an Asylum Research MFP 3D AFM system with Olympus AC240TS silicon cantilevers in tapping mode (AC, scan rate of 0.5 Hz).

Cell Toxicity Assay—Rat adrenal medulla cells (PC12, ATCC) were cultured in Dulbecco's modified Eagle's medium (5% fetal bovine serum, 10% horse serum, and 1% penicillin/streptomycin). The cell suspension (90 μ l) was incubated in 96-well microtiter plates (CellBIND, Corning) for 24 h. Afterward, A β or control samples (10 μ l) were added to microtiter plates, and the cells were further incubated for 48 h at 37 °C.

The cell viability was then evaluated using two assays. In the first method, the media was removed, and fresh media (200 μ l) and thiazolyl blue tetrazolium bromide (Sigma; 50 μ l of 2.5 mg/ml) were added to each well for 3 h at 37 °C. These solutions were then discarded, 250 μ l of DMSO was added, and the absorbance was measured at 562 nm. The toxicity values were normalized relative to the buffer (PBS).

The cell viability was also evaluated via the lactate dehydrogenase (LDH) assay (Sigma-Aldrich). The cell culture media was transferred to a clean 96-well, flat bottom plate. Equal volumes of LDH assay substrate, dye, and cofactor solutions were added to each well. The final volume of LDH assay solution added was equal to twice the volume of medium removed for testing. The microtiter plate was then covered and incubated at room temperature for 20 min, after which the reaction was terminated by the addition of 0.1 M HCl (final concentration). The absorbance was measured at 490 nm, and the LDH release values were normalized to the buffer (PBS).

A β toxicity was also evaluated for primary cultures of embryonic rat cortical neurons, as described previously (11). A β 42 peptide was added to the neuronal cell cultures at a concentration of 6 μ M. The cells were incubated for 24 h, and then the cell viability was analyzed using the MTT assay (Sigma). After 4 h of incubation with MTT, the media were removed and replaced with DMSO. The fraction of viable cells were quantified using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA) at a wavelength of 570 nm. The toxicity values were normalized relative to the buffer (PBS).

Gel Electrophoresis and Silver Staining—A β samples (25 μ M) were diluted into sample buffer (Novex LDS, Invitrogen), sonicated, analyzed using 10% BisTris gels (Invitrogen), and silver-stained (SilverXpress kit, Invitrogen).

Antibody Dot Blot Analysis—Each A β conformer (25 μ M) was spotted (2 μ l) on nitrocellulose membranes (Hybond ECL, GE Healthcare). Afterward, the blots were blocked overnight (10% nonfat dry milk in PBST) and then probed with A11 (Invitrogen), OC (Millipore), or 6E10 (Millipore) antibodies. The blots were washed, incubated with the appropriate horseradish peroxidase-conjugated secondary antibody, and developed (ECL Western blotting substrate, Thermo Fisher).

Circular Dichroism Spectroscopy—A β conformers (25 μ M in 0.1 \times PBS) were evaluated using a Jasco 815 spectrometer (1-mm path length cuvette) at 25 °C. Each sample spectrum is the average of at least 25 scans.

8-Anilinoanthralene-1-sulfonate (ANS) Fluorescence Analysis—ANS (Sigma-Aldrich) was used at 7.5–12.5 μ M to assay the conformation of A β (2.5 μ M). The ANS fluorescence spectra (λ_{ex} = 380 nm) were measured using a Tecan Safire² plate reader.

Lipid Bilayer Conductance Analysis—All lipid bilayers were formed using 1- α -phosphocholine (asolectin from soy, 20%; Sigma-Aldrich). Bilayers were formed using a modified version of the painting technique (26). A Delrin cup with a 250- μ m aperture was inserted into a holding chamber (Warner Instruments), and both the cup and chamber were filled (10 mM HEPES, 100 mM KCl, pH 7). Asolectin was dissolved in *n*-decane (200 mg/ml asolectin) and applied to the exterior of the cup aperture using a fine tipped brush. Bilayer formation was

² The abbreviations used are: ThT, thioflavin T; ANS, 8-anilinoanthralene-1-sulfonate; GdnHCl, guanidine hydrochloride; LDH, lactate dehydrogenase; AFM, atomic force microscopy; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

detected via an increase in capacitance and the formation of a seal in excess of 1 gigaohm. Bilayers were equilibrated for at least 10 min prior to the sample addition. A β samples (250 nM) were added to a chamber reservoir on one side of the bilayer and equilibrated for 20 min. For some experiments, the A11 antibody (0.2–3 μ M) was mixed with A β soluble oligomers (12.5 μ M) for 4 h and then diluted 50 times into the chamber reservoir. Voltage sweeps were performed from –100 to +100 mV at a rate of 40 mV/s. The data were collected through a BC535 patch clamp amplifier (Warner Instruments), digitized using a Digidata 1440A digitizer, and analyzed using Clampex 10.1 software (Axon Instruments).

Proteolytic Fragmentation Analysis—A β (25 μ M) was mixed with Proteinase K (0.5 μ g/ml) in PBS (pH 7.4), and A β samples (2 μ l) were deposited on nitrocellulose (Hybond ECL, GE Healthcare) periodically for 4 h. At the end of the fragmentation reaction, the blots were blocked overnight (10% nonfat dry milk in PBST) and then probed with sequence-specific antibodies against A β (6E10 against A β (3–10) from Sigma-Aldrich, BAM90.1 against A β (16–21) from Sigma-Aldrich, 4G8 against A β (18–22) from Covance, a polyclonal antibody against A β (30–36) from Sigma-Aldrich, 9F1 against A β (35–39) from Santa Cruz Biotechnology, Inc., and 12F4 against A β (37–42) from Abcam). The blots were then washed, incubated with the appropriate horseradish peroxidase-conjugated secondary antibody, and developed (ECL Western blotting Substrate, Thermo Fisher).

Size Exclusion Chromatography Analysis—Preformed A β oligomers (25 μ M) were mixed with a single domain antibody (2.5 μ M) specific for amyloid β presenting A β residues 33–42 in its third complementarity-determining region (24) and injected (100 μ l) into an analytical size exclusion column (TSK Gel G3000SWxl column, 0.78 \times 30 cm; Tosoh Bioscience). The elution profile of the A β -antibody complex was monitored at 280 nm.

RESULTS

Non-fibrillar A β Conformers Form at Low A β Concentrations—We first investigated the range of A β concentrations that promote formation of A β prefibrillar oligomers in PBS (pH 7.4). The assembly of A β 42 (1–75 μ M) can be readily evaluated via immunoblot analysis using antibodies specific for prefibrillar oligomers (A11) and fibrillar conformers (OC; Fig. 1) (12, 17, 18). We first confirmed that each conformation-specific antibody recognized prefibrillar oligomers and fibrils at submicromolar A β concentrations (supplemental Fig. S1). We also confirmed the proper loading of each A β conformer via the sequence-specific antibody 6E10, which recognizes the N terminus of A β (residues 3–10; Fig. 1).

At A β concentrations of <20 μ M, we find that both A11- and OC-positive conformers fail to form over 10 days (Fig. 1). In contrast, we find that higher concentrations of A β (20 and 25 μ M) lead to formation of A11-positive conformers after 1 day, and these oligomers persist for an additional 2 days (Fig. 1). Importantly, the A11-positive conformers appear to be non-fibrillar because the OC antibody does not recognize them. On the fourth day, the A11-positive oligomers formed at 20 and 25 μ M A β convert into an alternative conformer that is non-im-

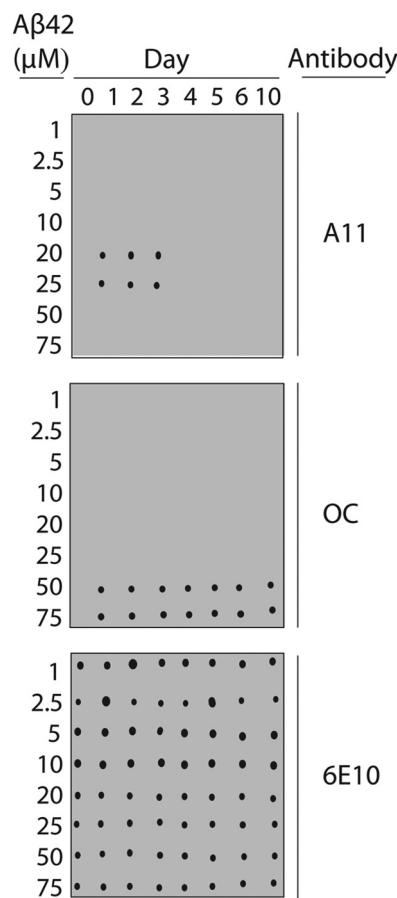


FIGURE 1. Conformation-specific antibody analysis of A β assembly. A β 42 conformers (1–75 μ M) were assembled for 10 days (without agitation), and periodically deposited on nitrocellulose membranes. Afterward, the membranes were probed with conformation-specific (A11, prefibrillar conformers (top), and OC, fibrillar conformers (middle)) and sequence-specific (6E10, N terminus of A β (bottom)) antibodies.

munoreactive with either conformation-specific antibody. Longer times (5–10 days) do not promote formation of A11- or OC-positive conformers for A β samples at 20–25 μ M (Fig. 1).

Because A β is well known to readily form amyloid fibrils, we suspected that A β concentrations above 25 μ M would lead to formation of fibrils without agitation. Indeed, we found that A β at 50 and 75 μ M formed OC-positive conformers after 1 day that were invariant over longer times (2–10 days; Fig. 1). These OC-positive conformers are A11-negative, consistent with fibrillar intermediates and mature amyloid fibrils (14, 18).

We also evaluated the homogeneity of A11-positive oligomers formed at 25 μ M A β (supplemental Fig. S1). We doped A β fibrils into preparations of A β A11-positive oligomers and evaluated the minimum fibril detection limit of the OC antibody. We found that fibrils could be detected at \geq 0.6 μ M (supplemental Fig. S1), which represents 5% of the A β peptide at 25 μ M. We observed the same sensitivity of the A11 antibody when we doped fibril samples with A11-positive oligomers. Therefore, we estimate that the fraction of A β fibrils in our non-fibrillar oligomer preparations was below 5%.

A β Oligomers Possess Size-independent Differences in Toxicity—The unique immunoreactivity of A β conformers formed at intermediate A β concentrations (20 and 25 μ M) led us to evaluate the size and toxicity of both A11-positive and

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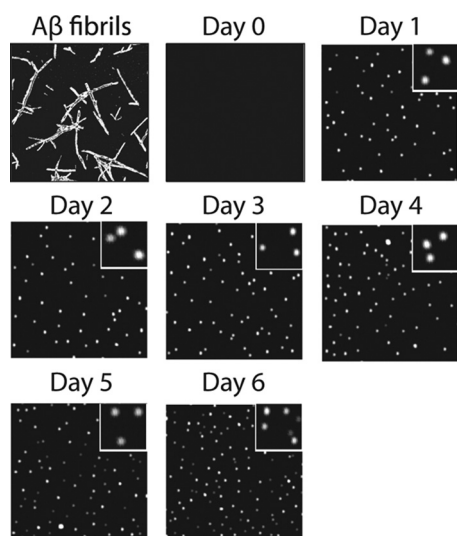


FIGURE 2. **AFM analysis of A β oligomerization.** A β 42 (25 μ M) was assembled without agitation, deposited on mica substrates, and imaged using AFM. Each image is 3 \times 3 μ m, and the inset images are 0.5 \times 0.5 μ m.

A11-negative A β conformers. Using AFM, we found that A11-positive oligomers (25 μ M A β) formed on days 1–3 were globular and possessed similar size (6.2 ± 0.5 nm in height; Fig. 2 and supplemental Fig. S2). Importantly, the A11-negative conformers formed on days 4–6 were also globular and of similar size (6.1 ± 0.6 nm in height) relative to A11-positive oligomers (Fig. 2 and supplemental Fig. S2). We also used size exclusion chromatography to evaluate the size of both A β oligomers. Because each A β oligomer sticks to the column matrix and fails to elute in non-denaturing buffers, we evaluated the size of A β oligomers when complexed to a small antibody domain specific for A β (supplemental Fig. S3). Importantly, we found that the elution times of A β oligomers bound to the same antibody were indistinguishable. We herein refer to A11-positive A β oligomers as A+ oligomers and oligomers of the same size that are non-reactive with either conformation-specific antibody as A– oligomers. Finally, AFM analysis revealed that higher concentrations of A β (50 μ M) led to the formation of fibrillar structures after 1 day (supplemental Fig. S4) as expected based on their reactivity with the OC antibody (Fig. 1).

We next evaluated the toxicity of each A β oligomer using multiple mammalian cell culture assays (14, 27, 28). We expected that A+ oligomers (formed at 20–25 μ M; days 1–3) would be more toxic than A– oligomers (formed at 20–25 μ M; days 4–10) and OC-positive conformers (formed at 50–75 μ M; days 1–6). We found that A+ oligomers were highly toxic to differentiated PC12 cells (Fig. 3A), whereas fibrils formed at elevated A β concentrations (50 μ M) were mildly toxic (supplemental Fig. S5). In contrast, A– oligomers formed at 25 μ M A β (days 4–6) were non-toxic relative to A β monomers (Fig. 3A). We also confirmed that these toxicity results were similar when evaluating metabolic activity (MTT reduction; Fig. 3A) or membrane integrity (LDH activity; Fig. 3B). Finally, we evaluated the toxicity of each A β oligomer to primary cultures of embryonic rat cortical neurons and also found that A+ oligomers were more toxic than A– oligomers and OC-positive fibrillar conformers (Fig. 3C).

A β Oligomers Display Dissimilar Activity for Disrupting Lipid Membranes—A β A+ oligomers have been shown to permeabilize reconstituted lipid membranes (29), which has been posited to be integral to their toxic activity *in vivo*. This observation led us to hypothesize that A– oligomers would be inactive at disrupting lipid membranes. To investigate this hypothesis, we evaluated the conductance of lipid bilayers in the absence and presence of both A β oligomers. We found that A+ oligomers permeabilized lipid bilayers and increased their conductance (Fig. 4A), as observed previously (29). In contrast, A– oligomers (as well as A β fibrils and monomers) failed to increase membrane conductance at the same A β concentrations (250 nM). We also evaluated whether the oligomer-specific (A11) antibody could antagonize the ability of A+ oligomers to permeabilize lipid bilayers (Fig. 4B). We found that the anti-oligomer antibody reduced membrane conductance in a dose-dependent manner. Our results reveal that the same A β conformer recognized by the A11 antibody is responsible for interacting with lipid bilayers and altering their structure.

A β A+ Oligomers Are More Hydrophobic than A– Oligomers—We next sought to define the biochemical and structural differences between A+ and A– A β oligomers. As a possible mechanism for the low toxicity of A– oligomers, we hypothesized that A– oligomers are folded in a manner in which their hydrophobic residues are less solvent-exposed than in A+ oligomers. To evaluate this hypothesis, we developed a proteolytic assay that uses sequence-specific antibodies to interrogate the relative degree of solvent exposure of multiple sequence epitopes within A β oligomers and fibrils. This assay is based on the premise that linear sequence epitopes within A β conformers are cleaved by the protease at a rate proportional to their degree of solvent exposure. Solvent-exposed sequences are more accessible to proteolysis and will exhibit more rapid loss of antibody binding with time. We used six sequence-specific A β antibodies directed against different epitopes distributed throughout A β (A β residues 3–10, 16–21, 18–22, 30–35, 35–39, and 37–42). We posited that the hydrophobic middle (A β (17–21)) and C-terminal (A β (30–42)) peptides within A β oligomers and fibrils would be cleaved more slowly than the hydrophilic N terminus (A β (1–16)).

We chose to use Proteinase K because it cuts at 19 positions that are distributed throughout the N-terminal, middle, and C-terminal regions of A β . We added Proteinase K (0.5 μ g/ml) to each A β conformer (25 μ M) and deposited A β samples on nitrocellulose membranes every 10–30 min during the fragmentation reaction (150 min). Because the deposited A β samples dry quickly (within seconds) once spotted on nitrocellulose, the proteolytic reaction is quenched rapidly and can be interrogated after the fragmentation reaction is complete. We found that the rate of proteolytic cleavage for the hydrophilic N terminus (A β (3–10)) was the same for A β monomers, oligomers, and fibrils (Fig. 5). In contrast, the fragmentation of the central A β peptide segment (A β (18–22)) was slower for A β oligomers and fibrils than for monomers, suggesting that this central region is more solvent-protected in aggregated A β conformers than in A β monomers. Importantly, this same region was more protected from proteolytic fragmentation within A– oligomers than within A+ oligomers and most protected

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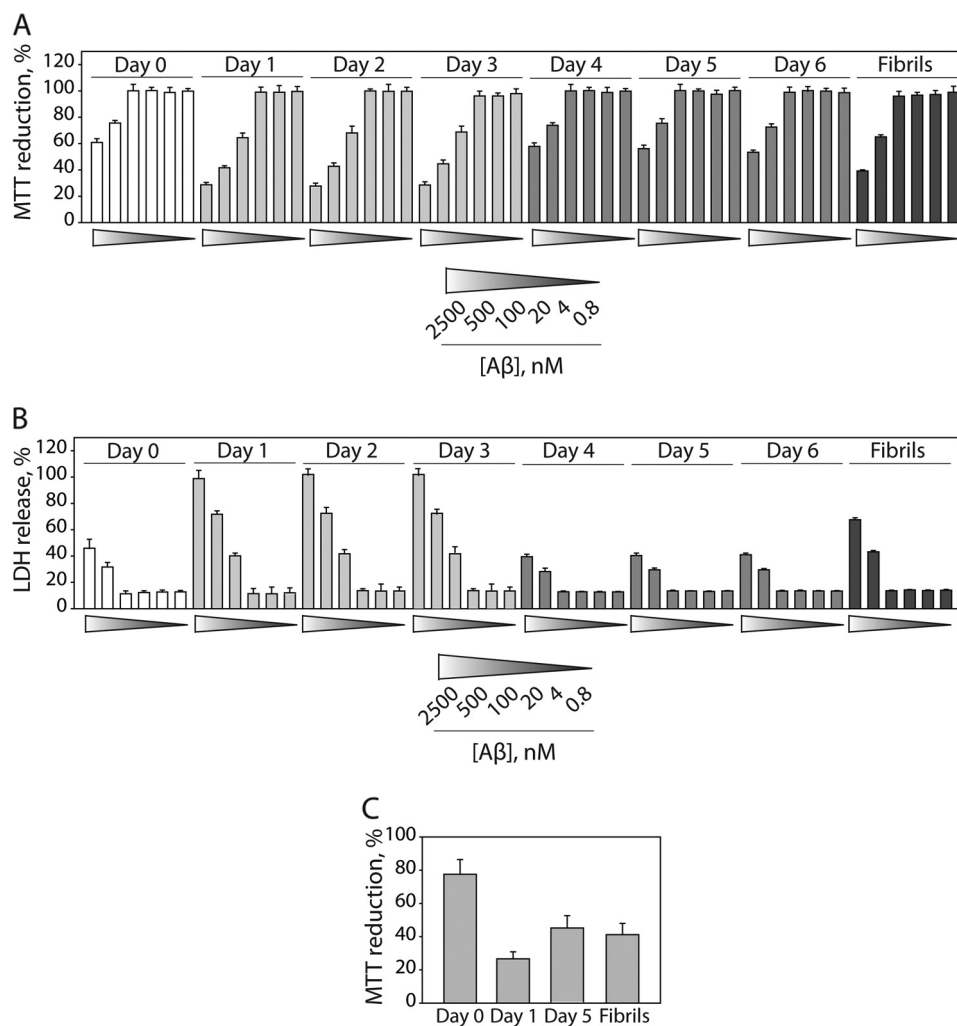


FIGURE 3. **Toxicity analysis of A β oligomers and fibrils.** A β 42 (25 μ M) was assembled without agitation and added to rat adrenal medulla cells (A and B) and rat primary cortical neuronal cells (C), and the relative toxicity was evaluated ($n = 3$). Error bars, S.D.

within fibrils (Fig. 5). We obtained identical results using a second antibody against an overlapping A β peptide segment (A β residues 16–21; supplemental Fig. S6). We also confirmed that both antibodies bound to each A β conformer with the same apparent affinity (supplemental Fig. S7).

Next we investigated the rate of proteolytic fragmentation of a C-terminal hydrophobic peptide segment of A β (residues 35–39) within each A β conformer (Fig. 5). Importantly, this C-terminal hydrophobic motif was more protected from the protease than the central (A β (18–22)) and N-terminal (A β (3–10)) peptide segments within A β oligomers and fibrils. Moreover, the same C-terminal A β peptide (A β (35–39)) was more protected within A $-$ oligomers than within A $+$ oligomers and most protected within A β fibrils. We obtained identical results for two additional antibodies directed against overlapping C-terminal A β peptides (A β (30–35) and A β (37–42); supplemental Fig. S6). Collectively, our results reveal that hydrophobic residues within the central and C-terminal regions of A β 42 are more solvent-protected within A $-$ A β oligomers than within A $+$ oligomers.

These proteolytic fragmentation results that suggest specific differences in the extent of solvent exposure of hydrophobic A β

peptides within A $+$ and A $-$ oligomers led us to evaluate the overall hydrophobicity of each A β conformer using the hydrophobic dye ANS (Fig. 6A and supplemental Fig. S8). As expected, we found that A β monomers (day 0) were most hydrophobic, as judged by their blue-shifted spectra ($\lambda_{\max} = 455 \pm 1$ nm). Moreover, we found that the hydrophobicity of the aggregated A β conformers was highest for A $+$ oligomers ($\lambda_{\max} = 483 \pm 1$ nm; days 1–3), intermediate for A $-$ oligomers ($\lambda_{\max} = 502 \pm 1$ nm; days 4–6), and lowest for fibrils ($\lambda_{\max} = 527 \pm 1$ nm). We also confirmed that the same patterns of hydrophobicity for each A β conformer were obtained at multiple ratios of ANS to A β (supplemental Fig. S8). Moreover, we confirmed that each A β conformer possesses a high level of homogeneity, as evidenced by a single peak in the ANS emission spectra (supplemental Fig. S8) relative to the multiple ANS peaks obtained when mixing A β oligomers and fibrils (supplemental Fig. S9). In summary, the increased hydrophobicity of A $+$ oligomers relative to A $-$ oligomers and fibrils revealed by ANS is consistent with our proteolytic fragmentation results (Fig. 5).

A β A $+$ Oligomers Are Less Stably Folded than A $-$ Oligomers— Based on our ANS and proteolytic results, we posited that A $+$

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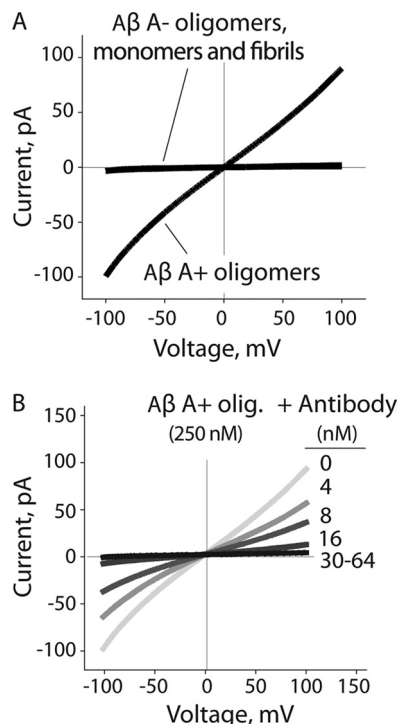


FIGURE 4. Impact of A β oligomers on lipid bilayer conductance. *A*, A β 42 conformers (250 nM) were added to a reservoir on one side of the lipid bilayer (L- α -phosphocholine), and the membrane conductance was measured. *B*, A β A+ oligomers (12.5 μ M) were mixed with the A11 antibody (0.2–3 μ M) and diluted into a reservoir on one side of the lipid bilayer (50 \times dilution), and the membrane conductance was measured.

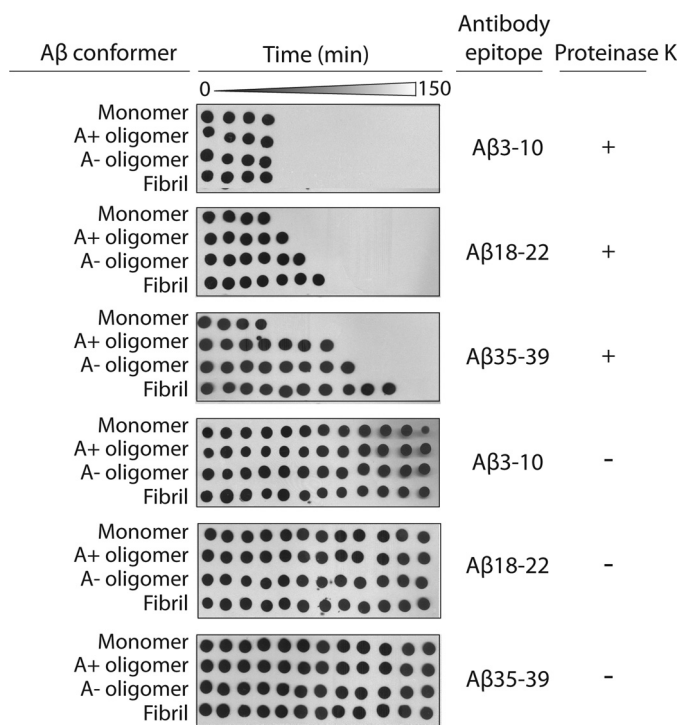


FIGURE 5. Rate of proteolytic fragmentation of A β peptide segments within A β oligomers. A β 42 (25 μ M) was incubated with Proteinase K (0.5 μ g/ml), deposited periodically on nitrocellulose (every 10–30 min), and probed with antibodies specific for N-terminal (A β (3–10); 6E10), central (A β (18–22); 4G8), and C-terminal (A β (35–39); 9F1) A β epitopes. The time intervals were 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 120, and 150 min.

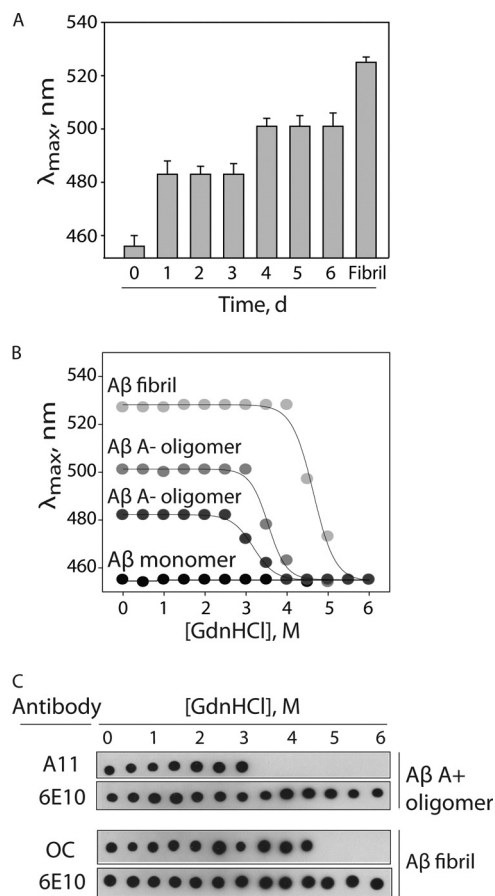


FIGURE 6. Analysis of hydrophobicity and conformational stability of A β oligomers. *A*, A β 42 (25 μ M) was assembled without agitation, and the hydrophobicity of A β conformers formed each day was evaluated using ANS fluorescence. The wavelength corresponding to the maximum ANS fluorescence is reported on the y axis. *B* and *C*, A β conformers (25 μ M) were incubated with variable amounts of guanidine hydrochloride, and then their relative degree of unfolding was evaluated using ANS fluorescence (*B*) and antibodies (A11, prefibrillar oligomers; OC, fibrillar conformers; 6E10, N terminus of A β) (*C*). Error bars, S.D.

oligomers are less stably folded than A- oligomers and fibrils. To evaluate this hypothesis, we analyzed the conformational stability of each A β conformer in guanidine hydrochloride (GdnHCl) using ANS fluorescence analysis (Fig. 6*B*). We found that A+ oligomers unfolded at lower denaturant concentrations ($D_{1/2} = 3.2 \pm 0.03$ M GdnHCl) than A- oligomers ($D_{1/2} = 3.5 \pm 0.03$ M GdnHCl) and fibrils ($D_{1/2} = 4.6 \pm 0.04$ M GdnHCl; Fig. 6*B*). The increased stability of A- oligomers relative to A+ oligomers is consistent with our finding that A- oligomers were insoluble in a strong surfactant (0.5% lithium dodecyl sulfate), whereas A+ oligomers were soluble in lithium dodecyl sulfate (supplemental Fig. S10).

It is also possible that A+ oligomers possess a small fraction of highly toxic oligomers with unique biochemical properties. This hypothesis would predict that the bulk biochemical properties of A+ oligomers (as judged by nonspecific dyes, such as ANS) would be different from those of the specific toxic oligomers themselves (as judged by specific probes, such as the A11 antibody). Therefore, we asked whether the conformational stability of A+ oligomers evaluated by ANS and the A11 antibody are similar (Fig. 6*C*). Indeed, we found that the A11

epitope was eliminated at 3.5 M GdnHCl, consistent with the ANS results ($D_{1/2} = 3.2 \pm 0.03$ M GdnHCl). We also performed a similar analysis for A β fibrils using the OC antibody. We found that the OC epitope was eliminated at 5 M GdnHCl (Fig. 6C), which is also consistent with the ANS results ($D_{1/2} = 4.6 \pm 0.04$ M GdnHCl). These results reveal that the folding stabilities of A11 (oligomer)- and OC (fibrillar)-positive conformers are weakly dependent on the method used to measure them.

A β Oligomers Lack β -Sheet Structure—We next investigated whether differences in secondary structure between A+ and A- oligomers explain their differential toxicity. We first asked whether A- oligomers are positive for the amyloid dye ThT, as would be expected for β -sheet-rich conformers (Fig. 7A). In contrast to A β fibrils, A- oligomers display low ThT fluorescence that is indistinguishable from the ThT fluorescence of A β monomers and A+ oligomers. We also evaluated the secondary structure of each A β oligomer using circular dichroism spectroscopy (Fig. 7B). Importantly, both A β oligomers possess random-coil structure that is indistinguishable from A β monomers and significantly different than the β -sheet structure of A β fibrils. Finally, we asked whether the A β oligomers are competent for seeding A β monomers into ThT-positive conformers in a manner similar to A β fibrils (Fig. 7C). Importantly, neither A β oligomer seeds A β monomers. We conclude that the secondary structure of A- oligomers is similar to that of A+ oligomers and significantly different from that of A β fibrils.

DISCUSSION

We have evaluated the conformational differences between two A β oligomers of similar size in order to carefully separate the contributions of aggregate size and conformation to cellular toxicity. An important finding of our studies is that increased solvent exposure of two hydrophobic A β peptide motifs within non-fibrillar A β oligomers is linked to increased cellular toxicity and disruption of lipid membranes. Our studies share important similarities with and differences from a previous study of two oligomers of a non-pathological bacterial protein (HypF-N) that possess similar size and dissimilar toxicity (13). Although HypF-N and A β possess little sequence similarity (6%), our finding that less well folded A β oligomers that are more toxic is consistent with the structural data for HypF-N oligomers. A notable difference between our work and this previous study is that the HypF-N oligomers are β -sheet-rich, whereas our A β oligomers lack β -sheets. The similar size-independent differences in toxicity for both β -sheet HypF-N oligomers and non- β -sheet A β oligomers may suggest that the toxicity of small amyloidogenic oligomers is governed primarily by the degree of solvent exposure of hydrophobic residues and is weakly influenced by their secondary structures. It will be important in the future to compare the toxicity of the non- β -sheet A+ oligomers formed in this work with β -sheet-rich A β oligomers reported previously (2, 11, 16).

Our identification of two A β 42 oligomers of similar size and dissimilar toxicity is reminiscent of two A β oligomers (A β -derived diffusible ligands) identified previously (8). An important advance in our work is the development of reproducible methods for forming low toxicity A β oligomers, which were reported previously to form sporadically (8). It is notable in this previous

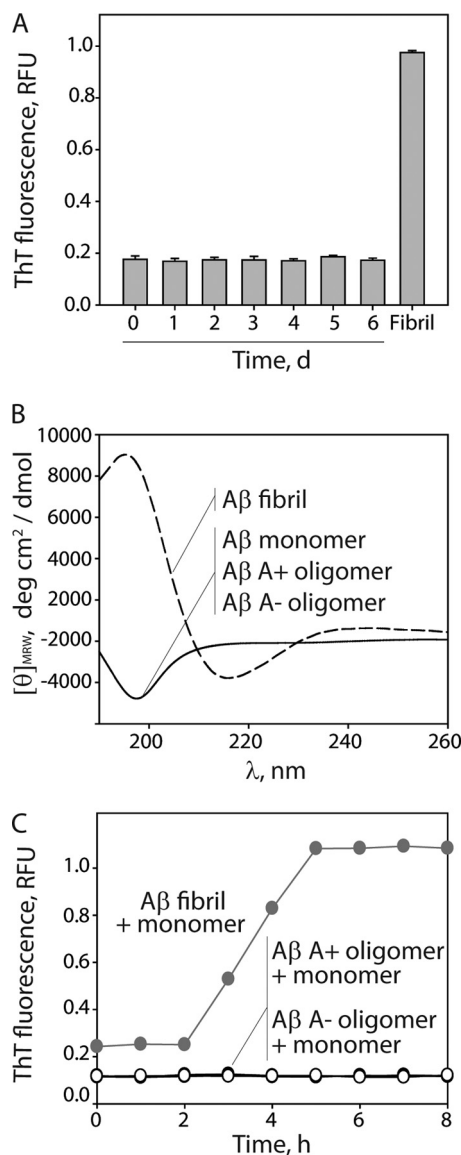


FIGURE 7. Characterization of the secondary structure and seeding activity of A β oligomers. A β 42 (25 μ M) was assembled without agitation (0–6 days), and its extent of fibrillization and secondary structure were evaluated using ThT fluorescence (A) and circular dichroism (B). C, A β fibrils and oligomers were mixed with A β monomers (25 μ M, 5% seed), and their ThT fluorescence was monitored. RFU, relative fluorescence units. Error bars, S.D.

study that A β 42 was assembled at high concentration (100 μ M) and low temperature (4 $^{\circ}$ C) in cell culture media. We find that lower A β concentrations (25 μ M) are essential to control the formation of A11- and OC-negative A β oligomers, and that the low temperature and cell culture media are unnecessary because we conducted our studies at 25 $^{\circ}$ C in PBS. We posit that the lack of reproducibility in the previous study was due to the elevated A β concentration and/or the use of cell culture media that would significantly accelerate aggregation and potentially promote heterogeneous nucleation with components in the media.

The intriguing concentration dependence we observed for A β oligomerization deserves further consideration. We find that A- oligomers form at A β concentrations of 20–25 μ M without agitation, whereas fibrils form at higher A β concentra-

Conformational Differences between A β Oligomers

tions (50–75 μM). Moreover, at A β concentrations of <20 μM , we find that A β fails to form oligomers or fibrils over 10 days in the absence of mixing. Our findings are consistent with two primary aggregation pathways for quiescent assembly: one that is dominant at A β concentrations near the threshold concentration necessary for aggregation and a second one that is dominant at elevated A β concentrations. At 20–25 μM A β , we find that A β A⁺ oligomers mature into “off-pathway” oligomers that possess similarities (e.g. insoluble in strong surfactant) and differences (e.g. non- β -sheet structure) relative to A β fibrils. We have previously shown that A⁺ oligomers at 25 μM mature into fibrils instead of A[−] oligomers when agitated (14, 28), revealing that quiescent assembly is necessary to favor formation of A[−] oligomers. We posit that relatively low A β concentrations and/or the lack of agitation favor A⁺ oligomers to undergo a modest conformational change that results in decreased solvent exposure of hydrophobic residues without fully maturing into β -sheet fibrils. In contrast, we posit that higher A β concentrations and/or agitation favor A⁺ oligomers to mature into β -sheet fibrils without being trapped in the A[−] oligomer conformation.

It will be interesting to evaluate if A β A[−] oligomers that we formed *in vitro* also form *in vivo*. Although the range of A β concentrations used in our study (20–75 μM) are much higher than the concentrations found in extracellular fluid (which are as low as 1 nM) (30–33), it is well established that A⁺ oligomers form *in vivo* (17, 34, 35). One possible explanation for the ability of A β to oligomerize *in vivo* is that A β can be concentrated significantly (>2.5 μM) within endosomes and lysosomes (36). High concentrations of A β within such intracellular compartments coupled with molecular crowding effects (37) may explain the ability of A β to oligomerize *in vivo* despite extremely low levels of A β in extracellular fluid. The antibody A11 has been invaluable for detecting A⁺ oligomers that form *in vivo* (17, 34, 35), and it will be necessary to also raise antibodies against A[−] oligomers to evaluate the biological relevance of such oligomers. Nevertheless, our characterization of a less toxic A β oligomeric isoform (A[−]) that is similar in size to the biologically relevant A⁺ A β oligomer provides important insights into size-independent mechanisms of A β -mediated cytotoxicity. We expect that our findings will motivate future site-specific structural studies to elucidate in more detail the conformational differences encoding the dissimilar toxicities of non-fibrillar A⁺ and A[−] A β oligomers.

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