Supporting Information

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SI Text

Chemicals and Reagents. Chemicals were obtained from Sigma–Aldrich and were of the highest purity available. Water was double-distilled and deionized by using a Milli-Q system (Millipore). Tris(2,2′-bipyridyl)dichlororuthenium(II) hexahydrate (Ru(Bpy)), ammonium persulfate (APS), SDS gels, and Simply Blue stain were purchased from Invitrogen.

Peptide Preparation. Aβ(1-40) (Aβ) was synthesized, purified, and characterized as described (1). Briefly, Aβ(1-40) synthesis was performed on an automated peptide synthesizer (model 433A; Applied Biosystems) using 9-fluorenylmethoxycarbonyl-based methods on preloaded Wang resins. Peptides were purified by RP-HPLC. Quantitative amino acid analysis and mass spectrometry yielded the expected compositions and molecular mass, respectively, for each peptide.

Aβ Aggregation. Uncross-linked Aβ solutions were prepared in PBS, pH 7.4, to produce concentrations of 25 μM. After sonication for 1 min with a bath sonicator, the peptide solution was centrifuged for 10 min at 16,000 × *g*. The resulting supernatant was used as an uncross-linked peptide control in subsequent aggregation assays. Unfractionated, cross-linked peptides, purified oligomers, and fibrils were dissolved in PBS, pH 7.4, at 25 μM concentration. Fibril seeds were produced by sonicating preformed fibrils for 45 min in a bath sonicator at 23 °C. Ten percent (vol/vol) of each assembly then was added to separate tubes of uncross-linked Aβ. The tubes were incubated at 37 °C for 0–7 days without agitation.

Preparation of Fibrils. Fifty micrometers of Aβ was dissolved in 10% (vol/vol) 60 mM NaOH and 90% (vol/vol) 10 mM phosphate buffer, pH 7.5. After sonication for 1 min with a bath sonicator, the peptide solution was centrifuged for 10 min at 16,000 × *g*. The resulting supernatant was incubated at 37 °C without agitation for 10 days. Fibril preparations were stored as lyophilizates and then were reconstituted at a concentration of 500 μM in PBS or cell culture medium.

PICUP. Cross-linking of Aβ was performed essentially as described (2). Lyophilized Aβ was dissolved at a nominal concentration of 90 μM in 10% (vol/vol) 60 mM NaOH and 90% (vol/vol) 10 mM phosphate buffer, pH 7.65. After sonication for 1 min in a bath sonicator (model 1510R-DTH; Branson Ultrasonics), the peptide solution was centrifuged for 10 min at 16,000 × *g*. The supernatant then was used immediately. Reagent concentrations were increased incrementally for every 30-μM increase in peptide concentration such that a 90-μM peptide solution would be cross-linked with 60 mM Ru(Bpy) and 3 mM APS. The mixture was irradiated for 1 s with visible light and then the reaction was quenched with Tricine sample buffer (Invitrogen) containing 5% (vol/vol) 2-mercaptoethanol. Samples were fractionated by using 1.5-mm-thick, 10–20% Tris-Tricine gradient gels. After SDS/PAGE, the bands were visualized by silver staining (SilverXpress; Invitrogen) or prepared for extraction (see next section).

Extraction of Aβ from SDS Gels. Gels were washed in water three times for 5 min each time and then they were incubated in Simply Blue stain (Invitrogen) for 1 h on a ZD-9556 orbital shaker (Madell Technology). The gel was destained in water for 1 h and then the stained bands were excised. Gel pieces were washed with 1 mL of double-distilled water three times before being subjected to three cycles of freezing and thawing in dry ice/ethanol and a warm water bath to make them more brittle. Pieces then were crushed in a microcentrifuge tube with a minipestle (Fisher Scientific) and incubated in 1 mL of 0.1% (vol/vol) ammonium hydroxide while rotating at 24 rpm for 30 min at room temperature on a Mini LabRoller Rotator (Labnet International). The tubes were briefly centrifuged (16,000 × *g*) and the supernates were collected. The gel slurry was then subjected to a second round of incubation and centrifugation. To remove SDS, gel extracts were treated with SDSOut (Pierce) and then were dialyzed against water for 48 h by using Spectra/Por Biotech CE Dialysis Membranes (2,000 MWCO [molecular weight cutoff], 7.5 mm diameter). The dialyzates then were was lyophilized. The purity of the lyophilizates was determined by SDS/PAGE and silver staining.

Determination of Oligomer Frequency Distributions. Samples were analyzed by SDS/PAGE and silver staining, as described (2). To produce intensity profiles and calculate the relative amounts of each oligomer type, densitometry was performed and One-Dscan software (version 2.2.2; BD Biosciences Bioimaging) was used to determine peak areas of baseline-corrected data.

ThT Fluorescence. ThT and EM studies were performed essentially as described (3). Ten microliters of sample was added to 190 μL of ThT dissolved in 10 mM PBS, pH 7.4, and then the mixture was vortexed briefly. Fluorescence was determined three times at intervals of 10 s with a Hitachi F-4500 fluorometer. Excitation and emission wavelengths were 450 nm (slit width = 5 nm) and 482 nm (slit width = 10 nm), respectively. Sample fluorescence was determined by averaging the three readings and subtracting the fluorescence of a ThT blank.

CD Spectroscopy. Samples were placed in a 1-mm path-length cuvette (Hellma) and spectra were acquired in a J-810 spectropolarimeter (JASCO). The CD cuvettes were maintained on ice before introduction into the spectrometer. After temperature equilibration, spectra were recorded at 22 °C from ≈190–260 nm at 0.2-nm resolution with a scan rate of 100 nm/min. Ten scans were acquired and averaged for each sample. Raw data were manipulated by smoothing and subtraction of buffer spectra, according to the manufacturer's instructions. To determine the fractional content of each secondary structure element, experimental data were fitted with a linear combination of reference spectra of α-helix, β-sheet, and SC structures (4).

EM. A 10-μL aliquot of each sample was spotted onto a glow-discharged, carbon-coated Formvar grid (Electron Microscopy Sciences) and incubated for 20 min. The droplet then was displaced with an equal volume of 2.5% (vol/vol) glutaraldehyde in water and incubated for an additional 5 min. Finally, the peptide was stained with 8 μL of 1% (vol/vol) filtered (0.2 μm) uranyl acetate in water (Electron Microscopy Sciences). This solution was wicked off and then the grid was air-dried. Samples were examined with a JEOL CX100 transmission electron microscope.

AFM. Peptide solutions were characterized with a Nanoscope V Dimension 5000 scanning probe microscope (Veeco Digital Instruments). All measurements were carried out in "tapping mode" under ambient conditions with single-beam silicon can-

tilever probes. A 10-μL aliquot of each sample was spotted onto freshly cleaved mica (Ted Pella, Inc.), incubated at room temperature for 5 min, rinsed with water, and then blown dry with air. At least four regions of the mica surface were examined to ensure that an accurate sample of the structures on the mica was obtained.

Toxicity Assays. Rat pheochromocytoma PC12 cells were cultured in 75-cm^2 flasks (430641, Corning) in F-12K medium (ATCC) containing 15% (vol/vol) horse serum, 2.5% (vol/vol) FBS, 100 units/mL penicillin, 0.1 mg/mL of streptomycin, and 25 μg/mL amphotericin B at 37 °C in an atmosphere of 5% (vol/vol) CO_2 in air. To prepare cells for assay, the medium was removed and the cells were washed once gently with F-12K medium, containing 0.5% (vol/vol) FBS, 100 units/mL penicillin, 0.1 mg/mL streptomycin, and 25 μg/mL amphotericin B. A cell suspension then was prepared by addition of this latter medium, but containing 100 μg/mL of nerve growth factor (Invitrogen), followed by agitation of the flask. Cell concentration was determined by trypan blue staining, after which cells were plated at a density of 30,000 cells per well (90 μL of total volume per well) in 96-well assay plates (Costar 3610; Corning). The nerve growth factor-induced differentiation of the cells was allowed to proceed for 48 h. To perform toxicity assays, Aβ solutions of concentration 2.5–500 μM were prepared in F-12K medium containing 0.5% (vol/vol) FBS, 100 units/mL penicillin, 0.1 mg/mL streptomycin, and 25 μg/mL amphotericin B. Ten-microliter aliquots of Aβ solution were added to the wells to yield final Aβ concentrations of 0.25, 0.5, 1, 5, 10, 25, and 50 μM. The cells then were incubated for 24 h. To determine the effects of Aβ on MTT metabolism, 15 μL of MTT solution (Promega) was added to each well and the plate was incubated in the CO_2 incubator for an additional 3.5 h. The cells then were lysed by the addition of 100 μL of solubilization solution (Promega) followed by overnight incubation. MTT reduction was assessed by measuring absorption at 570 nm (corrected for background absorbance at 630 nm) with a BioTek Synergy HT microplate reader. For gel-isolated samples (unfractioned, cross-linked peptides or purified oligomers), the negative control sample was produced identically to the oligomer samples, except that a piece of polyacrylamide gel containing no protein was excised and then subjected to the same extraction and preparation procedures. For uncross-linked and fibrillar Aβ, the negative control was medium. The positive toxicity control was 1 μM staurosporine. Six replicates were done for each treatment group, and the data from 3 independent experiments were combined and reported as mean ± SE. Percentage toxicity $T = ((A_{A\beta} - A_{\text{medium}})/(A_{\text{staurosporine}}) - A_{\text{medium}})) \times 100$, where $A_{A\beta}$, A_{medium}, and $A_{\text{staurosporine}}$ were absorbance values from Aβ-containing samples, negative control (blank gel isolate or medium alone), or staurosporine alone, respectively. Effective concentration (EC_{50}) was defined as the concentration of each Aβ assembly that produced an assay value of 50%. EC_{50} were calculated by the sigmoidal curve fitting of the data as shown in Fig. S5, using GraphPad Prism software (version 4.0a).

LDH activity was determined by the Promega CytoTox-ONE Homogeneous Membrane Integrity assay. To do so, peptide samples and negative control samples were prepared as described above and then were incubated with the cells for 48 h. The positive toxicity control was lysis solution provided by the manufacturer. One-hundred microliters of LDH reagent then was added to each well and the plate was incubated in the dark for 10 min, after which 50 μL of stop solution was added and the fluorescence was measured by using the BioTek Synergy HT microplate reader with excitation wavelength of 560 nm and emission wavelength of 590 nm. Six replicates were done for each treatment group and the data from three independent experiments were combined and reported as mean ± SE. Percentage toxicity was calculated according to the formula above, except the term $A_{\text{staurosporine}}$ was replaced with A_{lysis}.

Statistical Analysis. One-way factorial ANOVA followed by Bonferroni post hoc comparisons were used to determine statistical significance among datasets. These tests were implemented within GraphPad Prism software (version 4.0a). Significance was defined as $P < 0.05$.

1. Walsh DM, Lomakin A, Benedek GB, Condron MM, Teplow DB (1997) Amyloid β-protein fibrillogenesis: Detection of a protofibrillar intermediate. *J Biol Chem* 272:22364–22372.
2. Bitan G, Lomakin A, Teplow DB (2001) Amyloid β-protein oligomerization: Prenucleation interactions revealed by photo-induced cross-linking of unmodified proteins. *J Biol Chem* 276:35176–35184.
3. Ono K, et al. (2008) Effects of grape seed-derived polyphenols on amyloid β-protein self-assembly and cytotoxicity. *J Biol Chem* 283:32176–32187.
4. Chang CT, Wu CS, Yang JT (1978) Circular dichroic analysis of protein conformation: Inclusion of the β-turns. *Anal Biochem* 91:13–31.

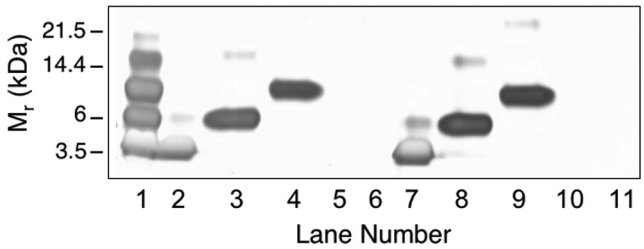

Fig. S1. SDS/PAGE of Aβ assemblies before and after incubation with F12K medium. Aβ was subjected to PICUP and then the resulting products were analyzed by SDS/PAGE on a 10–20% gradient gel. After staining with Simply Blue, visualized bands were excised and the protein was extracted under alkaline conditions. The extracts then were dissolved at a concentration of 25 μM in F12K medium, pH 7.4. After incubation at 37 °C for 2 days, the samples were reanalyzed by SDS/PAGE and silver staining. Lane 1, cross-linked Aβ before incubation; lane 2, monomer before incubation; lane 3, dimer before incubation; lane 4, trimer before incubation; lane 5, a gel piece equivalent in size to the protein-containing pieces, but without protein; lane 6, F12K medium; lane 7, monomer after incubation; lane 8, dimer after incubation; lane 9, trimer after incubation; lane 10, gel piece after incubation; lane 11, F12K medium after incubation. The gel is representative of each of three independent experiments. No bands were observed at $M_r > 25$ kDa.

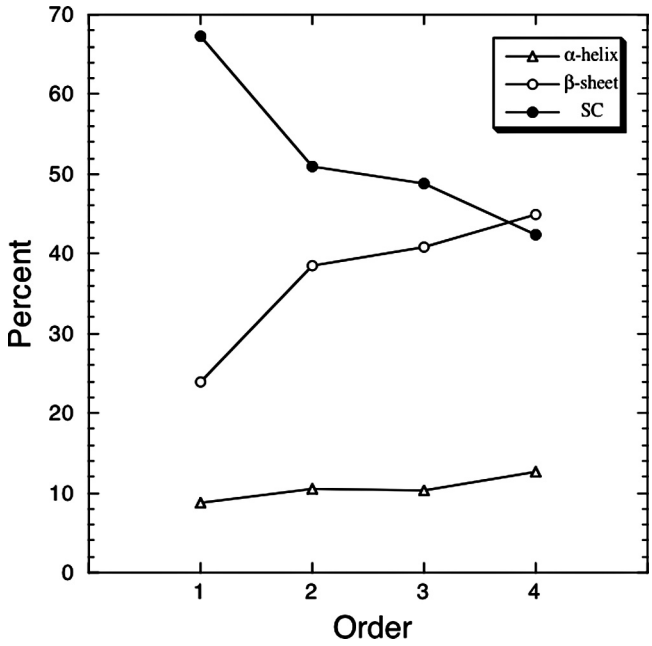

Fig. S2. Secondary structure of pure oligomers. Gel-purified Aβ monomers, dimers, trimers, and tetramers were studied by CD. The relative amounts (percentage) of α-helix, β-sheet, and SC are plotted as a function of oligomer order.

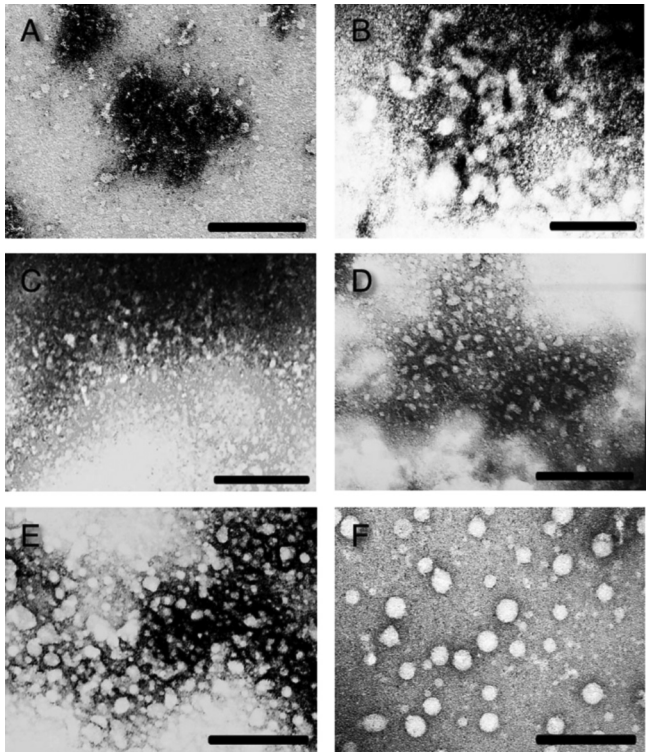

Fig. S3. Comparative analysis of Aβ oligomer morphology by EM. Uncross-linked (*A*), cross-linked (unfractionated) (*B*), monomeric (*C*), dimeric (*D*), trimeric (*E*), or tetrameric (*F*) Aβ were examined by EM. (Scale bars: 100 nm.)

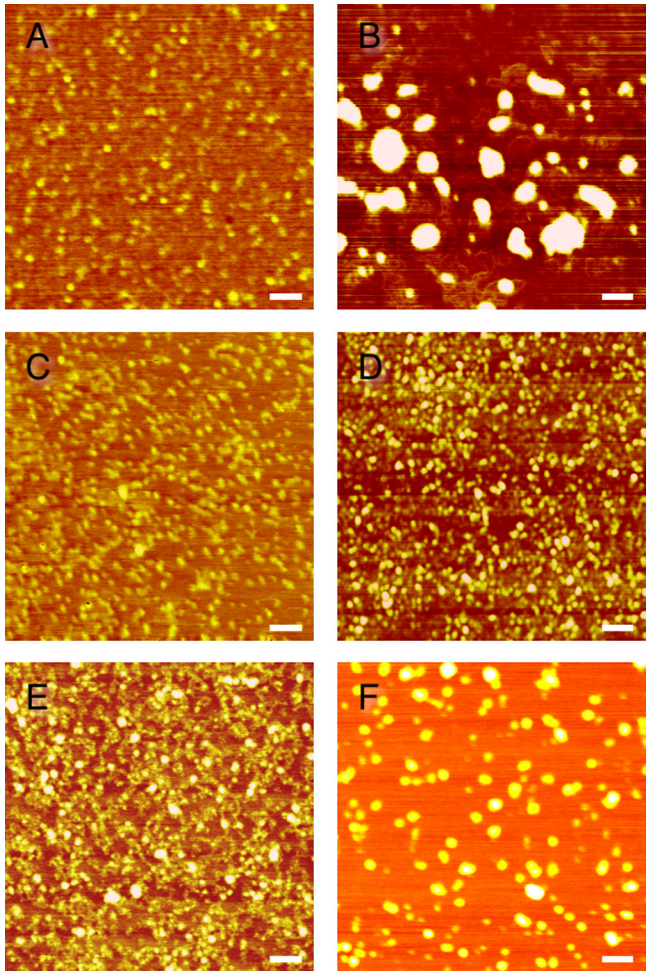

Fig. S4. Comparative analysis of Aβ oligomers by AFM. Uncross-linked (*A*), cross-linked (unfractionated) (*B*), monomeric (*C*), dimeric (*D*), trimeric (*E*), or tetrameric (*F*) Aβ were examined by AFM. (Scale bars: 100 nm.)

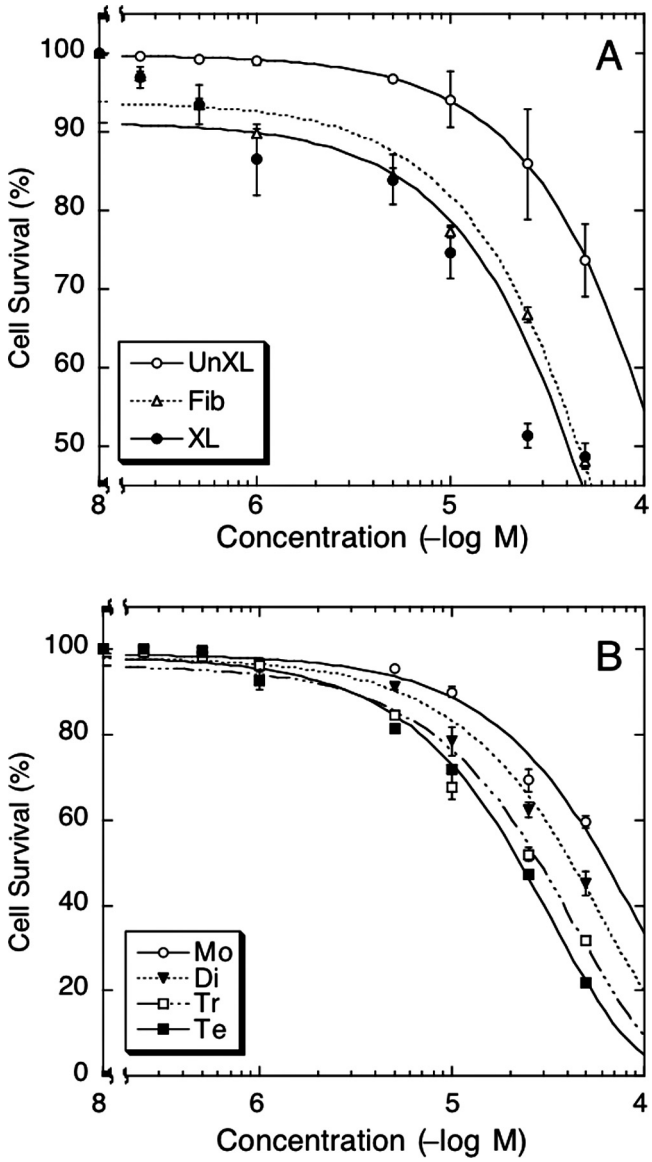

Fig. S5. MTT metabolism. Uncross-linked Aβ (○, UnXL), Aβ fibrils (△, Fib), or cross-linked Aβ (●, XL) (*A*) or Aβ monomer (○, Mo); dimer (▼, Di); trimer (□, Tr), or tetramer (■, Te) (*B*) were added at final nominal concentrations of 0.25–50 μM to differentiated PC12 cells. MTT assays were done after 24 h. Data are expressed as percentage toxicity. The data are representative of those obtained in each of three independent experiments.

Table S1. Nucleation activity of oligomers and preformed fibrils

Oligomer Order	$t_{1/2}$ (h)	Relative activity
1	65	1
2	10	13
3	2	98
4	2	130
Fibrils	1	$>$ 100

Nucleation activity was calculated by direct inspection of ThT progress curves and determination of the time, in h, at which the ThT level was half maximal ($t_{1/2}$) or as the activity of the oligomer relative to monomer, where relative activity = $t_{1/2}^{\text{monomer}}/(t_{1/2}^{\text{oligomer } i}/i)$ with oligomer order = i. Fibril activity cannot be determined precisely because of the existence of complex frequency distributions of quaternary and lower-order structure.

Table S2. Toxicity of pure Aβ oligomers

Oligomer order	EC_{50} (μM)[a]	Relative activity
Uncross-linked	102.5 ± 5.6	0.7
1	67.3 ± 8.7	1
2	41.6 ± 3.9	3
3	24.5 ± 1.9	8
4	20.5 ± 0.4	13
Cross-linked, unfractionated	43.0 ± 2.7	3*
Fibrils	57.6 ± 2.2	ND

Cytotoxicity was determined as effective concentration at which 50% of maximal toxicity was observed (EC_{50}) in MTT assays or as the activity of the oligomer relative to monomer, where relative activity = $EC_{50}^{monomer}/(EC_{50}^{oligomer\ i}/i)$ with oligomer order = i. Fibril activity cannot be determined precisely because of the existence of complex frequency distributions of quaternary and lower-order structure. ND, not determined.

*Oligomer order i in this sample was calculated as the number average for monomer through tetramer, according to $\bar{i} = \Sigma_{i=1}^{4} f_i \cdot i$.