

Multifunctional peptide-assembled micelles for simultaneously reducing amyloid- β and reactive oxygen species

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Table of Contents

Experimental section.....	S3-12
Fig. S1, S2.....	S-13
Fig. S3, S4.....	S-14
Fig. S5, S6.....	S-15
Fig. S7, S8.....	S-16
Fig. S9, S10.....	S-17
Fig. S11, S12.....	S-18
Fig. S13, S14.....	S-19
Fig. S15, S16.....	S-20
Fig. S17, S18.....	S-21
Fig. S19.....	S-22

Experimental section

Chemicals and Reagents.

The peptide LPFFD-NH₂ (LD), Ac-TGFQGSWHIHF_{TANFVNTK}-NH₂ (TK), and A β ₁₋₄₂ were purchased from GL Biochem, Ltd. (Shanghai, China). 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) was bought from Tokyo Chemical Industry (TCI) Co., Ltd. (Shanghai, China). N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDCI), N-hydroxysuccinimide (NHS), Thioflavin T (ThT), 3,5,3',5'-tetramethylbenzidine (TMB), glutathione (GSH), hemin, 2', 7'-dichlorodihydro-fluorescein diacetate (DCFH-DA) and IR-780 iodide dye (IR780) were all purchased from Sigma-Aldrich Ltd. (Shanghai, China). Acridine orange (AO) and cyclosporine (CSA) were purchased from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Polysialic acid (PSA, molecular weight is about 12000) was supplied by Zhejiang Changxing Pharmaceutical Co., Ltd. (Zhejiang, China). CCK-8 Cell (Counting Kit-8) was obtained from Beyotime Biotechnology Co., Ltd. (Shanghai, China). Dialysis bags with a molecular weight cut off (MWCO) of 500 Da and 3500 Da were obtained from Yuanye Biotechnology Co., Ltd. (Shanghai, China). 4',6-diamidino-2-phenylindole (DAPI) were purchased from Thermo Fisher Scientific Inc. (California, USA). All other chemicals are of analytical grade and used directly without further purification. Water (18.2 M Ω ·cm) from a laboratory water purification system (Hitech Instruments Co., Ltd.) was used throughout the experiment. (Shanghai, China).

Measurements.

Transmission electron microscopy (TEM) measurements were performed on HT7700 electron microscope (Hitachi, Tokyo, Japan). The dynamic light scattering (DLS) size measurement was measured at 25 °C using a Malvern Zetasizer Nanoseries (Malvern, England). UV-vis absorption and fluorescence spectra were measured with Cary 60 UV-vis spectrophotometer and Cary Eclipse spectrofluorophotometer, respectively (Agilent Technologies, Palo Alto, CA, USA). Proton nuclear magnetic resonance (¹H NMR) was measured by using a Bruker-500 MHz NMR spectrometer at 25 °C. The mass spectra were measured with LC-MS spectrometer (Bruker, America). The data of fluorescence images and CCK-8 assay were collected using Cytation 3 multi-mode reader (BioTek Instruments Inc. Winooski, VT, USA). The flow cytometric (FCM) data was obtained from a FACS Calibur flow cytometric system (BD Biosciences, Oxford, UK). Confocal laser scanning microscopy (CLSM) was collected by using a Leica TCS-SP8 confocal scanning microscope (Solms, Germany).

Synthesis of tk-GSH.

The synthesis of tk-GSH refers to the method of Yuan et al.¹ As followed, 800 mg of GSH (2.6 mmol) was dissolved in 5.0 mL of anhydrous methanol, then 100 μL (1.3 mmol) of anhydrous acetone was slowly added under stirring, and 500 μL of concentrated sulfuric acid was carefully dropped into above solution with ice bath conditions. After reacting for 48 h at room temperature, NaHCO₃ saturated solution and dichloromethane (DCM) were used to extract the product, which was purified by a dialysis bag with a molecular weight of 500 Da. The product was collected and

dried overnight in a freeze-drying oven to obtain a white solid tk-GSH. Yield: 797.9 mg (1.1 mmol, 86.3 %). MS calculated for $C_{27}H_{47}N_6O_{12}S_2$, 710.26, found, 711.2677 (M + H). 1H NMR (500 MHz, D_2O), δ (ppm): 4.58–4.56 (m, 2H), 4.06 (s, 4H), 3.78 (m, 12H), 3.64–3.62 (m, 2H), 3.17–3.14 (m, 2H), 3.13–2.99 (m, 2H), 2.47–2.46 (m, 4H), 2.10–2.05 (m, 2H), 1.99–1.94 (m, 2H), 1.66 (dd, $J = 8.2, 5.5$ Hz, 6H).

Synthesis of PSA-LD-TK (PLT).

Firstly, the peptides LD and TK were dissolved in HFIP, dried with N_2 and placed in a refrigerator at -30 °C for further use. Then, 500 mg (0.04 mmol) of PSA was dissolved in 2.0 mL deionized water, and activated with 11.5 mg (0.04 mmol) of EDCI and 6.9 mg (0.04 mmol) of NHS at room temperature for 4 h. Subsequently, 0.06 mM peptides (LD and TK were dissolved in 2.0 mL DMSO) in total with a series of molar ratios (1:0, 0.75:0.25, 0.5:0.5, 0.25:0.75, 0:1) were dropwise added to the PSA solution, followed by stirring and reacting at 37 °C for 48 h, and then it was dialyzed (MWCO, 3500 Da) in DMSO and deionized water. Later, white solid were obtained by freeze-drying and designated as PLT1, PLT2, PLT3, PLT4, and PLT5 respectively.

Synthesis of PSA-tk-GSH-LD-TK (PGLT).

500 mg (0.04 mmol) PSA was dissolved in 2.0 mL deionized water, then 11.5 mg (0.06 mmol) EDCI and 6.9 mg (0.06 mmol) NHS were added and activated at room temperature for 4 h. Then, 142 mg (0.2 mmol) of tk-GSH aqueous solution was dropped into the mixture, and the mixture was stirred at room temperature for 48 h. After the reaction, the product was purified in deionized water with 3500 Da dialysis

bag. The product was carefully collected and lyophilized to obtain a white solid PSA-tk-GSH (640 mg).

520 mg (0.04 mmol) of PSA-tk-GSH was first dissolved in 2.0 mL deionized water and activated by 11.5 mg (0.04 mmol) EDCI and 6.9 mg (0.04 mmol) NHS for 4 h. Then, the mixture was placed at room temperature and stirred rapidly for 48 h by adding short peptide LD and TK (LD:TK = 1:1). After the reaction, the product was purified in DMSO/water (1:1, v/v) using dialysis bags (MWCO, 3500 Da), and then purified in water. The product was collected and lyophilized overnight to obtain a white solid PGLT.

Preparation of Multifunctional Peptide Micelles.

5 mg of PLT1, PLT2, PLT3, PLT4, PLT5 or PGLT were dissolved in 300 μ L DMSO respectively, and then added to deionized water (700 μ L) rapidly under tip ultrasound. The ultrasound condition is: 78 W, 10 s/time, 3 times. Finally, the products were purified in deionized water with 3500 Da dialysis bag, and the multi-functional peptide micelles including MPLT1, MPLT2, MPLT3, MPLT4, MPLT5 and MPGLT were characterized by TEM and DLS.

Preparation procedure of MPGLT-IR780 was as follows. 5 mg PGLT was dissolved in 200 μ L DMSO, mixed with 100 μ L of IR780 (1 mg/mL, dissolved in DMSO) and rapidly injected into 700 μ L deionized water under tip ultrasound, and then purified in deionized water with 3500 Da dialysis bag. The loading efficiency (LE) and loading ratio of IR780 was determined by UV-vis spectrometry. The LE was calculated by the following formula:

LE (%) = (weight of loaded IR780) / (total weight of MPGLT-IR780) × 100.

Inhibition of A β ₁₋₄₂ Aggregation by Multifunctional Peptide Micelles.

The A β ₁₋₄₂ (1 mg/mL) peptide powder was dissolved in HFIP. After that, a thin film was obtained by removing HFIP with nitrogen and kept at -30 °C for use.

The above A β ₁₋₄₂ film was dissolved in 10 mM PBS (pH=7.4). Then, the A β ₁₋₄₂ solutions with and without micelles (MPLT1, MPLT2, MPLT3, MPLT4, MPLT5 and MPGLT) were incubated at 37 °C for different time. Then they were co-incubated with ThT in PBS to the final concentration of A β peptide at 20 μ M, micelles at 20 μ g/mL, ThT at 20 mM, and the fluorescence intensities were monitored at 485 nm by fluorescence spectrophotometer. Besides, the morphologies of A β fibril and the co-assembly solutions at 24 h were observed by TEM after negative staining with uranyl acetate solution.

Investigation on ROS Removal by MPGLT.

The ROS scavenging experiment by MPGLT was carried out at room temperature using the ultraviolet absorption changes of TMB substrate in the presence of H₂O₂. Firstly, 0.25 mM hemin, 30% H₂O₂, 10 mg/mL TMB, MPGLT, tk-GSH, and GSH diluted with 25 mM PBS (pH = 7.4) to different concentrations (1-50 μ g/mL, identical concentration of GSH) were prepared. Then, hemin, H₂O₂, TMB and tk-GSH (GSH or MPGLT) were mixed and placed on the microplate reader for testing after 5 min of reaction. The absorption value of TMB at 652 nm was recorded by using UV-vis spectrometry and Cytation 3 multi-mode reader.

Cell Culture.

SHSY-5Y neuroblastoma (SHSY-5Y) cells were obtained from the Cell Bank of Chinese Academy of Science (Shanghai, China). The cells were cultured and maintained in RPMI medium (1640) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin and streptomycin), and then were grown in a cell culture incubator at 37 °C and humidified atmosphere with 5% CO₂ supply.

In Vitro Cytotoxicity Assay.

In vitro cytotoxicity assay was carried out using CCK-8 assay. Briefly, a density of 2×10^4 SHSY-5Y cells per well were seeded in 96-well plates and cultured overnight. And then, the following steps were performed. (1) To evaluate the cytotoxicity of the peptide micelles, the cells were incubated with fresh 1640 medium containing MPLT1, MPLT2, MPLT3, MPLT4, MPLT5, and MPGLT with a series of different concentrations, 0, 10, 20, 30, 40, 50, 60, 80, 100 µg/mL for additional 24 h. (2) To study A β -induced cytotoxicity, the cells were treated with fresh 1640 medium containing 20 µM A β or A β_{1-42} (20 µM) + Cu²⁺ (20 µM) with and without MPLT1, MPLT2, MPLT3, MPLT4, MPLT5, and MPGLT sample solutions at the concentration of 20 µg/mL were co-incubated with cells for another 24 h. (3) To investigate the H₂O₂-induced cytotoxicity, the cells were cultured with PBS containing different concentrations of H₂O₂ (25, 50, 75, 100, 125, 150, 200 and 250 µM) and further placed in an incubator for 1-4 h. (5) To explore H₂O₂ cytotoxicity in the presence of MPGLT, the cells were preincubated with the medium containing GSH, tk-GSH, and MPGLT (10 µg/mL, identical concentration of GSH) for 3 h.

Subsequently, the cells were incubated with PBS containing 100 μM H_2O_2 for another 4 h. Finally, 10 μL of CCK-8 solutions was added to each well and cultured for 4 h. The absorbance at 490 nm was performed by using a microplate reader. All the experiments were performed in triplicate. The cell viability was calculated by the formula below:

Cell viability (%) = the absorbance of experimental group / the absorbance of blank control group \times 100%.

Acridine Orange (AO) Assay.

SHSY-5Y cells (2×10^4) were plated on a 35 mm Petri dish with a 20 mm bottom well. After attachment overnight, the cells were cultured with MPLT1, MPLT2, MPLT3, MPLT4, and MPLT5 (20 $\mu\text{g}/\text{mL}$) for 4 h, followed by washing with PBS, and then were stained with 5 $\mu\text{g}/\text{mL}$ AO in 1640 medium for another 10 min. After washed with PBS and the fluorescence of the cells were observed using CLSM with the excitation wavelength at 488 nm.

Cellular Uptake and Distribution of MPGLT In Vitro.

The cell uptake and intracellular distribution of MPGLT were evaluated by fluorescence imaging and flow cytometry (FCM) examinations, respectively. Encapsulation of IR780 into micelles was used to prepare MPGLT-IR780 for micelle uptake monitoring. SHSY-5Y cells were seeded in a 35 mm Petri dish with a 20 mm bottom well. After 24 h incubation, the cells were incubated with MPGLT-IR780 at the concentration of 20 $\mu\text{g}/\text{mL}$ for different time durations (i.e., 0, 0.25, 0.5, 1, 2, or 3 h). After thoroughly washed with PBS, stained with DAPI, followed by imaging using

Cytation 3 cell imaging multimode reader (DAPI: $\lambda_{\text{Ex}} = 377 \text{ nm}$, $\lambda_{\text{Em}} = 420 \text{ nm}$; IR780: $\lambda_{\text{Ex}} = 647 \text{ nm}$, $\lambda_{\text{Em}} = 794 \text{ nm}$). Otherwise, the cells were trypsinized and resuspended in 500 μL PBS for flow cytometry (FCM) analysis.

Intracellular Determination of ROS.

2×10^4 cells/well of SHSY-5Y cells were first seeded in a 35 mm Petri dish with a 20 mm bottom well and cultured for 24 h. After that, cell medium was removed and the adherent cells were incubated with MPGLT, tk-GSH, or GSH (10 $\mu\text{g}/\text{mL}$, identical concentration of GSH). The cells were washed three times with PBS, and then incubated at 37 °C for another 1 h with 60 μM H_2O_2 . To determine $\text{A}\beta_{1-42}$ and Cu^{2+} induced ROS, the cells were treated with $\text{A}\beta_{1-42}$ (20 μM) + Cu^{2+} (20 μM) with and without 20 $\mu\text{g}/\text{mL}$ of MPLT3 and MPGLT, and then incubated at 37 °C for 8 h. The cells were further incubated with DCFH-DA (5 μM) at 37 °C for 1 h. After being washed three times with PBS, stained with DAPI, intracellular ROS levels were imaged by a Cytation 3 cell imaging multimode reader (DCF: $\lambda_{\text{Ex}} = 469 \text{ nm}$, $\lambda_{\text{Em}} = 525 \text{ nm}$). As for the FCM assay, the cells were washed with PBS, trypsinized, and collected for FCM measurement ($\lambda_{\text{Ex}} = 488 \text{ nm}$, $\lambda_{\text{Em}} = 525 \text{ nm}$).

Animals.

C57B/L6 mice (male; 25-30 g body weight; age, 25 weeks old) and Balb/c (female; 13-15 g body weight; age, 5-weeks old) were obtained from Laboratory Animal Center of East China of Normal University (Shanghai, China). APP^{swe}/PS1^{dE9} transgenic mice (male; 28-32 g body weight; age, 25 weeks old) were purchased from Shanghai Model Organisms Center, Inc. (Shanghai, China). All animal studies were

performed in accordance with the guidelines approved by the Institutional Animal Care and Use Committee of East China Normal University.

In Vivo Imaging.

Balb/c mice were randomly divided into three groups as PBS, MPGLT-IR780, and CSA/MPGLT-IR780. Firstly, the mice were intravenously injected 10 μ M CSA for 30 min in the group of CSA/MPGLT-IR780. Then, the untreated and CSA-treated mice were followed by i.v. administration of PBS and MPGLT-IR780 (220 μ g/mL), respectively. The fluorescent images at different time ($\lambda_{\text{Ex}} = 745$ nm and $\lambda_{\text{Em}} = 820$ nm for IR780) were obtained using a Caliper IVIS Lumina II imaging system (PerkinElmer, USA). After that, the mice were sacrificed and the brains as well as major organs (i.e., heart, liver, spleen, lung, and kidney) were collected and examined for fluorescence imaging ex vivo ($\lambda_{\text{Ex}} = 745$ nm and $\lambda_{\text{Em}} = 820$ nm for IR780). All the images were obtained with the same parameter settings and scale. Furthermore, the brains were frozen sectioned immediately and stained with DAPI after IVIS image, and then examined by using Cytation 3 cell imaging multimode reader.

Clearance of A β and ROS In Vivo.

C57B/L6 mice and APP^{swe}/PS1^{dE9} transgenic mice were regarded as wild type mice (WT mice) and AD mice, respectively. All mice were given food and water, and kept in a colony room at 22 \pm 2 $^{\circ}$ C and 45 \pm 10% humidity under a 12: 12 h light/dark cycle. AD mice were divided into two groups termed as AD group and MPGLT group. All mice were firstly intravenously administered 10 μ M CSA for 30 min. Then, WT mice were treated by PBS and AD mice were treated by 220 μ g/mL of MPGLT. The

treatment was repeated every other day for two weeks.

For the ROS evaluation experiment, the mice were intravenously injected with DCFH-DA (2.5 mg/kg). After 30 min, the mice were immediately sacrificed and the brains were collected and imaged for IVIS *ex vivo* (DCF: $\lambda_{\text{Ex}} = 485$ nm, $\lambda_{\text{Em}} = 520$ nm). All the images were obtained with the same parameter settings and scale. And the brains were frozen sectioned immediately and stained with DAPI after IVIS image, and then examined using a Cytation 3 cell imaging multimode reader.

For the A β plaques evaluation experiment, the animals were euthanized, followed by heart perfusion with 60 mL of cold saline. The whole brains were harvested and fixed in 10% formalin, embedded in paraffin, and sectioned at 3 μm . These sections were incubated with anti-A β antibody (1:500, GB13414-1, Servicebio) for 18 h at 37 $^{\circ}\text{C}$ and then with a Thioflavine S (ThS) -conjugated Goat antirabbit IgG polyclonal antibody (1:400, GB25303, Servicebio) at room temperature for 2 h. Cell nuclei were stained with DAPI, and coverslips were mounted on glass slides. All images were taken with Cytation 3 cell imaging multimode reader (ThS: $\lambda_{\text{Ex}} = 469$ nm, $\lambda_{\text{Em}} = 525$ nm). Nissl staining sections were used for quantitative analysis of neuronal injury in the cortex of mice. Furthermore, to analyze the biosecurity of MPGLT, the brains and major organs (i.e., heart, liver, spleen, lung, and kidney) were collected for the H&E staining.

References

- [1] F. Y. Zhou, B. Feng, T. Wang, D. Wang, Z. Cui, S. Wang, C. Ding, Z. Zhang, Liu, J. Yu, Y. Li, *Adv. Funct. Mater.*, 2017, **27**, 1703674.

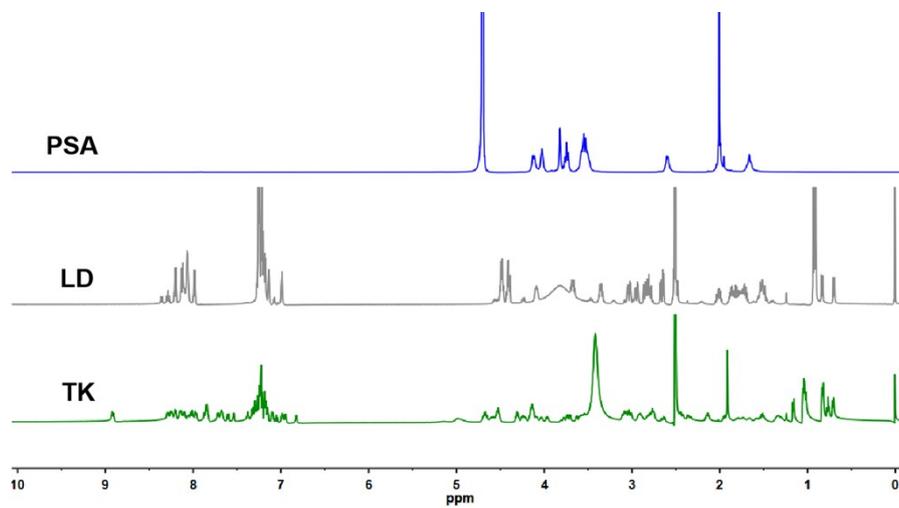


Fig. S1 The ^1H NMR spectra of PSA, LD, and TK.

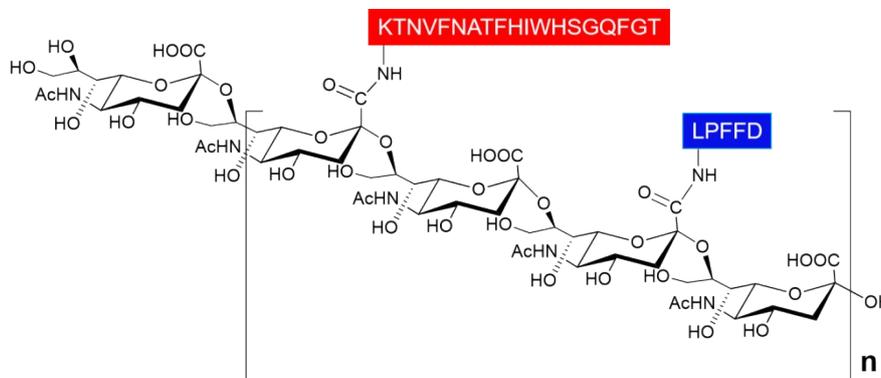


Fig. S2 The structure of polymer-peptide conjugates (PLT).

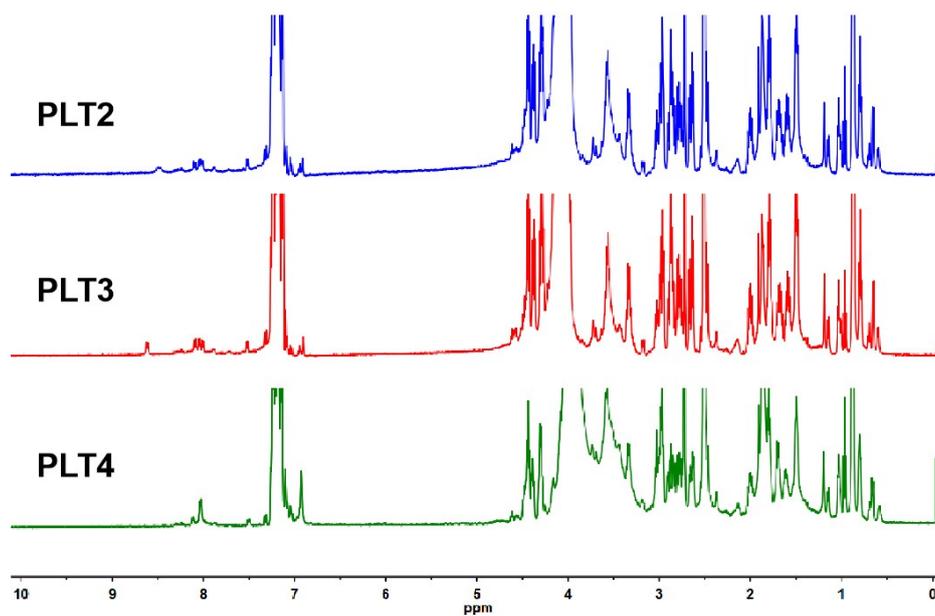


Fig. S3 The ^1H NMR spectra of PLT2, PLT3, and PLT4.

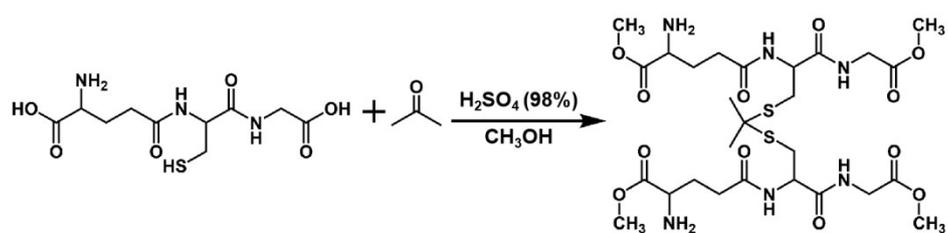


Fig. S4 The synthesis route of tk-GSH.

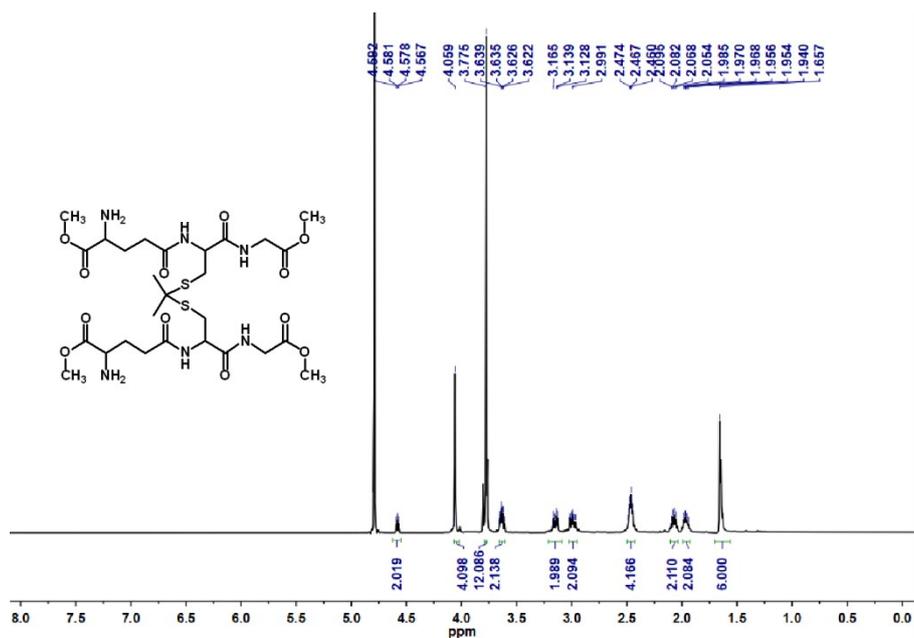
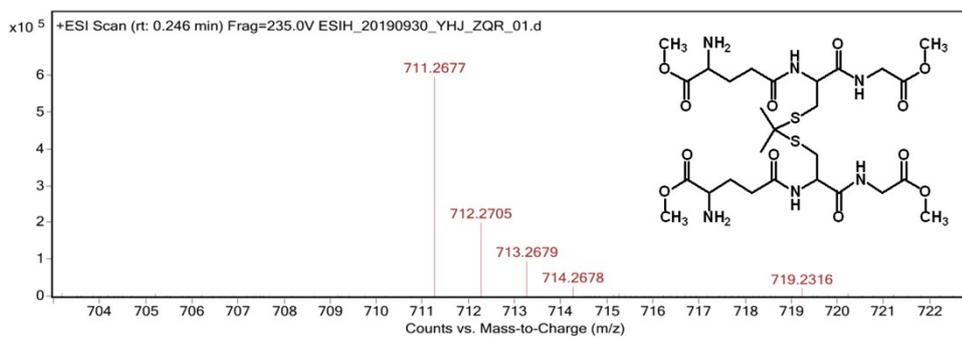


Fig. S5 ^1H NMR spectra of tk-GSH in D_2O .



Formula Calculator Results					
m/z	Calc m/z	Diff (mDa)	Diff (ppm)	Ion Formula	Ion
711.2677	711.2688	1.13	1.59	C ₂₇ H ₄₇ N ₆ O ₁₂ S ₂	(M+H) ⁺

Fig. S6 HRMS spectrum of tk-GSH.

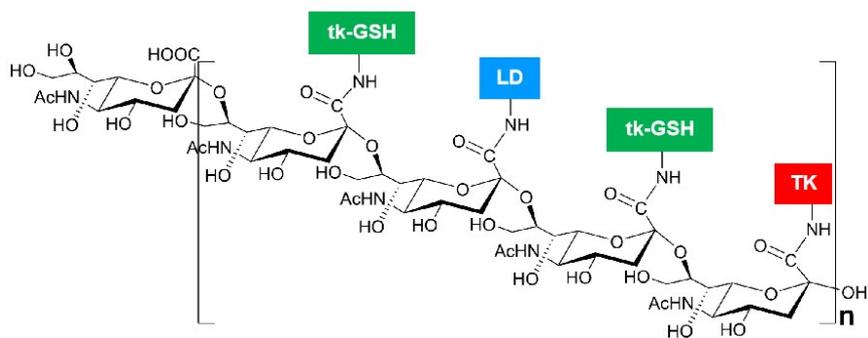


Fig. S7 The structure of polymer-peptide PGLT.

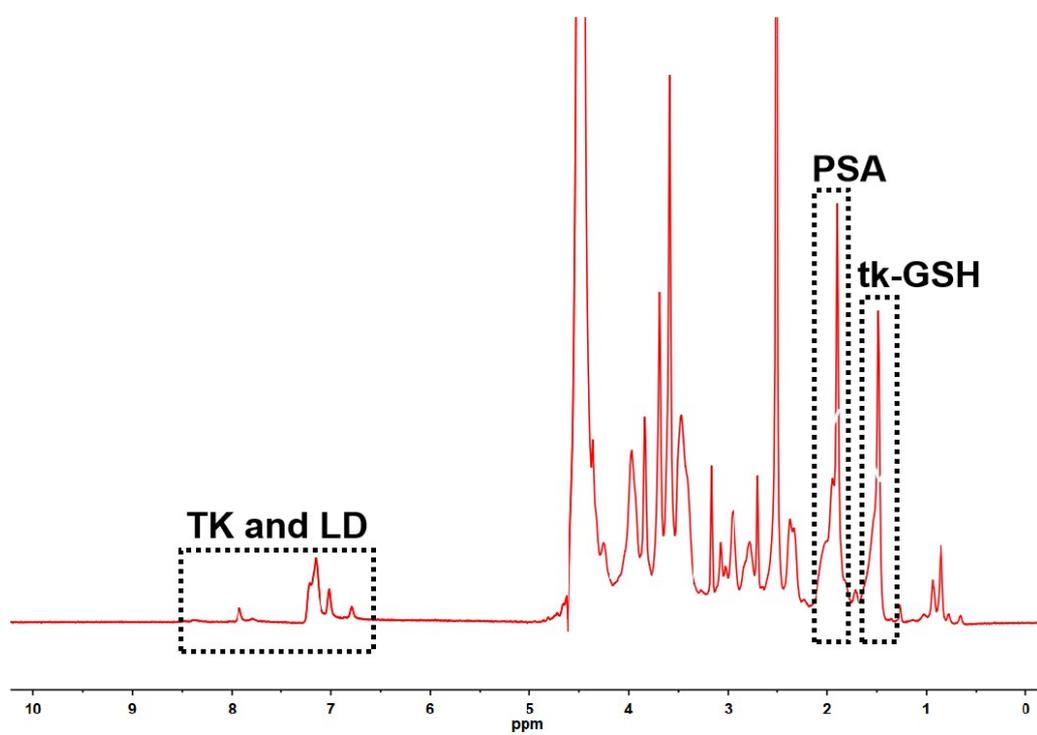


Fig. S8 ^1H NMR spectra of polymer-peptide PGLT.

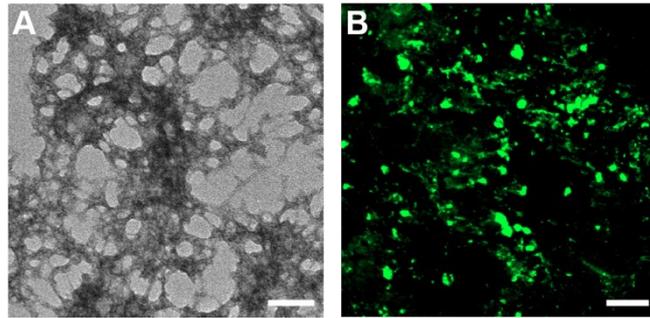


Fig. S9 (A) TEM and (B) ThT assay of $A\beta_{1-42}$ incubated for 48 h. Scale bars = 500 nm and 100 μm .

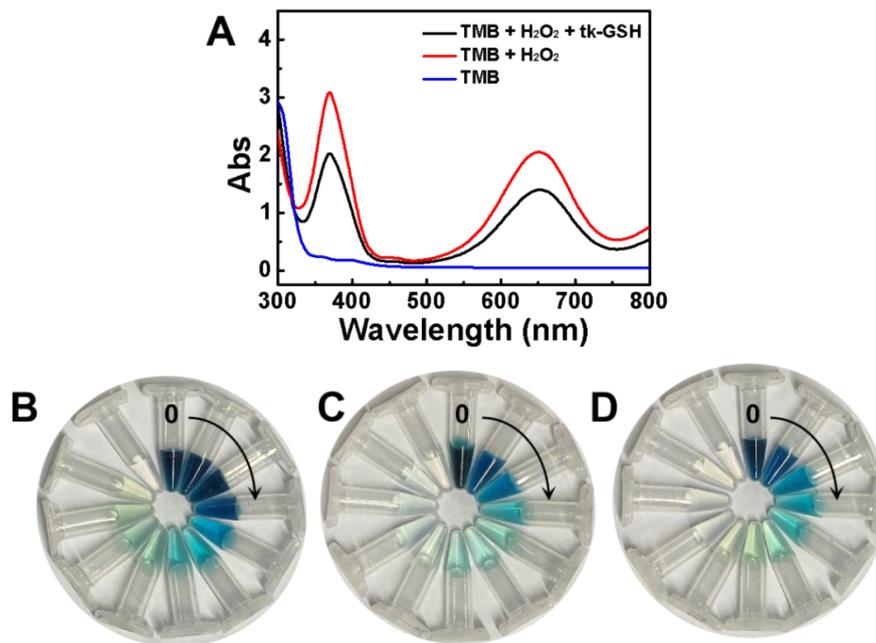


Fig. S10 (A) The UV-vis absorption spectra of TMB, TMB + H_2O_2 , TMB + H_2O_2 + tk-GSH (20 $\mu\text{g}/\text{mL}$) in the presence of 0.25 mM hemin. TMB: 1.7 mM, H_2O_2 : 1.2 M, the incubation time of H_2O_2 and tk-GSH is 5 min. (B-D) The photographs of TMB + H_2O_2 + GSH (0–50 $\mu\text{g}/\text{mL}$) (B), TMB + H_2O_2 + tk-GSH (0–50 $\mu\text{g}/\text{mL}$) (C), and TMB + H_2O_2 + MPGLT (0–50 $\mu\text{g}/\text{mL}$) (D) in the presence of 0.25 mM hemin. TMB: 1.7 mM, H_2O_2 : 1.2 M, respectively.

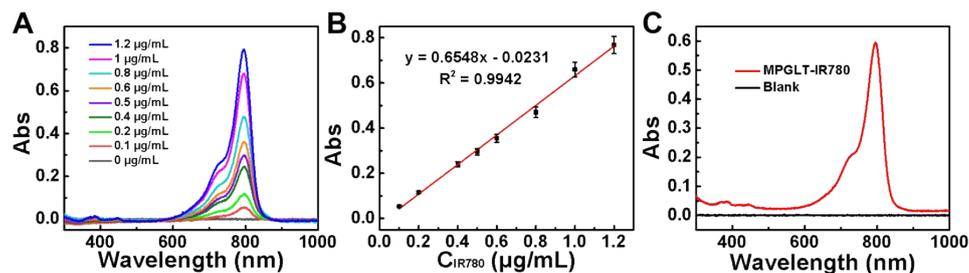


Fig. S11 (A) The UV-vis spectra of IR780 at different concentrations. (B) The calibration curve of the adsorption peak at 795 nm upon different concentrations of IR780 with a concentration ranging from 0 to 1.2 µg/mL. Data are presented as mean \pm s.d. (n = 3). (C) The UV-vis spectra of MPGLT-IR780 (red curve) and blank (DMSO, black curve).

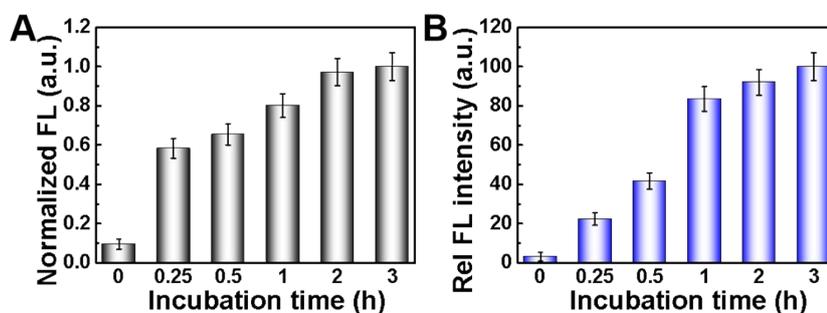


Fig. S12 The average fluorescence intensity (A) from the images of fluorescence imaging and (B) from the FCM in the study that MPGLT-IR780 was internalized into SHSY-5Y cells with different time. Data are presented as mean \pm s.d. (n = 3).

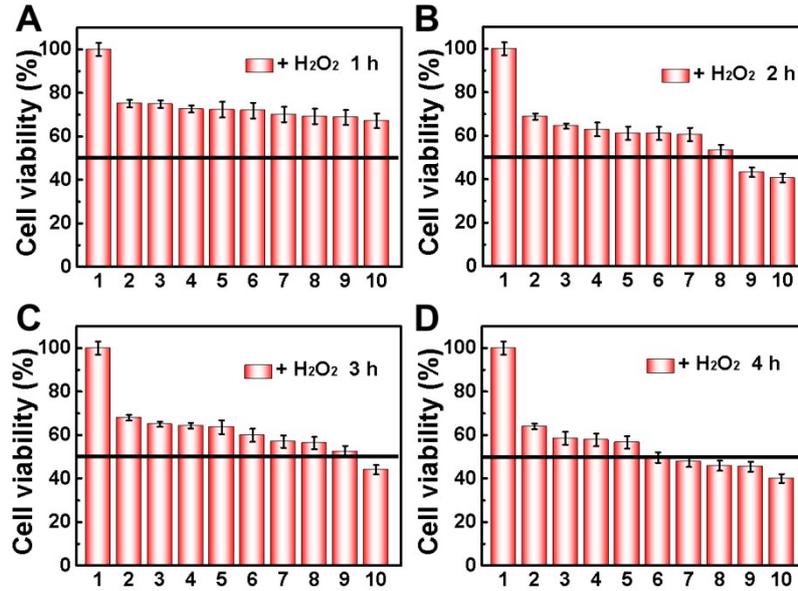


Fig. S13 Cells viability of SHSY-5Y cells upon the incubation of H_2O_2 (1: DMEM, 2: PBS, 3: 25 μM , 4: 50 μM , 5: 75 μM , 6: 100 μM , 7: 125 μM , 8: 150 μM , 9: 200 μM , 10: 250 μM) for (A) 1 h, (B) 2 h, (C) 3 h, and (D) 4 h by CCK-8 test. Data are presented as mean \pm s.d. ($n = 3$).

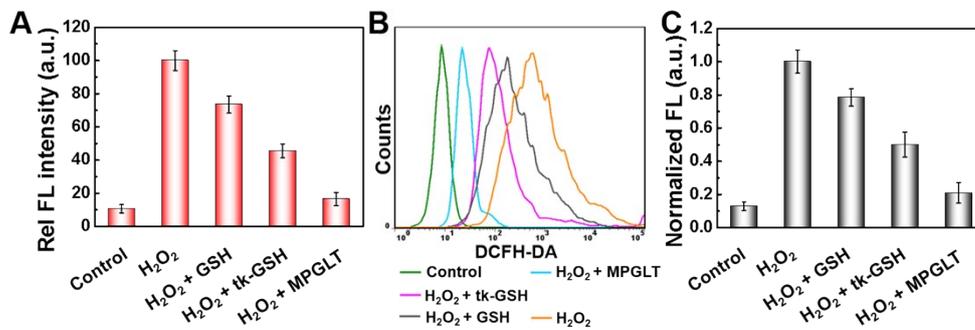


Fig. S14 (A) The relative fluorescence intensity from the fluorescence images of Figure 4C. (B) FCM of ROS in SHSY-5Y cells following various ROS scavengers treatment with GSH, tk-GSH, and MPGLT. (C) The average fluorescence intensity from the FCM measurement in (B). Data are presented as mean \pm s.d. ($n = 3$).

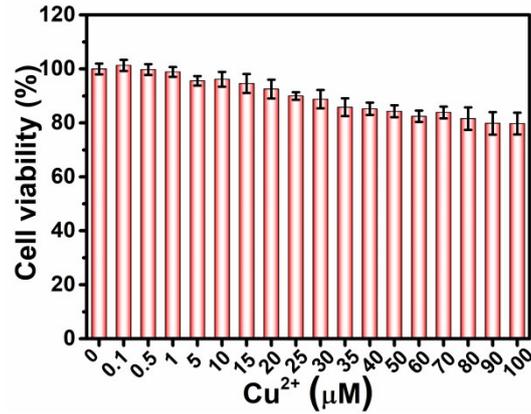


Fig. S15 Cells viability estimated by CCK-8 assay. SHSY-5Y cells were incubated with 0, 0.1, 0.5, 1, 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100 μM of Cu^{2+} .

Data are presented as mean \pm s.d. (n = 3).

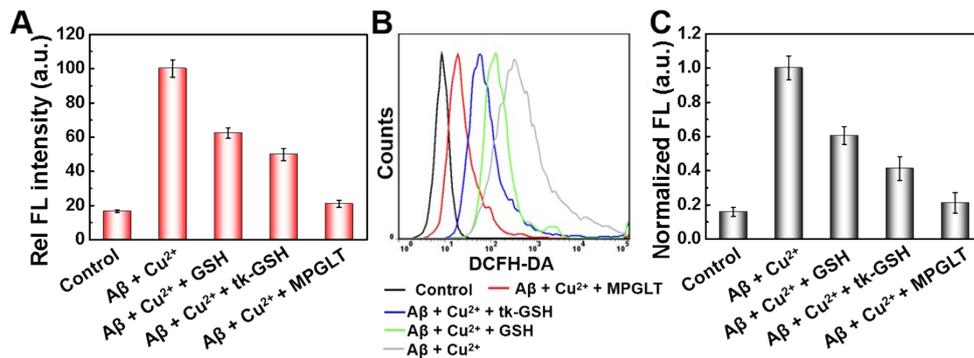


Fig. S16 (A) The relative fluorescence intensity from the fluorescence images of Figure 4E. (B) FCM of the effect of GSH, tk-GSH and MPGLT on A β_{1-42} (20 μM) + Cu^{2+} (20 μM) induced production of ROS. (C) The average fluorescence intensity from the FCM measurement in (B). Data are presented as mean \pm s.d. (n = 3).

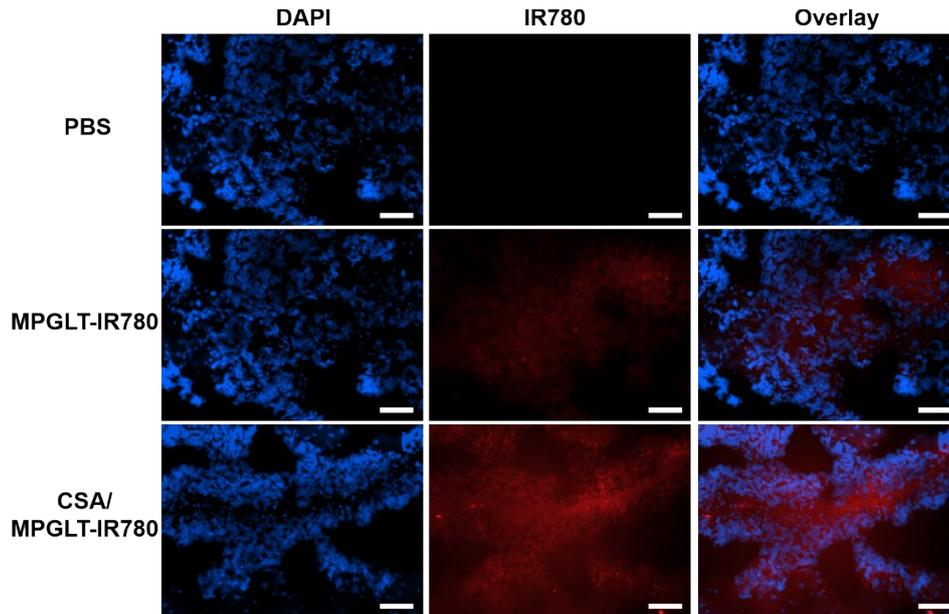


Fig. S17 Fluorescence analysis of PBS and 220 $\mu\text{g}/\text{mL}$ of MPGLT-IR780 with or without cyclosporine (10 μM) distribution in the brain sections (DAPI: $\lambda_{\text{ex}} = 377 \text{ nm}$; IR780: $\lambda_{\text{ex}} = 647 \text{ nm}$), scale bars = 500 μm .

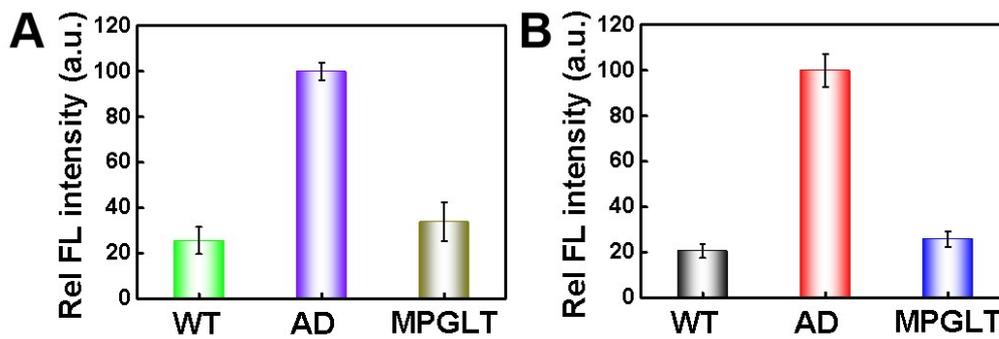


Fig. S18 The relative fluorescence intensity from the fluorescence images of Figure 6C (A) and Figure 6D (B). Data are presented as mean \pm s.d. (n = 3).

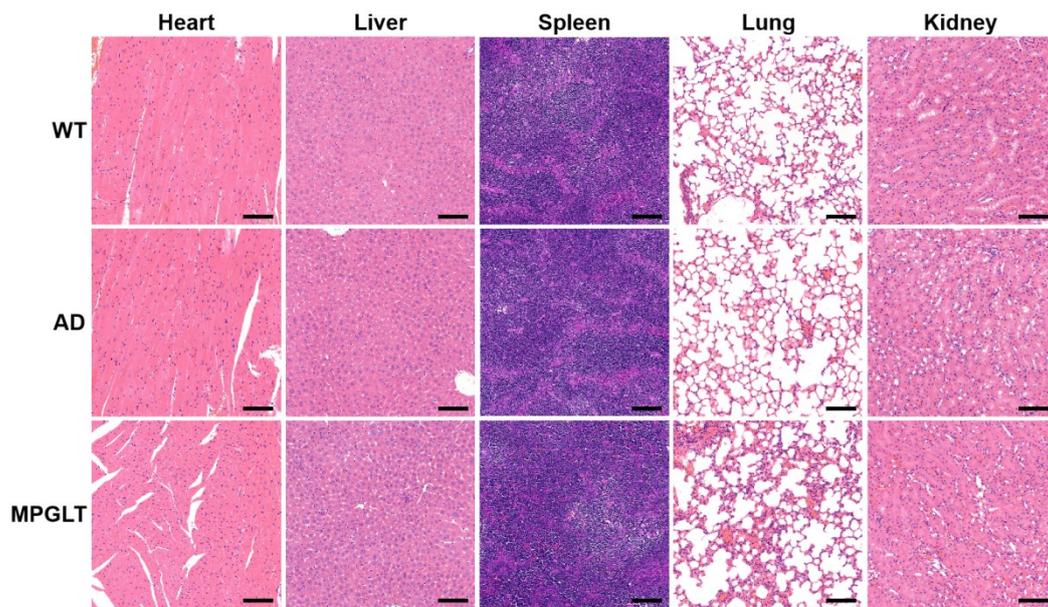


Fig. S19 Hematoxylin and eosin (H&E) images of organs of AD mice treated by PBS (AD group) or MPGLT (MPGLT group), and WT mice treated with PBS as a control. Scale bar = 50 μm.