

A Disulfide-Stabilized A β that Forms Dimers but Does Not Form Fibrils

Sheng Zhang, Stan Yoo, Dalton T. Snyder, Benjamin B. Katz, Amy Henrickson, Borries Demeler, Vicki H. Wysocki, Adam G. Kreutzer,* and James S. Nowick*



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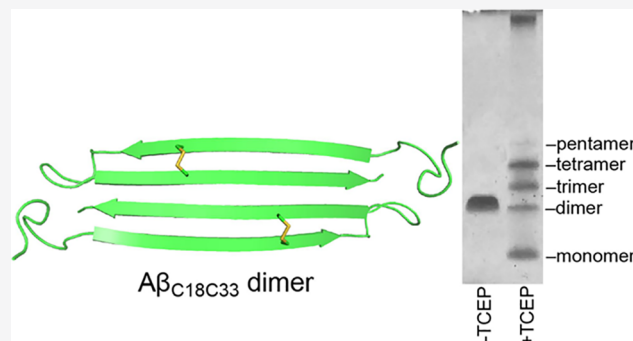


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ABSTRACT: A β dimers are a basic building block of many larger A β oligomers and are among the most neurotoxic and pathologically relevant species in Alzheimer's disease. Homogeneous A β dimers are difficult to prepare, characterize, and study because A β forms heterogeneous mixtures of oligomers that vary in size and can rapidly aggregate into more stable fibrils. This paper introduces A β_{C18C33} as a disulfide-stabilized analogue of A β_{42} that forms stable homogeneous dimers in lipid environments but does not aggregate to form insoluble fibrils. The A β_{C18C33} peptide is readily expressed in *Escherichia coli* and purified by reverse-phase HPLC to give ca. 8 mg of pure peptide per liter of bacterial culture. SDS-PAGE establishes that A β_{C18C33} forms homogeneous dimers in the membrane-like environment of SDS and that conformational stabilization of the peptide with a disulfide bond prevents the formation of heterogeneous mixtures of oligomers. Mass spectrometric (MS) studies in the presence of dodecyl maltoside (DDM) further confirm the formation of stable noncovalent dimers. Circular dichroism (CD) spectroscopy establishes that A β_{C18C33} adopts a β -sheet conformation in detergent solutions and supports a model in which the intramolecular disulfide bond induces β -hairpin folding and dimer formation in lipid environments. Thioflavin T (ThT) fluorescence assays and transmission electron microscopy (TEM) studies indicate that A β_{C18C33} does not undergo fibril formation in aqueous buffer solutions and demonstrate that the intramolecular disulfide bond prevents fibril formation. The recently published NMR structure of an A β_{42} tetramer (PDB: 6RHY) provides a working model for the A β_{C18C33} dimer, in which two β -hairpins assemble through hydrogen bonding to form a four-stranded antiparallel β -sheet. It is anticipated that A β_{C18C33} will serve as a stable, nonfibrilizing, and noncovalent A β dimer model for amyloid and Alzheimer's disease research.



INTRODUCTION

Dimers are among the most important oligomers of the β -amyloid peptide A β . Although A β fibrils are the most commonly found A β species in Alzheimer's disease-affected brains, A β oligomers are key contributors to neurodegeneration.^{1–6} Small oligomers of A β have been shown to be far more neurotoxic than large oligomers.^{7–9} A β dimers are thought to be the basic building block of many larger A β oligomers and are one of the most neurotoxic and pathologically relevant species in Alzheimer's disease.^{10–18} Homogeneous A β dimers are difficult to prepare, characterize, and study because A β forms heterogeneous mixtures of oligomers that vary in size and can rapidly aggregate into more stable fibrils.^{19–27} Plentiful sources of homogeneous and stable A β dimers would be valuable in amyloid and Alzheimer's disease research.

Although biogenic A β oligomers are arguably more biologically relevant than synthetic A β oligomers, biogenic A β oligomers are difficult to study because they only occur in minute concentrations and must be isolated from brain tissue.^{7,12–15,28} When A β oligomers are prepared by allowing

A β to aggregate in vitro, the resulting A β oligomers are heterogeneous in size, morphology, and toxicity.^{29,30} To obtain A β dimers, researchers have covalently cross-linked A β monomers through several types of intermolecular linkages, including isopeptide bonds,^{28,31,32} dityrosine bonds,^{28,31,33–35} disulfide bonds,^{16,18,34,36–38} homocysteine disulfide bonds,³⁸ glycine–serine-rich linkers,^{39,40} alkyl linkers,^{38,41–43} 4-hydroxynonenal-induced cross-linking,⁴⁴ and photoinduced cross-linking of unmodified proteins (PICUP).^{23,24,45–47} These covalent A β dimers generally aggregate further with time into a heterogeneous mixture of larger oligomers, protofibrils, or fibrils (Table S8).

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Noncovalent $A\beta$ dimers may be more biologically relevant than covalent dimers, because they mimic native $A\beta$ dimers that are formed through noncovalent interactions (Figure 1).^{5,33,48–50} Previous approaches to generate noncovalent

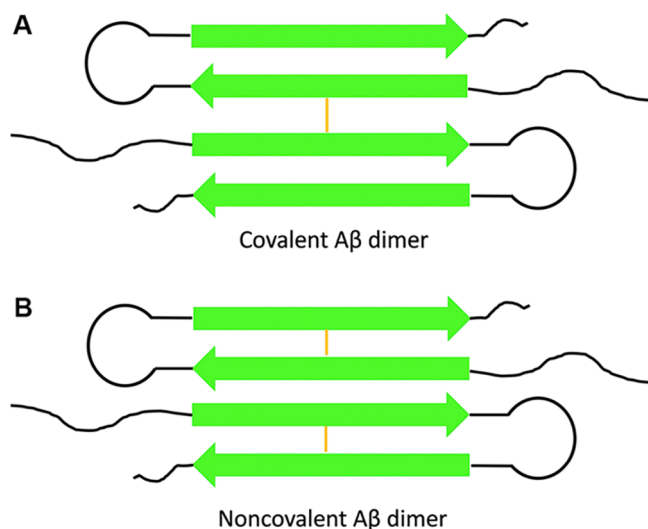


Figure 1. Cartoons of dimers of $A\beta$ β -hairpins illustrating the differences between covalent and noncovalent dimers. (A) Covalent $A\beta$ dimer generated by an intermolecular disulfide bond. (B) Noncovalent $A\beta$ dimer containing two $A\beta$ monomers stabilized by intramolecular disulfide bonds. Disulfide bonds are shown in solid orange lines.

oligomers have relied on stabilizing intramolecular linkages, including intramolecular disulfide bonds and oxime linkages, to facilitate noncovalent $A\beta$ oligomer formation.^{51–57} These intramolecular linkages are designed to induce a β -hairpin conformation, stabilizing $A\beta$ in the oligomeric state and thus delaying or preventing fibril formation. $A\beta$ β -hairpins are thought to be the building blocks of many $A\beta$ oligomers and thus are of special biological relevance.^{52,54,57–59} These modified $A\beta$ peptides bearing intramolecular linkages form various sizes of oligomers upon incubation in solution (Table S8).

$A\beta$ oligomers formed by full-length $A\beta$ peptides bearing intermolecular or intramolecular linkages have proven useful in amyloid and Alzheimer's disease research.^{60–64} These artificial oligomers have been used as chemical models to study a variety of aspects of $A\beta$ oligomers, including neurotoxicity,^{16,37,38,41,51,52,55} aggregation kinetics and pathways,^{23,24,28,31–34,36,42,44,52,56} effects on behavioral deficits and neuroplasticity,¹⁸ antibody-binding activities,^{43,57} and oligomer structures.⁵³ Even though these efforts have provided useful chemical model systems, most of the oligomers produced are heterogeneous mixtures comprising multiple oligomerization states. There are, to our knowledge, no model systems based on full-length $A\beta$ that form stable homogeneous dimers.

Our laboratory has recently developed conditions that permit the efficient expression and purification of $A\beta_{42}$ peptides and mutants thereof.^{65,66} In the current study, we set out to explore the effect of intramolecular disulfide linkages in $A\beta_{42}$ peptides by preparing and studying a variety of mutant $A\beta_{42}$ peptides containing two cysteine residues. Here, we report the discovery and preparation of $A\beta_{C18C33}$, a mutant $A\beta_{42}$ peptide containing an intramolecular disulfide bond that forms stable homogeneous dimers in lipid environments.

MATERIALS AND METHODS

The materials and procedures for molecular cloning, peptide expression, purification, and characterization as well as SDS-PAGE, mass spectrometry, CD, ThT, TEM, AUC, and DLS studies are described in detail in the Supporting Information.

RESULTS AND DISCUSSION

Design of Disulfide-Stabilized $A\beta_{42}$ Peptides. In previous studies, our laboratory has observed that β -hairpin peptides derived from $A\beta$ that contain different residue pairings from the central and C-terminal regions of $A\beta$ form different types of oligomeric assemblies.²⁰ Inspired by this observation as well as by earlier studies of $A\beta$ peptides containing disulfide linkages,^{51–53,57} we expressed and purified five mutant $A\beta_{42}$ peptides containing pairs of cysteine residues at positions 21 and 30, 18 and 33, 21 and 32, 24 and 29, and 21 and 31 (Figure 2). In each peptide, we mutated one

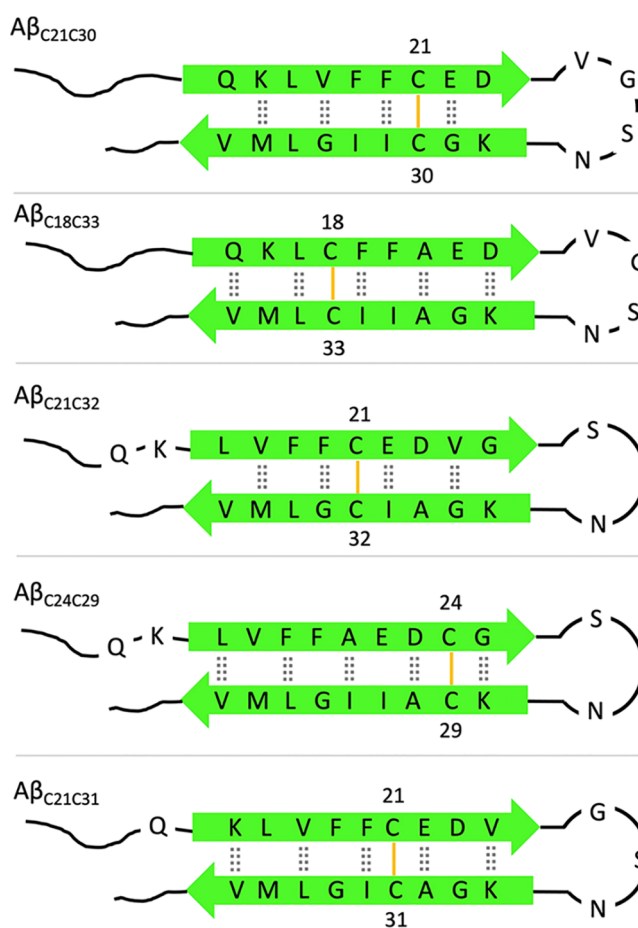


Figure 2. Cartoons of mutant $A\beta_{42}$ peptides containing intramolecular disulfide bonds that stabilize the peptides in a β -hairpin conformation. Cartoons illustrate β -hairpin structures with different disulfide linkages and residue pairings. Disulfide bonds are shown in solid orange lines. Hydrogen bonds are shown in gray dashed lines.

nonpolar residue from the hydrophobic central region of $A\beta$ and one nonpolar residue from the hydrophobic C-terminal region with the goal of inducing β -hairpin formation with different residue pairings or hydrogen-bonding patterns. For example, while peptides $A\beta_{C21C30}$ and $A\beta_{C18C33}$ share the same residue pairing, the peptides differ in hydrogen-bonding pattern. In peptide $A\beta_{C21C30}$, amino acids K₁₆, V₁₈, F₂₀, and

E₂₂ hydrogen-bond with amino acids M₃₅, G₃₃, I₃₁, and G₂₉, while in peptide A β _{C18C33}, amino acids Q₁₅, L₁₇, F₁₉, A₂₁, and D₂₃ hydrogen-bond with amino acids V₃₆, L₃₄, I₃₂, A₃₀, and K₂₈. A β _{C21C30} has previously been reported to form a mixture of dimers and trimers in SDS-PAGE and hexamers in the solid state.^{52,53} The other four peptides, A β _{C18C33}, A β _{C21C32}, A β _{C24C29}, and A β _{C21C31}, have not been previously reported.

Preparation of Disulfide-Stabilized A β Peptides. We prepared the mutant A β peptides by expression in *Escherichia coli* followed by reverse-phase HPLC purification.^{65,66} We first prepared the corresponding recombinant plasmids encoding mutant A β peptides bearing double cysteine mutations through molecular cloning (Figures S1 and S2). We then transformed the recombinant plasmids into *E. coli* and expressed the mutant A β peptides through isopropyl β -D-1-thiogalactopyranoside (IPTG) induction. After expression, we lysed the cells and solubilized the inclusion bodies with urea to obtain cell lysates containing the mutant A β peptides. We then added dimethyl sulfoxide (DMSO) to the cell lysate to oxidize the cysteines to form the intramolecular disulfide bond in each mutant A β peptide. Finally, we purified the disulfide-stabilized A β peptides by preparative reverse-phase HPLC using a C8 column at 80 °C with water and acetonitrile containing 0.1% trifluoroacetic acid (TFA). This procedure afforded ca. 8 mg of each pure mutant peptide from 1 L of bacterial culture (Table S7). Each of the peptides contains an N-terminal methionine residue that results from the start codon for the expression. For comparison, we also prepared A β _(M1–42), which is a homolog of full-length A β ₄₂ with properties similar to native A β ₄₂.^{65,67}

SDS-PAGE Studies of the Mutant A β ₄₂ Peptides: Discovery of the A β _{C18C33} Dimer. To evaluate the propensities of the disulfide-stabilized A β peptides to form oligomers, we performed sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and used silver staining to visualize the species formed by each peptide (Figure 3A). In the SDS-PAGE, A β _(M1–42) runs with the main band just above the 5 kDa ladder band at ca. 6 kDa, which corresponds to the monomer, as well as a pair of weaker bands at ca. 13 kDa and 15 kDa, which are generally attributed to trimer and tetramer.^{68,69} The previously reported A β _{C21C30} peptide gives a pair of bands with similar appearance that are slightly lower in position. These bands have previously been attributed to dimer and trimer,⁵² although the trimer and tetramer cannot be rigorously excluded. The A β _{C21C32} and A β _{C21C31} peptides give bands just above the 5 kDa ladder band at ca. 6 kDa and thus form monomers. The A β _{C24C29} peptide gives a pattern of monomer, trimer, and tetramer bands that are similar in position and appearance to those of A β _(M1–42). The A β _{C18C33} peptide stands out among the mutant peptides, giving an intense band midway between the 10 and 15 kDa ladder bands at ca. 12 kDa, which corresponds to a dimer.

To further explore the dimerization of the A β _{C18C33} peptide, we ran SDS-PAGE at concentrations ranging from 7.8 to 500 μ M (Figure 3B). At each concentration, the dimer band predominates with small bands corresponding to monomer and higher-order oligomers appearing at higher concentrations. In contrast, A β _(M1–42) shows a predominance of the monomer at all concentrations, with the trimer and tetramer bands appearing at higher concentrations.

To investigate the effect of the disulfide bond of A β _{C18C33} upon dimer formation, we reduced the disulfide bond with tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and studied the oligomerization of the reduced A β _{C18C33} peptide

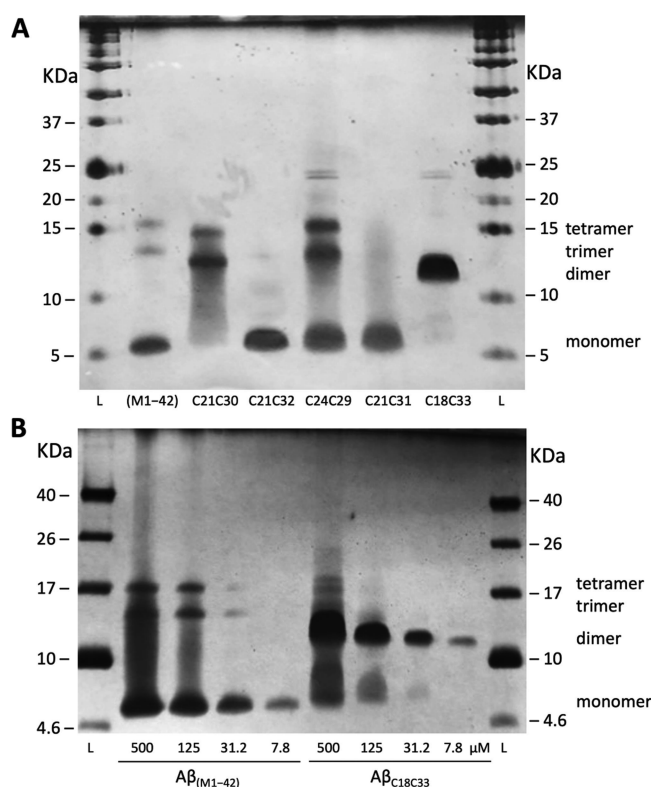


Figure 3. SDS-PAGE studies of the oligomerization propensities of the disulfide-stabilized A β peptides. (A) Oligomerization patterns of disulfide-stabilized A β peptides at 80 μ M concentration. A 10 μ L aliquot of each peptide was run on the gel. (B) Comparison of the oligomerization patterns of A β _(M1–42) and A β _{C18C33} at various concentrations. A 10 μ L aliquot of each peptide was run on the gel.

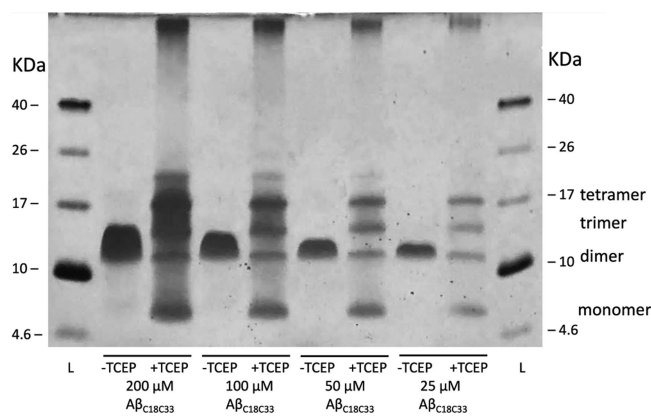


Figure 4. SDS-PAGE studies of A β _{C18C33} before and after TCEP reduction at various concentrations (200, 100, 50, and 25 μ M). A 10 μ L aliquot of each peptide was run on the gel.

by SDS-PAGE (Figure 4). The reduced A β _{C18C33} peptide behaves more like A β _(M1–42), showing distinct bands corresponding to monomer, dimer, trimer, and tetramer at lower concentrations as well as an additional band corresponding to a pentamer at higher concentrations. The reduced A β _{C18C33} peptide also shows a band at the very top of the gel, indicating that the reduced peptide also assembles to form fibrils or other high-molecular weight aggregates. These results demonstrate that the disulfide bond is essential for homogeneous dimer formation and establish that the cysteine

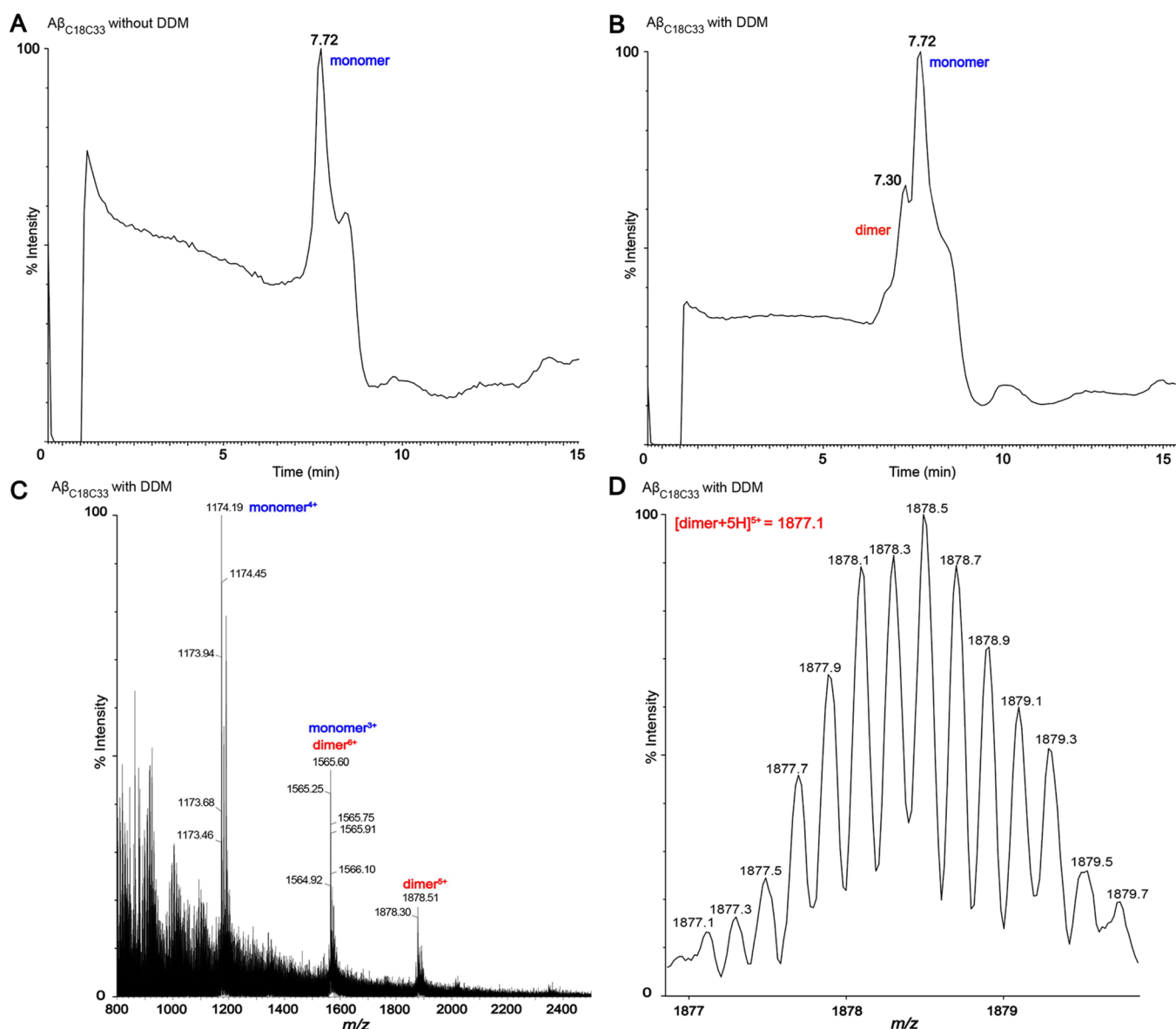


Figure 5. SEC-MS studies of $A\beta_{C18C33}$. (A) SEC chromatogram showing $A\beta_{C18C33}$ monomers. (B) SEC chromatogram showing the formation of $A\beta_{C18C33}$ dimer after incubation with DDM. (C) Mass spectrum of the SEC peaks corresponding to $A\beta_{C18C33}$ dimer and monomer. (D) Mass peak corresponding to the 5+ ion associated with $A\beta_{C18C33}$ dimer. The $A\beta$ peptide was dissolved in 10 mM ammonium acetate buffer with or without 400 μ M DDM (pH 7.3) to a final peptide concentration of 0.25 mg/mL and incubated at 37 °C for 2 h. SEC-MS was run in 1:1 ammonium formate:acetonitrile running buffer (pH 10.0).

mutations alone do not induce the formation of a pronounced dimer band in SDS-PAGE.

Mass Spectrometric Studies of $A\beta_{C18C33}$. To further corroborate the formation of noncovalent dimers and to exclude the possibility of covalent dimer formation through a pair of intermolecular disulfide bonds, we performed mass spectrometry. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry shows exclusively the $A\beta_{C18C33}$ monomer (Figure S14). This observation is significant because it establishes that the dimer observed for $A\beta_{C18C33}$ in SDS-PAGE is noncovalent rather than a catenane or a covalent dimer formed through a pair of intermolecular disulfide bonds. The $A\beta_{C18C33}$ peptide also shows monomer in the ESI mass spectrum in SEC-MS eluting with ammonium formate and acetonitrile at pH 10 (Figure 5A). Incubation of $A\beta_{C18C33}$ with the nonionic detergent dodecyl maltoside (DDM), followed by

SEC-MS analysis, gives a new peak in the total ion current chromatogram associated with the dimer (Figure 5B). Analysis of this chromatographic peak reveals [dimer +5H]⁵⁺ and [dimer +6H]⁶⁺ ions associated with the dimer (Figure 5C,D). The observation of noncovalent dimers in SEC-MS experiments with DDM provides further evidence that Aβ_{C18C33} forms well-defined dimers in the presence of detergent.

To further characterize the noncovalent $A\beta_{\text{C18C33}}$ dimers, we performed native ion mobility–mass spectrometry (IM-MS).⁶⁹ In this technique, the sample is directly injected into the mass spectrometer, and species are separated in the gas phase on the basis of mobility and mass-to-charge ratio. A solution of 25 μM $A\beta_{\text{C18C33}}$ and 300 μM DDM in 200 mM ammonium acetate buffer at pH 7.4 directly injected into the instrument gave an ion mobility chromatogram with two main peaks with arrival times at 7.8 and 11.1 ms (Figure 6). Analysis of the isotope

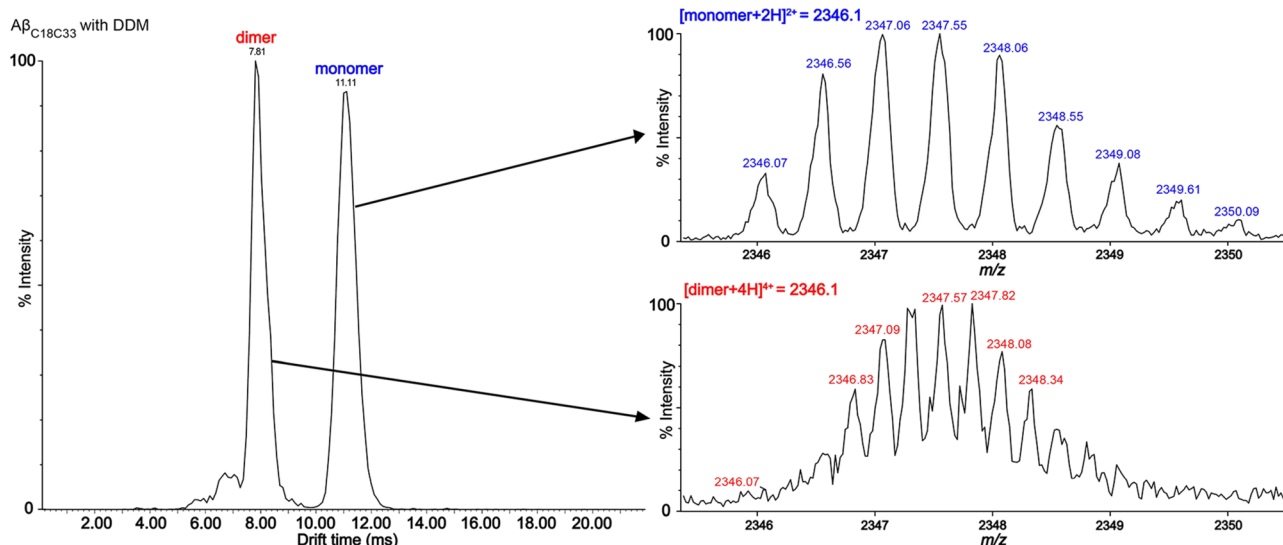


Figure 6. IM-MS studies of $A\beta_{C18C33}$ in the presence of DDM. The $A\beta$ peptide was dissolved in 200 mM ammonium acetate buffer containing 300 μ M DDM (pH 7.4) to a final concentration of 25 μ M and then directly injected on the IM-MS system at 100 μ L/min.

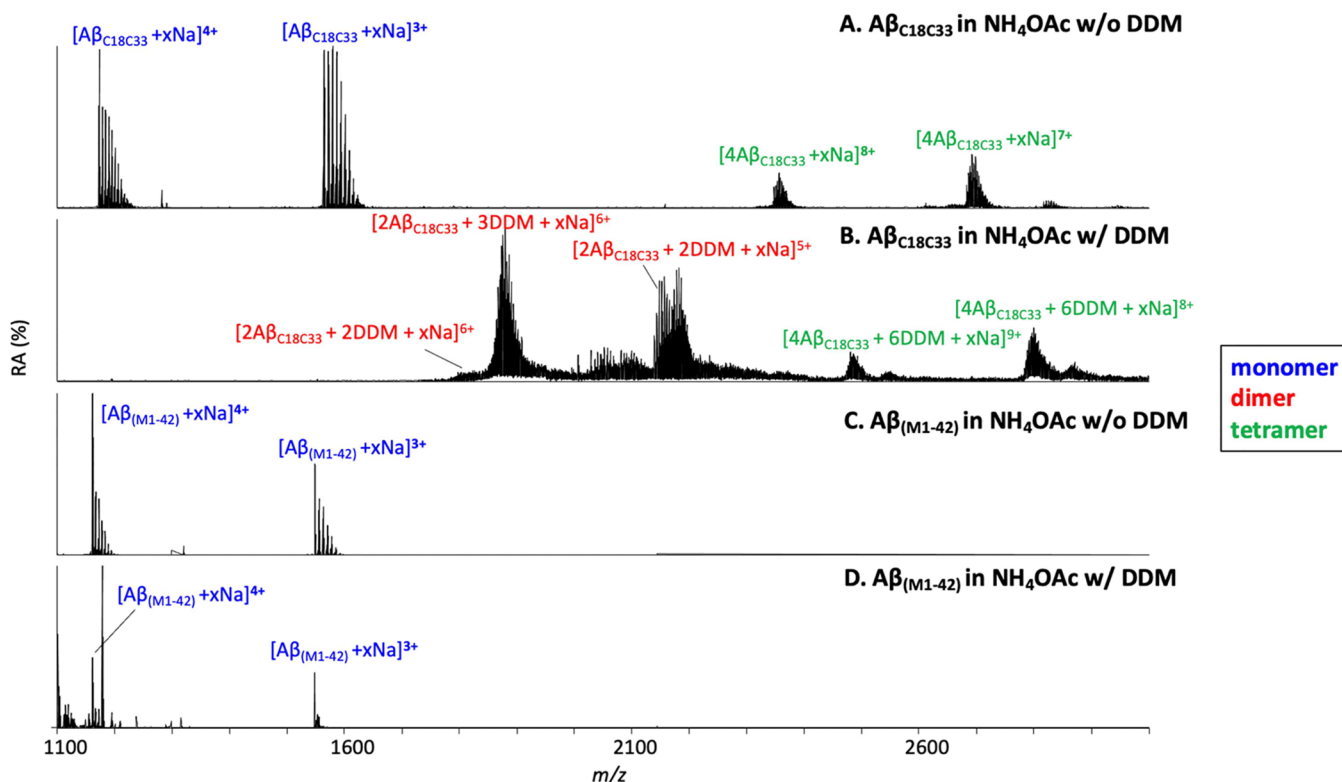


Figure 7. High-resolution native mass spectrometric analysis of $A\beta_{C18C33}$ and $A\beta_{(M1-42)}$ in the absence or presence of DDM. (A) $A\beta_{C18C33}$ (25 μ M) in 200 mM ammonium acetate solution. (B) $A\beta_{C18C33}$ (25 μ M) in 200 mM ammonium acetate solution with 300 μ M DDM. (C) $A\beta_{(M1-42)}$ (25 μ M) in 200 mM ammonium acetate solution. (D) $A\beta_{(M1-42)}$ (25 μ M) in 200 mM ammonium acetate solution with 300 μ M DDM. Spectra were acquired at a resolution setting of 100,000.

patterns and m/z ratios of the mass spectrometric peaks associated with the 7.8 ms ion mobility peak revealed a distinct signature for the $[dimer + 4H]^{4+}$, indicating that this peak corresponds to the dimer. A similar analysis of the 11.2 ms ion mobility peak revealed a distinct signature for $[monomer + 2H]^{2+}$, indicating that this peak corresponds to the monomer. The SEC-MS and IM-MS results thus agree with the SDS-PAGE results that $A\beta_{C18C33}$ forms well-defined dimers in the presence of detergent micelles.

We conducted high-resolution native mass spectrometry on an Orbitrap Exactive Ultrahigh Mass Range (UHMR) mass spectrometer—a high-sensitivity instrument with excellent mass range and high m/z transmission—to provide additional insights into the dimers formed by $A\beta_{C18C33}$ in DDM. When solutions of $A\beta_{(M1-42)}$ in 200 mM ammonium acetate were nano-electrosprayed into the instrument, only monomers were observed regardless of whether 300 μ M DDM was included (Figure 7C,D).

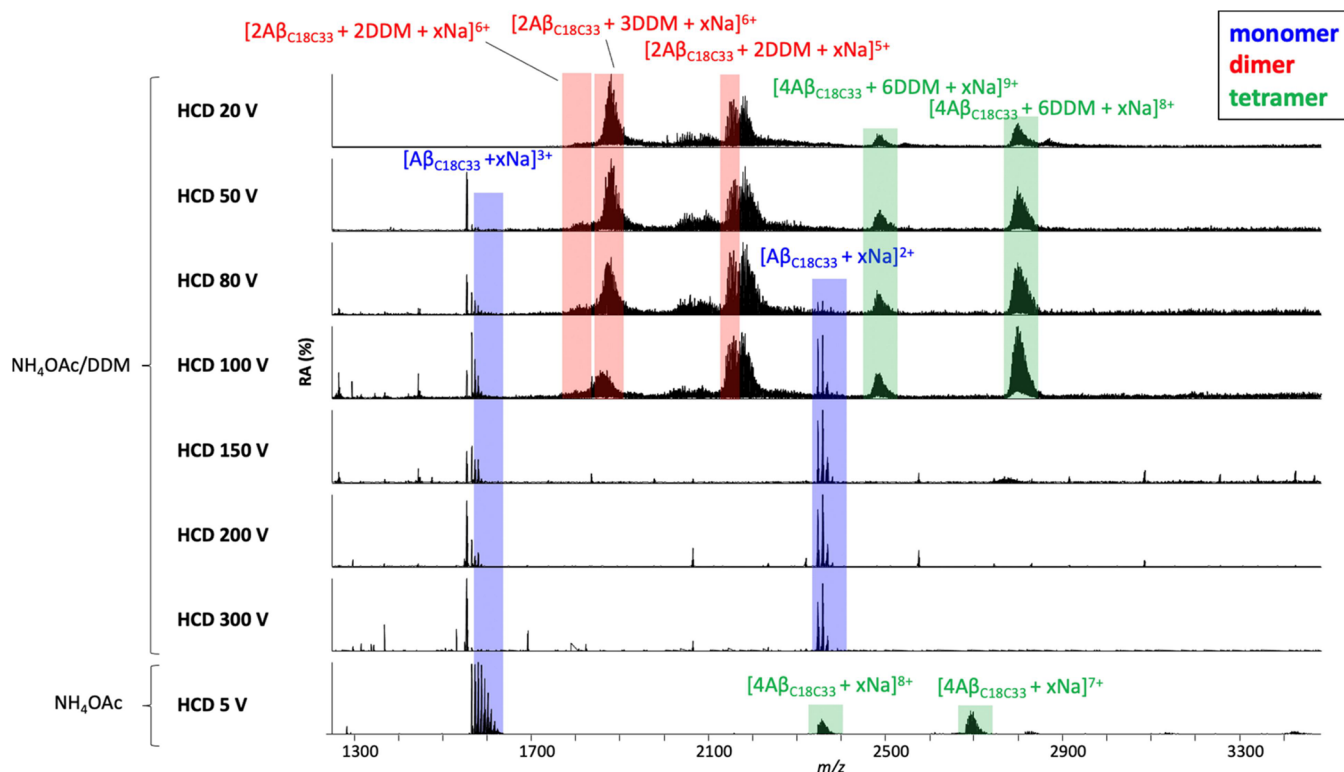


Figure 8. Native mass spectra of 25 μM $\text{A}\beta_{\text{C18C33}}$ in 200 mM ammonium acetate with 300 μM DDM as a function of HCD (CID) collision energy and comparison to the native spectrum from ammonium acetate. Spectra were acquired at a resolution setting of 100,000.

$\text{A}\beta_{\text{C18C33}}$ exhibits very different behavior than $\text{A}\beta_{(\text{M1-42})}$ in high-resolution native mass spectrometry. When a solution of $\text{A}\beta_{\text{C18C33}}$ in 200 mM ammonium acetate (without DDM) was nano-electrosprayed into the instrument, monomers and tetramers were observed, with the monomer peaks having substantially greater intensity than the tetramer peaks (Figure 7A). The peaks for the monomers and the tetramers each appear as broad ladders reflecting the presence of multiple sodium ions, which likely arise from the sodium hydroxide treatment during the preparation of the $\text{A}\beta$. The stoichiometry of the tetramers was confirmed by MS/MS, which produced monomer and trimer fragment ions (data not shown).

The inclusion of DDM in the $\text{A}\beta_{\text{C18C33}}$ sample resulted in the formation of dimers. When a solution of $\text{A}\beta_{\text{C18C33}}$ in 200 mM ammonium acetate containing 300 μM DDM was nano-electrosprayed into the instrument, dimers and tetramers were observed with little or no monomer (Figure 7B). The dimers and tetramers were observed as DDM adducts, with 2–3 DDM molecules per dimer and 6 DDM molecules per tetramer. All species were assigned based on charge states from isotopic patterns available using the 100,000 resolution setting on the UHMR.

To confirm the presence of $\text{A}\beta_{\text{C18C33}}$ in the broad ladders observed in ammonium acetate containing DDM, we collected native mass spectra with collision-induced dissociation (CID) at increasing collision voltages on an Orbitrap Exactive UHMR instrument (Figure 8). Collision-induced dissociation of the entire distribution of dimers and tetramers was accomplished in the higher-energy C-trap dissociation (HCD) cell.⁷⁰ At low HCD energy (e.g., 20–50 V), broad ladders corresponding to dimers and tetramers were observed. As the HCD energy was increased to 80 V and beyond, the dimers and tetramers decreased in relative abundance, which is concurrent with an

increase in monomer abundance. The monomers have sharper features and are thus especially easy to discern. The increase in HCD energy causes the disruption of the noncovalent interactions within the dimer and tetramer complexes and dissociation to monomers, providing evidence that these broad features do contain $\text{A}\beta_{\text{C18C33}}$ peptides. Mass spectra were recorded at high resolution so that isotope spacings could be used to confidently assign charge states and, thus, stoichiometry. The fact that the dimer and tetramer remain adducted with several molecules of DDM in the gas phase even with significant gas-phase collisional activation is noteworthy in that it suggests strong association of these oligomers with the DDM detergent. Interestingly, the DDM molecules are lost only when the noncovalent dimer and tetramer dissociate to monomers.

Circular Dichroism Studies of $\text{A}\beta_{\text{C18C33}}$. To explore the structure of the dimers formed by $\text{A}\beta_{\text{C18C33}}$, we turned to circular dichroism (CD) spectroscopy. In the absence of SDS, a 30 μM solution of $\text{A}\beta_{\text{C18C33}}$ in 10 mM sodium phosphate buffer at pH 7.4 exhibits a broad negative band below 230 nm with a shallow minimum at 203 nm (Figure 9A). With 20 mM SDS, the CD spectrum displays a well-defined minimum at 212 nm and maximum at 191 nm (Figure 9A). These data indicate that the $\text{A}\beta_{\text{C18C33}}$ peptide adopts a β -sheet structure in the presence of SDS but that random coil structure predominates in the absence of SDS. The spectrum in the presence of SDS differs dramatically from that of $\text{A}\beta_{(\text{M1-42})}$, which exhibits minima at 207 and 222 nm and a maximum at 190 nm in SDS and thus adopts an α -helical structure (Figure 9B). These results highlight the effect of the disulfide bond upon the conformation of $\text{A}\beta_{\text{C18C33}}$, preventing α helix formation and enforcing β -sheet formation under conditions most relevant to SDS-PAGE.

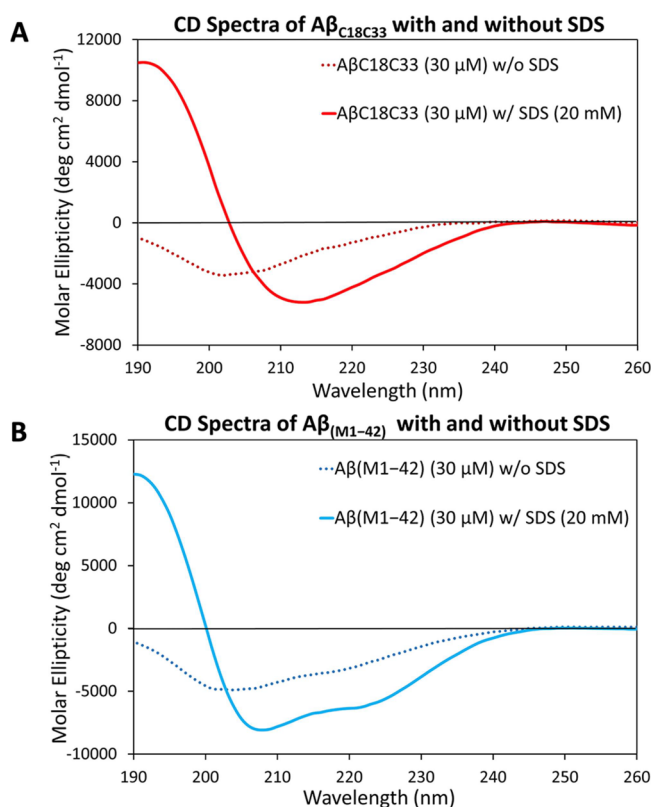


Figure 9. CD spectra of (A) $A\beta_{C18C33}$ and (B) $A\beta_{(M1-42)}$ in the absence or presence of SDS. CD experiments were performed in 10 mM sodium phosphate buffer at pH 7.4.

To gain further insight into the structure of the $A\beta_{C18C33}$ dimers under conditions relevant to the mass spectrometric experiments described above, we collected CD spectra of $A\beta_{C18C33}$ and $A\beta_{(M1-42)}$ with various concentrations of DDM. The CD spectra of $A\beta_{C18C33}$ with DDM at concentrations well above the critical micelle concentration (CMC, 0.17 mM) resemble the CD spectrum of $A\beta_{C18C33}$ with 20 mM SDS (CMC 8 mM), exhibiting a well-defined minimum at 214 nm and a maximum at ca. 190 nm (Figure 10A). The CD spectrum of $A\beta_{(M1-42)}$ with DDM displays a broader minimum centered at 215 nm and a weaker maximum at ca. 190 nm (Figure 10B). Analysis of the $A\beta_{C18C33}$ CD spectra using the secondary structure analysis server BeStSel^{71,72} indicates the formation of ca. 50% antiparallel β -sheet structure as the concentration of DDM is raised above the CMC (Figure 10C). In contrast, the $A\beta_{(M1-42)}$ spectra show only ca. 20% antiparallel β -sheet structure under these conditions (Figure 10C). These results further support a model in which the disulfide bond stabilizes a β -hairpin conformation in $A\beta_{C18C33}$ in the membrane-like environments provided by detergents.^{52,73}

ThT, TEM, AUC, and DLS Studies of $A\beta_{C18C33}$. To determine the effect of the disulfide bond upon fibril formation, we performed thioflavin T (ThT) fluorescence assays. Solutions of $A\beta_{C18C33}$ and $A\beta_{(M1-42)}$ were incubated in PBS buffer at pH 7.4 in the presence of ThT at 37 °C. Under these conditions, $A\beta_{(M1-42)}$ showed a characteristic lag time followed by a rapid onset of fluorescence at ca. 30 min (Figure 11B). In contrast, $A\beta_{C18C33}$ showed no appreciable increase in fluorescence over 24 h (Figure 11A). To rule out the possibility that the $A\beta_{C18C33}$ peptide resists fibrilization because

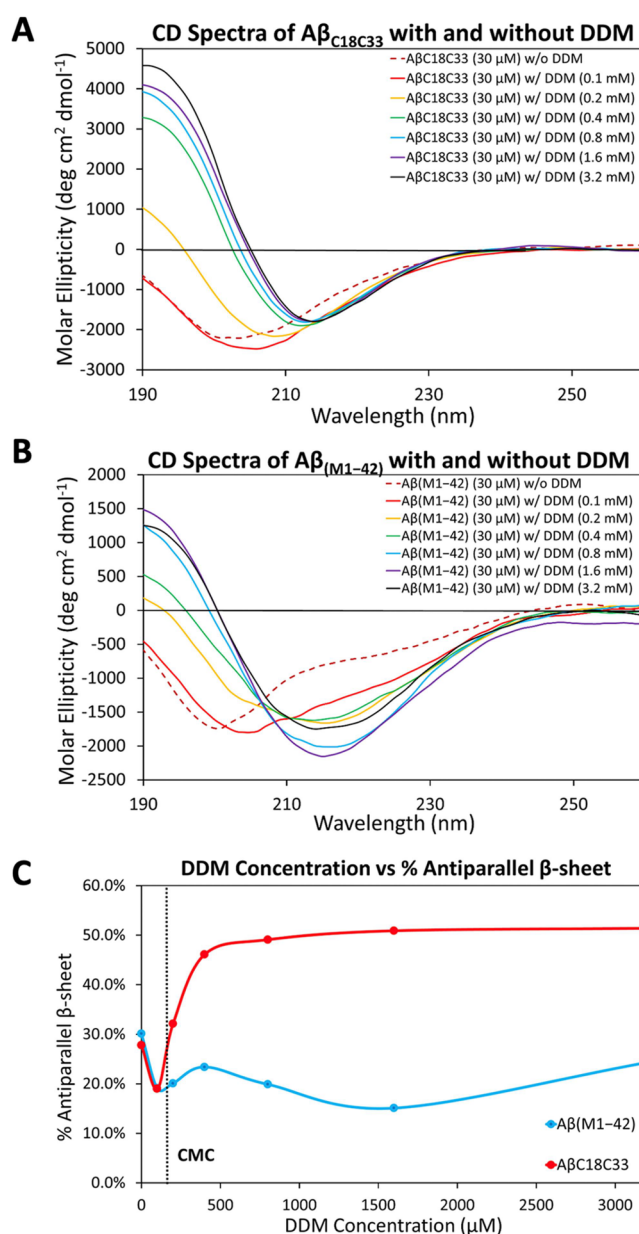


Figure 10. CD spectra of $A\beta_{C18C33}$ and $A\beta_{(M1-42)}$ with DDM. (A) CD spectra of $A\beta_{C18C33}$ with various concentrations of DDM. (B) CD spectra of $A\beta_{(M1-42)}$ with various concentrations of DDM. CD experiments were performed in 10 mM sodium phosphate buffer at pH 7.4. (C) Percentage of antiparallel β -sheet structure calculated for the $A\beta_{C18C33}$ and $A\beta_{(M1-42)}$ peptides with various concentrations of DDM. The CD spectra were analyzed using the secondary structure analysis server BeStSel.^{71,72} The critical micelle concentration (CMC) of DDM (ca. 0.17 mM) is marked with a dashed line.

of the cysteine mutations, rather than the intramolecular disulfide bond, we performed a ThT assay using an $A\beta_{C18C33}$ analogue containing two alanine mutations, $A\beta_{A18A33}$. Under comparable conditions, the $A\beta_{A18A33}$ peptide showed a lag time of ca. 4.5 h followed by a rapid onset of fluorescence (Figure 11C). These observations demonstrate that the disulfide bond prevents fibril formation. The longer lag time associated with the $A\beta_{A18A33}$ mutant likely reflects the effect of the valine-to-alanine mutation at position 18 of the critical¹⁷ LVFFA²¹ amyloidogenic region of $A\beta$.

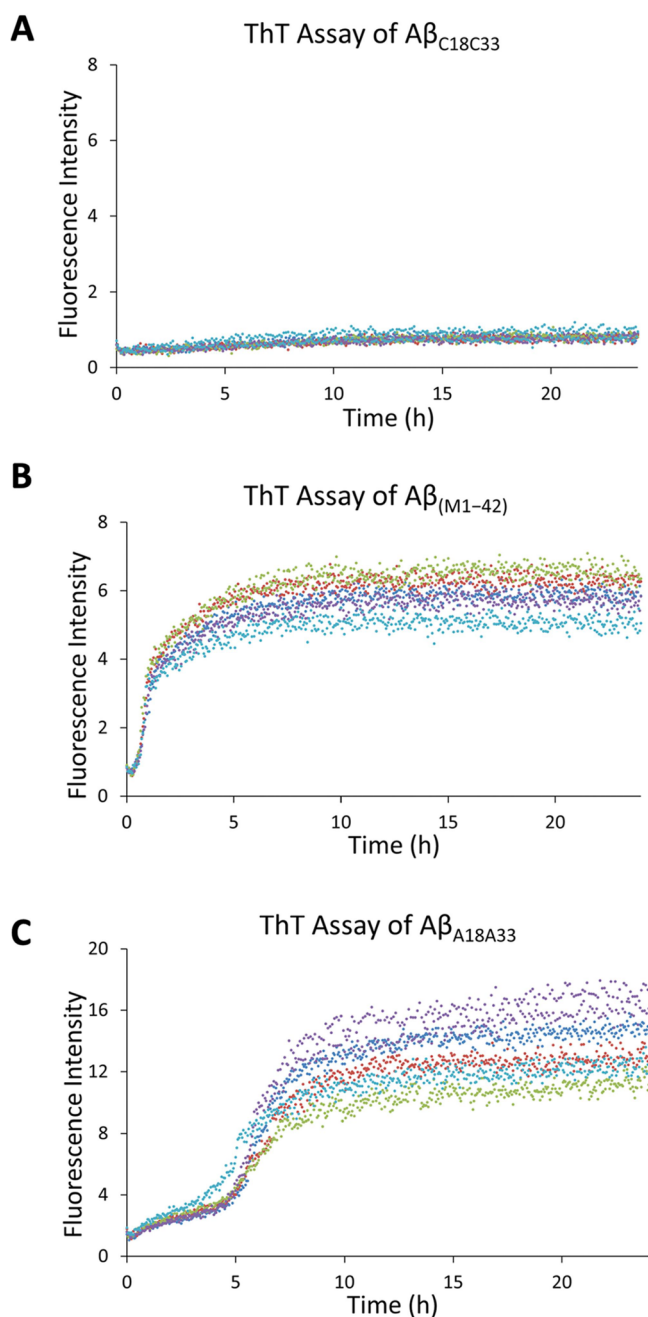


Figure 11. ThT fluorescence assays of (A) $A\beta_{C18C33}$, (B) $A\beta_{(M1-42)}$, and (C) $A\beta_{A18A33}$. Assays were performed with 40 μ M $A\beta$ and 40 μ M ThT in pH 7.4 PBS buffer at 37 °C without shaking (quiescent conditions). Fluorescence was monitored with 440 nm excitation and 485 nm emission. The ThT assays were performed in five technical replicates (purple, dark blue, red, light blue, and green).

We performed transmission electron microscopy (TEM) to corroborate that $A\beta_{C18C33}$ does not fibrilize. Incubation of $A\beta_{C18C33}$ at 37 °C for 24 h in PBS buffer, followed by TEM imaging, showed no fibrils (Figure 12A). In contrast, $A\beta_{(M1-42)}$ showed long fibrils typical of $A\beta$ (Figure 12B). $A\beta_{A18A33}$ also showed fibrils, albeit shorter than the characteristic $A\beta_{(M1-42)}$ fibrils (Figure 12C). Upon treatment with TCEP to reduce the disulfide bond and incubation in PBS buffer, $A\beta_{C18C33}$ showed fibrils (Figure 12D). Collectively, these results confirm that the intramolecular disulfide bond is critical to arresting the $A\beta_{C18C33}$ peptide in the oligomeric state.

To further assess the aggregation of the $A\beta_{C18C33}$ peptide in aqueous solution without detergent, we performed analytical ultracentrifugation (AUC) and dynamic light scattering (DLS) experiments. Sedimentation velocity AUC experiments in 10 mM sodium phosphate buffer with 25 mM NaCl at pH 7.4 on samples of $A\beta_{C18C33}$ at 5–160 μ M showed a range of oligomeric species up to 70–80 kDa as well as monomer, with larger oligomeric species becoming more prevalent at higher concentrations (Figure S4). Larger aggregates that settled rapidly were also observed to pellet in these 60,000 rpm experiments and constituted 52–76% of the sample. In DLS experiments, a freshly prepared solution of $A\beta_{C18C33}$ (30 μ M in 10 mM sodium phosphate buffer at pH 7.4) showed a peak centered at a hydrodynamic diameter of 24.4 nm (Figure 13). Upon incubation for 24 h at 37 °C, the peak shifted to 32.7 nm. These results show that $A\beta_{C18C33}$ forms large stable oligomeric assemblies in aqueous solution in the absence of detergent and are consistent with the pelleting observed in the AUC experiments. Monomer and dimer are likely in equilibrium with these assemblies but are too small to be observed by DLS. In contrast, a freshly prepared solution of $A\beta_{(M1-42)}$ showed a peak centered at 58.8 nm, which shifted to 955 nm upon incubation for 24 h (Figure 13). These observations are consistent with the fibril formation observed in the ThT and TEM experiments.

Structure of the $A\beta_{C18C33}$ Dimer. Thus far, we have not been able to determine the structure of the $A\beta_{C18C33}$ dimer. The ^1H NMR spectrum of $A\beta_{C18C33}$ is broad in aqueous solution, and efforts to obtain a ^1H – ^{15}N HSQC spectrum of ^{15}N -labeled $A\beta_{C18C33}$ in the presence of detergents using simple solution-phase NMR techniques have not been successful. Nevertheless, we believe that the recently published NMR structure of an $A\beta_{42}$ tetramer by Carulla and coworkers⁵⁸ (Figure 14A; PDB: 6RHY) holds clues into the potential structure of the $A\beta_{C18C33}$ dimer. The tetramer consists of two $A\beta_{42}$ β -hairpins that flank two C-terminal $A\beta_{42}$ β -strands. In each β -hairpin, valine 18 and glycine 33 are proximal and form a non-hydrogen-bonded pair.

In our working model for the $A\beta_{C18C33}$ dimer, the cysteine 18–cysteine 33 disulfide linkage stabilizes this β -hairpin conformation. Two $A\beta_{C18C33}$ β -hairpins hydrogen-bond together without the intervening C-terminal $A\beta_{42}$ β -strands that make up the Carulla tetramer. Figure 14B illustrates this working model for the structure of the $A\beta_{C18C33}$ dimer. An attractive feature of this model is that the C terminus of each monomer subunit is proximal to the side chain of Lys₂₈ in the other monomer subunit, allowing for a salt bridge. We think that this model for the dimer of full-length $A\beta$ is reasonable because our laboratory has previously observed similar hydrogen-bonded dimers of other constrained β -hairpin peptides^{74–77} and because we have found that the lipophilic environment provided by SDS micelles supports hydrogen-bonding interactions.^{78–80}

CONCLUSIONS

$A\beta_{C18C33}$, a double mutant of the expressed peptide $A\beta_{(M1-42)}$ containing an intramolecular disulfide bond, forms stable noncovalent dimers in the presence of anionic and nonionic detergents. The dimers exhibit a high degree of β -sheet character and can readily be observed by SDS-PAGE and mass spectrometry. In aqueous buffer without detergent, the $A\beta_{C18C33}$ peptide does not aggregate to form fibrils but instead forms solutions of large oligomers as well as smaller oligomers

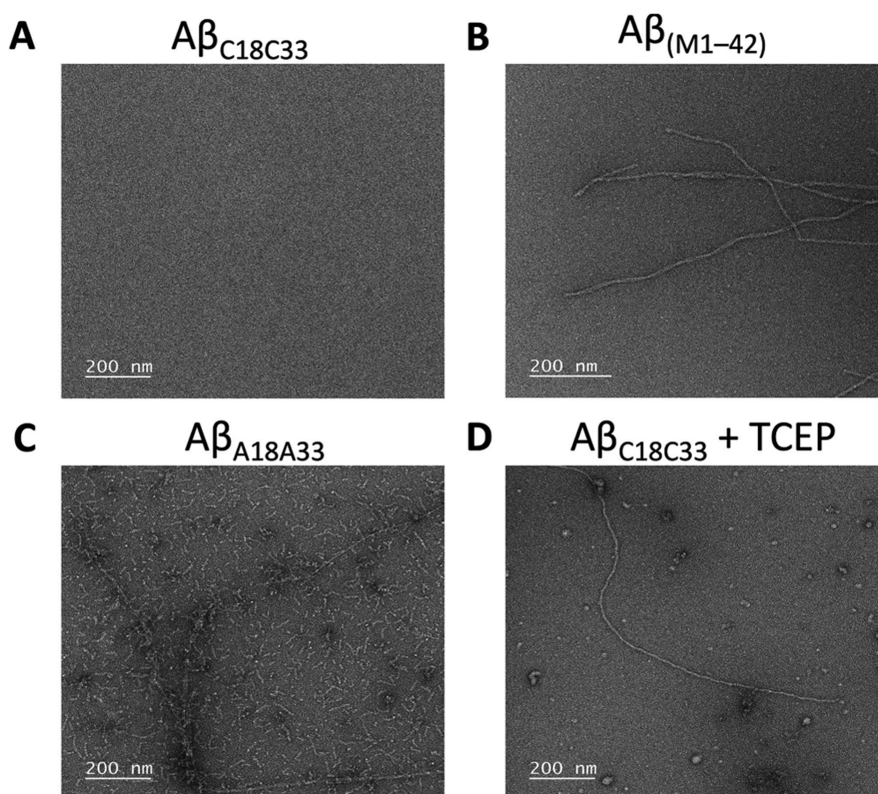


Figure 12. Transmission electron micrographs of A β peptides: (A) A β_{C18C33} , (B) A $\beta_{(M1-42)}$, (C) A β_{A18A33} , and (D) A β_{C18C33} with TCEP reduction. Solutions of the A β peptides were incubated in pH 7.4 PBS buffer for 24 h at 37 °C and then imaged by TEM with uranyl acetate staining.

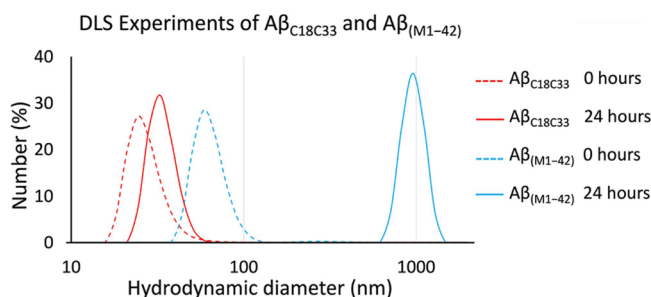


Figure 13. Dynamic light scattering experiments of A β_{C18C33} and A $\beta_{(M1-42)}$. DLS experiments were performed on 30 μ M solutions of the A β peptides in 10 mM sodium phosphate buffer at pH 7.4. DLS data were recorded for freshly prepared solutions, and then afterward, the solutions were incubated for 24 h at 37 °C.

and monomer. The membrane-like environment provided by the detergents thus facilitates the formation of dimers. The A β_{C18C33} peptide is readily prepared by expression in *E. coli* and can be purified by preparative reverse-phase HPLC. We have deposited the plasmid to express A β_{C18C33} at Addgene to make it available to other researchers.⁸¹

A β oligomers continue to be studied intensively through a variety of experimental and computational methods.^{82–90} We anticipate that A β_{C18C33} will be a valuable tool in biological and biophysical experiments by providing ready access to stable, homogeneous, and noncovalent A β dimers. Oligomers of native A β peptides (A β_{1-40} , A β_{1-42} , A $\beta_{(M1-42)}$, etc.) are heterogeneous and prone to further aggregation to form fibrils. Oligomeric preparations of these peptides can thus be problematic to use in biological and biophysical studies. The A β_{C18C33} peptide provides an attractive alternative to these

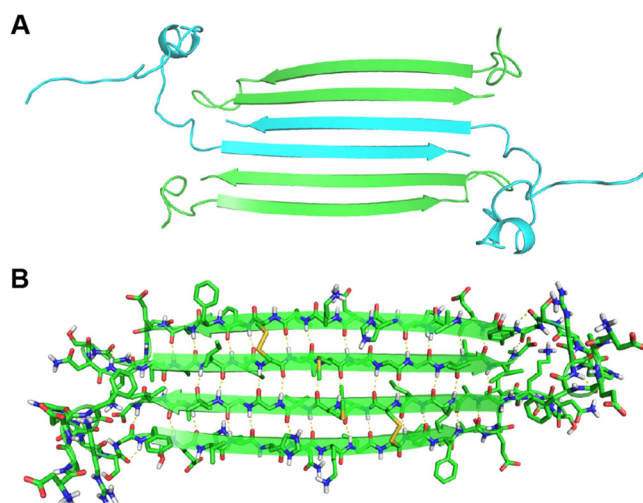


Figure 14. Structural model of the A β_{C18C33} dimer. (A) The recently published NMR structure of an A β_{42} tetramer by Carulla and coworkers⁵⁸ (PDB: 6RHY). (B) Working model for the structure of the A β_{C18C33} dimer.

preparations because of the stability and homogeneity of the dimers that it forms in membrane-like environments.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.biochem.1c00739>.

Detailed procedures for molecular cloning, peptide expression, purification, and characterization, as well as

SDS-PAGE, mass spectrometry, CD, ThT, TEM, AUC, and DLS studies; supplementary figures, tables, and characterization data for the expressed peptides (PDF)

AUTHOR INFORMATION

Corresponding Authors

Adam G. Kreutzer – Department of Chemistry, University of California Irvine, Irvine, California 92697-2025, United States; orcid.org/0000-0002-9724-6298;
Email: akreutze@uci.edu

James S. Nowick – Department of Chemistry and Department of Pharmaceutical Sciences, University of California Irvine, Irvine, California 92697-2025, United States; orcid.org/0000-0002-2273-1029; Email: jsnowick@uci.edu

Authors

Sheng Zhang – Department of Chemistry, University of California Irvine, Irvine, California 92697-2025, United States; orcid.org/0000-0002-2105-0627

Stan Yoo – Department of Chemistry, University of California Irvine, Irvine, California 92697-2025, United States; orcid.org/0000-0002-8982-7631

Dalton T. Snyder – Resource for Native Mass Spectrometry Guided Structural Biology, The Ohio State University, Columbus, Ohio 43210, United States; orcid.org/0000-0003-2154-5002

Benjamin B. Katz – Department of Chemistry, University of California Irvine, Irvine, California 92697-2025, United States

Amy Henrickson – Department of Chemistry and Biochemistry, University of Lethbridge, Lethbridge, Alberta T1K 3M4, Canada; orcid.org/0000-0003-3266-5202

Borries Demeler – Department of Chemistry and Biochemistry, University of Lethbridge, Lethbridge, Alberta T1K 3M4, Canada; orcid.org/0000-0002-2414-9518

Vicki H. Wysocki – Resource for Native Mass Spectrometry Guided Structural Biology, The Ohio State University, Columbus, Ohio 43210, United States; orcid.org/0000-0003-0495-2538

Complete contact information is available at:
<https://pubs.acs.org/10.1021/acs.biochem.1c00739>

Notes

The authors declare no competing financial interest.

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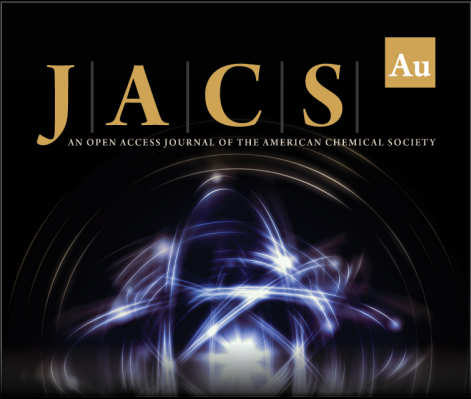
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