Supporting Information

Photoactive chlorin e6 is a multifunctional modulator of amyloid-β aggregation and toxicity via specific interactions with its histidine residues

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Materials and Methods

All chemicals and reagents were of analytical grade. Ce6 and $A\beta_{40}$ were purchased from Frontier Scientific (Logan, Utah) and GL Biochem (Shanghai, China) respectively and used as received. Ce6 was analyzed with inductively coupled plasma atomic emission spectroscopy (ICP-AES) to confirm the absence of any metal contamination. The purity of Aβ (>95%) was confirmed by HPLC and high-resolution mass spectroscopy. To ensure the monomeric state of the peptide, it was dissolved in high-grade 1,1,1,3,3,3-hexafluoroisopropanol (HFIP). The solvent was then removed by a stream of nitrogen, and the sample was dried under high vacuum for 1 h. The obtained film was dissolved in 10 mM NaOH and its concentration was determined by UV absorption at 280 nm using the extinction coefficient of tyrosine. The monomerized Aβ was stored as lyophilized powder at -80 ºC until use. All photoirradiations were carried out with a 100 W halogen lamp (Philips, Hamburg, Germany) with an appropriate cutoff filter ($\lambda > 350$ nm). Light intensity was measured before every experiment using a power meter. The fluent rate for all of the irradiations was calculated to be 6.5 mW cm^{-2}.

Thioflavin T (ThT) aggregation assay. The antiamyloidogenic activity of Ce6 was determined in black 96-well flat-bottom plates as described previously.[1] Briefly, monomerized solutions of $A\beta_{40}$ (66 μM, 15 μL) were incubated with increasing concentrations of Ce6 (5 μL) in 25% dimethyl sulfoxide (DMSO) in PBS (50 mM, pH 7.4). The volume of each well was then completed to 50 μL using PBS, giving a final Aβ

concentration of 20 μM. The plates were sealed with clear polyolefin foils and were either immediately irradiated for 1 h or kept in dark. Plates were then incubated at 37 ºC with constant shaking (750 rpm) for 72 h to induce aggregation. Following incubation, a solution of ThT (24 μM, 150 μL) in glycine buffer (50 mM, pH 8.5) was added to each well and the fluorescence of amyloid-bound ThT was immediately measured using a plate reader (Infinite M200, Tecan, Switzerland) at excitation and emission wavelengths of 430 and 492 nm, respectively. The ThT solution was freshly prepared by diluting a ThT stock solution (1 mM, in water) with the glycine buffer.

For kinetic ThT assays examining the effect of Ce6 and Cu^{2+} on aggregation, Aβ (5 μM) was incubated in the absence or presence of Cu^{2+} (1.5 μM) and Ce6 (0.125 μM) in a HEPES solution (50 mM, 160 mM NaCl, pH 7.4) containing ThT (18 μM). HEPES was used due to its low affinity for Cu ions.[2] The plates were sealed with clear polyolefin foil and incubated at 37 °C without shaking, while the fluorescence of amyloid-bound ThT was monitored for 72 h using a plate reader (Infinite M200, Tecan, Switzerland) as described.

Transmission electron microscopy (TEM) analysis. Samples were prepared for TEM studies by spotting aliquots (5 μL) of the aggregation assay onto glow-discharged, carbon-coated Formvar/copper grids (SPI supplies, West Chester, PA). The samples were then blotted with a filter paper and allowed to dry. The dried samples were negatively stained with 2% uranyl acetate in water (5 μL) for 30 s, washed with water, blotted with a filter paper, and dried again. Samples were then analyzed by a Tecnai G2 TEM (FEI Tecnai™ G2, Hillsboro, Oregon) operated at 120 kV.

Photo-induced cross-linking of unmodified proteins (PICUP). PICUP experiments were performed as previously described.[3] Samples were prepared for PICUP in a similar manner to the aggregation assay. The concentrations of Aβ and Ce6 in all samples were 50 μM, and each sample contained 5% DMSO. After each time interval, samples (18 μL) were mixed with ammonium persulfate (APS; 1 μL, 20 mM) and [tris (2,2'-bipyridyl)-ruthenium(II)] (1 μL, 1 mM) both in PBS (10 mM). The mixture was then photoirradiated for 7 s in a 1.5 mL microcentrifuge test tube, using a 150 W lamp positioned 15 cm from the bottom of the reaction tube. In order to evaluate the cross-linking property of Ce6 and the effect of potential quenchers (NaN_3, His, Tyr), freshly prepared mixtures of Aβ (50 μM) and Ce6 (50 μM) in the presence or absence of the quenchers were irradiated for 1 min. Cross-linking of Aβ by Ce6 was also

studied under anaerobic conditions. The solution of Aβ (50 μM) and Ce6 (100 μM) in PBS (10 mM) in a total volume of 50 μL (containing 5% DMSO) was degassed, using the freeze-pump-thaw method. The mixture was then illuminated for 1 min under vacuum in a 1.5 mL microcentrifuge test tube. Cross-linking reactions were quenched immediately with sample buffer (Invitrogen, 8 μL for each 20 μL of sample) containing 5% β-mercaptoethanol. Samples were then analyzed using a 12.5% tricine SDS-poly acrylamide gel and visualized by the silver stain method.

Circular dichroism (CD) spectroscopy. CD measurements were carried out using a Chirascan spectrometer (Applied Photophysics, UK). Samples were prepared in PB (in absence of DMSO) in a manner similar to the ThT aggregation assay, photoirradiated for 1 h, and analyzed daily while incubated in dark. Measurements were performed at room temperature in a 2 mm optical path length cell without dilution, and the spectra were recorded from 260 to 190 nm with a step size and a bandwidth of 1 nm. The spectra are the average of three measurements after background subtraction.

Dot blot assay. Samples (4 μL) from different aggregation assays either subjected to 1 h photoirradiation, or kept in the dark, were spotted onto nitrocellulose membrane (0.2 μm) and dried at room temperature. The membranes were then blocked for 1 h with 5% nonfat milk in tris buffered saline (TBS, 10 mM) containing 0.01% Tween 20 (TBST), washed three times (5 min) with TBST, and incubated at 4 °C overnight with polyclonal antibody A11 (Millipore, at 1:1000) or 6E10 antibody (Covance, at 1:1000) in 0.5% nonfat milk in TBST. The membranes were washed with TBST and incubated for 1 h with horseradish peroxidase (HRP) conjugated antirabbit IgG at 1:5000 (A11) or with HRP-conjugated antimouse IgG at 1:10000 (6E10) in 0.5% nonfat milk in TBST at room temperature. The blots were then washed five times with TBST and developed using the ECL reagent kit (Pierce).

Cell culture experiments. Neuronal-like pheochromocytoma cells (PC-12) derived from rat adrenal medulla were maintained in low-glucose Dulbecco's modified eagle medium (DMEM) supplemented with horse serum (10%) and fetal bovine serum (FBS; 5%), L-glutamine, penicillin, and streptomycin (full medium) in a 5% CO_2 atmosphere at 37 ºC. Cell survival experiments were carried out as described previously.[4] In brief, cells (10,000 per well) were grown in 96-well tissue culture plates in 0.1 mL of full medium and incubated at 37 ºC overnight for attachment. Aβ40 (100 μM) was incubated in the absence or presence of Ce6 (100 and 1000 μM) in 2.5% DMSO in PBS in a total volume of 50 μL and immediately

photoirradiated for 1 h or kept in the dark. Samples were then aged for two days at 37 °C without shaking. On the day of the experiment, the medium was replaced with fresh medium (90 μL), and the aged samples (10 μL) were then diluted by a factor of 10 in the new medium. Control wells received PBS (10 μL) containing 0.25% DMSO (100% cell viability). Cells were incubated for an additional 48 h, and cell viability was then determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Each experiment was performed in quadruplicate or quintuplicate and repeated three times.

The effect of Ce6 on Cu^{2+}-induced Aβ toxicity was determined as described previously with some modification.[2] Briefly, Aβ (400 μM, 16.5 μL) was aged at 37º C in the dark for 48 h with $CuCl_2$ (40 μM, 16.5 μL in DDW) in the presence or absence of Ce6 (4–200 μM, 16.5 μL in 10% DMSO in DDW). The total volume was adjusted to 495 μL using HEPES buffer (50 mM, NaCl 160 mM, pH 7.4). For the toxicity experiments, PC12 cells were seeded into wells of 96-well plates at 10,000 cell/well, using full DMEM medium. In the next day, the medium was replaced with OPTI-MEM (50 μL) containing 12% of FBS and the aged Aβ samples (150 μL) were then diluted in the wells to afford a final concentration of Aβ (10 μM), Cu^{2+} (1 μM), and Ce6 (0.1–5 μM). After four days of incubation, cells were collected by light trypsinization (50 μL), centrifuged and suspended in 100 μL of fresh complete DMEM medium. Cell viability was then determined by MTT assay as described.

EPR Spectroscopy. X-band EPR spectra were obtained at room temperature on a Bruker ELEXSYSE500cw X-band EPR spectrometer (Bruker, Karlsruhe, Germany) equipped with a standard rectangular Bruker EPR cavity (ER 4119 HS). Spin-trap EPR measurements experiments were performed as described previously.[5] In brief, a solution of Ce6 (180 μM, 22 μL) in NaOH/D_2O (20 mM) and 2,2,6,6-tetramethylpiperidine (TEMP, 400 μM, 10 μL) in D_2O was incubated without or with $Aβ_{40}$ (250 μM, 16 μL) in D_2O. The total volume was adjusted to 100 μL using PBS/D_2O to afford final concentrations of Aβ (40 μM), Ce6 (40 μM) and TEMP (40 μM) in PBS/D_2O (1:9). The mixtures were then photoirradiated for 5 min or kept in the dark, and introduced into gas-permeable Teflon capillary tubes (Zeus Industries, Raritan, NJ) folded twice into a narrow quartz tube that was kept open at both ends. The tubes were then placed in the EPR cavity, and the spectra were recorded.

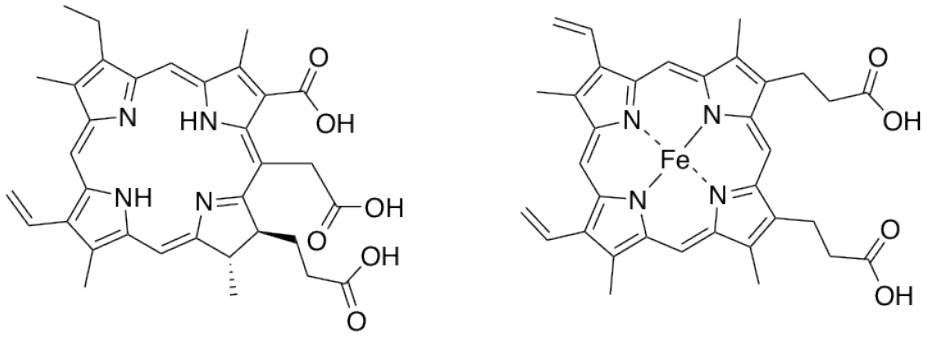
NMR Experiments. NMR experiments were carried out on a Bruker 500 MHz instrument equipped with a cryogenically cooled probehead. Unlabeled, ^{15}N-labeled, and ^{13}C-labeled

$A\beta_{40}$ peptide was purchased from AlexoTech AB (Sweden) and prepared in D_2O:H_2O (1:9; v:v) according to a previously published protocol[3a] to obtain monomeric Aβ (75 μM, 0.6 mL) samples in PB buffer (20 mM, pH 5.0 or 7.3). NMR spectra were recorded at +5 °C as 2D ^{1}H,^{15}N-HSQC or ^{1}H,^{13}C-HSQC spectra, where the peaks were identified using our previously published assignment.[6]

To study the effect of Ce6 on Aβ aggregation under dark or light conditions, Ce6 (1 mg) was added to each Aβ sample and tested with NMR after one hour of incubation either in darkness or under light irradiation.

The effect of Ce6 on Cu^{2+}-induced Aβ aggregation was probed by first measuring the NMR spectrum of Aβ (80 μM) in the presence of Cu^{2+} (40 μM) in HEPES buffer (40 mM, pH 7.3), followed by addition of Ce6 (0.8 mM) under dark conditions and subjecting the mixture to NMR measurement.

Statistical analysis. Results are given as mean ± SD. Statistical significance ($p < 0.05$) among experimental groups was calculated using the two-tailed Student's t test.



Scheme S1. Chemical structures of Ce6 and closely related heme, which is shown to highlight the difference between chlorin and porphyrin structures.

Table S1. Effect of photoexcited Ce6 on the amino acid content of Aβ.[a]

Amino acid	Aβ	Aβ + Ce6
Tyr	1	1
His	3	**0**
Phe	3	3

[a] *A mixture of Aβ (20 μM) and Ce6 (20 μM) in PBS was irradiated for 1 h or kept in the dark. The solutions were then diluted 20-fold with H_2O, lyophilized, and analyzed for their amino acid content. Aβ was used as the control.*

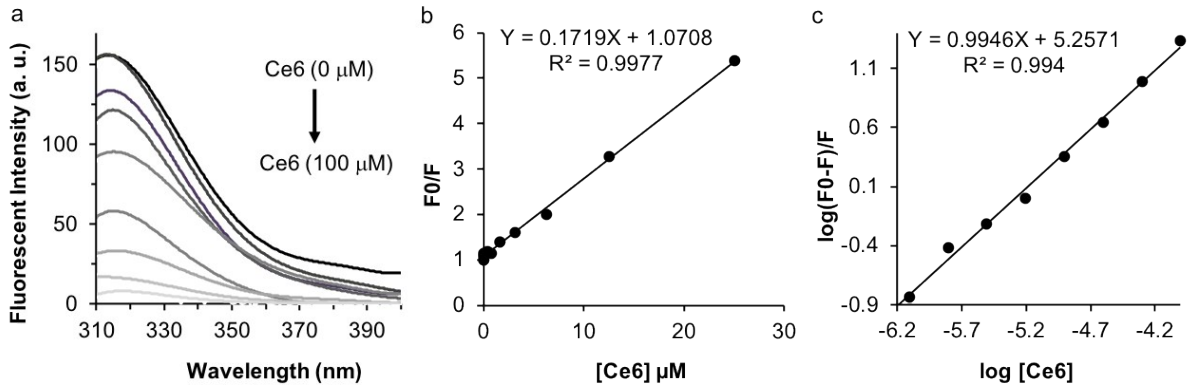
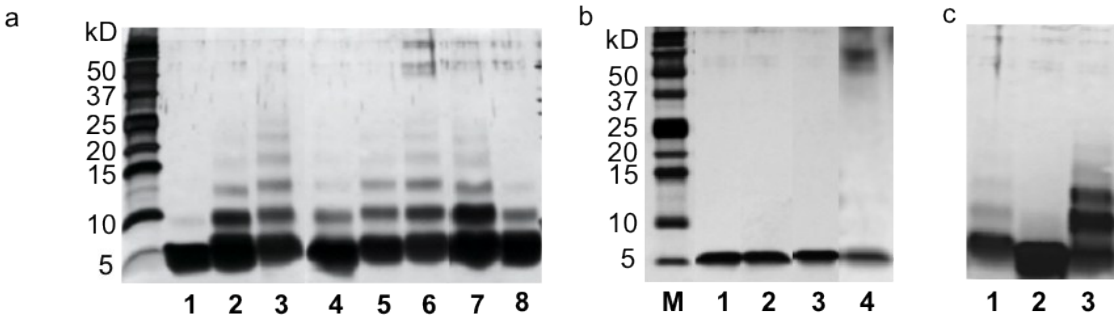


Figure S1. Fluorescence studies to determine the binding affinity of Ce6 to Aβ and the number of the binding sites. a) Intrinsic fluorescence of Aβ in the presence of increasing concentrations of Ce6. Aβ (50 μM) was titrated against different concentrations of Ce6 (1–100 μM) in PBS (20 mM, pH 7.4), and the emission spectra were measured from 310–400 nm with excitation of 280 nm. b) Stern–Volmer plot demonstrating linear quenching curve and indicating single class of interaction. c) Double logarithm plot of log(F0-F)/F vs. log[Ce6] to calculate the binding affinity between Ce6 and Aβ. Aβ (50 μM) was titrated against increasing concentration of Ce6 in PBS (20 mM, pH 7.4) and the binding affinity was calculated according to:

$$\log(F_0\text{-}F)/F = \log K + n\log[\text{Ce6}]$$

where F_0 and F are the fluorescence emission intensities of Aβ's Tyr at 316 nm ($\lambda_{ex.}$ = 280 nm) in the absence and presence of Ce6. *K* is the binding association constant and *n* the number of the binding sites.



Aβ (50 μM)	+	+	+	+	+	+	+	+
Ru^{2+}/APS	-	+	-	-	-	-	+	+
Ce6 (100 μM)	-	-	+	+	+	+	-	-
His (2.5 mM)	-	-	-	+	-	-	-	-
NaN_3 (2.5 mM)	-	-	-	-	+	-	+	-
Tyr (0.25 mM)	-	-	-	-	-	+	-	+

Figure S2. Mechanism of Aβ cross-linking by photoexcited Ce6. a) SDS-PAGE image of Aβ (50 μM) irradiated with Ce6 (100 μM) in the absence (lane 3) or presence of His (2.5 mM; lane 4), NaN_3 (2.5 mM; lane 5) and Tyr (0.25 mM; lane 6). b) SDS-PAGE image of Aβ (50 μM, lane 1) incubated with excess of H_2O_2 (1 mM, lane 2), H_2O_2 (1 mM) and $FeSO_4$ (10 μM, lane 3) and H_2O_2 (1 mM) following irradiation (lane 4). c) SDS-PAGE image of Aβ (50 μM) irradiated with Ce6 (100 μM) in the presence (lane 1) or absence of oxygen (lane 2). Lane 3 represents control SDS-PAGE image of Aβ (50 μM) after PICUP cross-linking using Ru^{2+} complex.

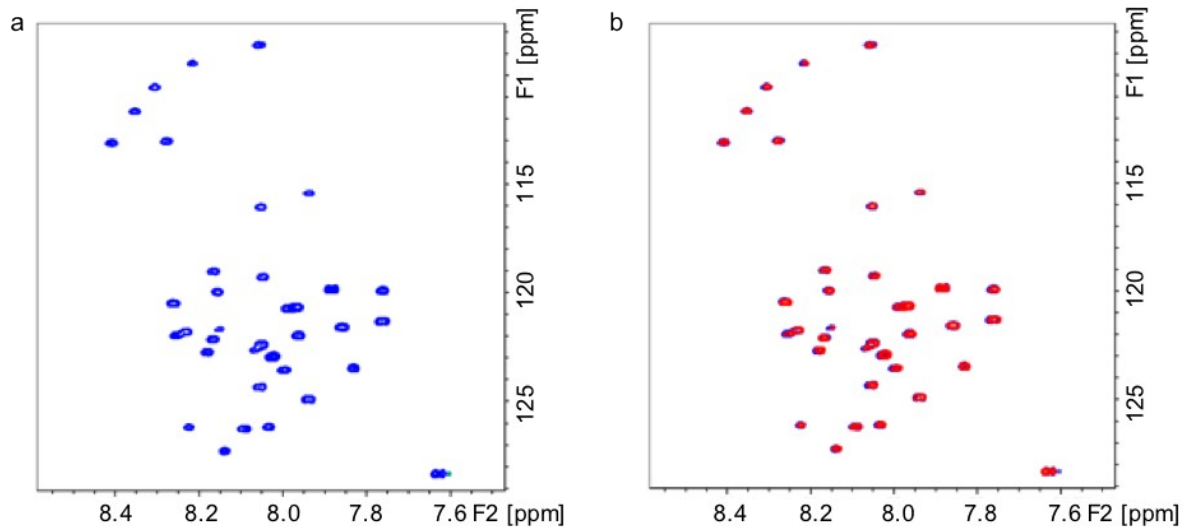


Figure S3. $^{1}H,^{15}N$-HSQC NMR spectra of [H6A, H13A and H14A]-mutated $A\beta_{40}$ before and after incubation with Ce6. a) HSQC NMR spectrum of mutated $A\beta_{40}$ in HEPES buffer (40 mM, pH 7.3). b) HSQC spectrum of mutated $A\beta_{40}$ after addition of Ce6 (1.27 mg) under dark conditions (blue peaks), and following 1 h of irradiation (red peaks). There is no significant difference between the two spectra.

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