Supporting Information for

Original article

ROS-removing nano-medicine for navigating inflammatory microenvironment to enhance anti-epileptic therapy

Zheng Zhou, **Keying Li**, **Yongchao Chu**, **Chao Li**, **Tongyu Zhang**, **Peixin Liu**, **Tao Sun**, **Chen Jiang***

Department of Pharmaceutics, School of Pharmacy, Fudan University; Key Laboratory of Smart Drug Delivery, Ministry of Education; State Key Laboratory of Medical Neurobiology and MOE Frontiers Center for Brain Science, Shanghai 201203, China

Received 22 June 2022; received in revised form 29 August 2022; accepted 15 September 2022

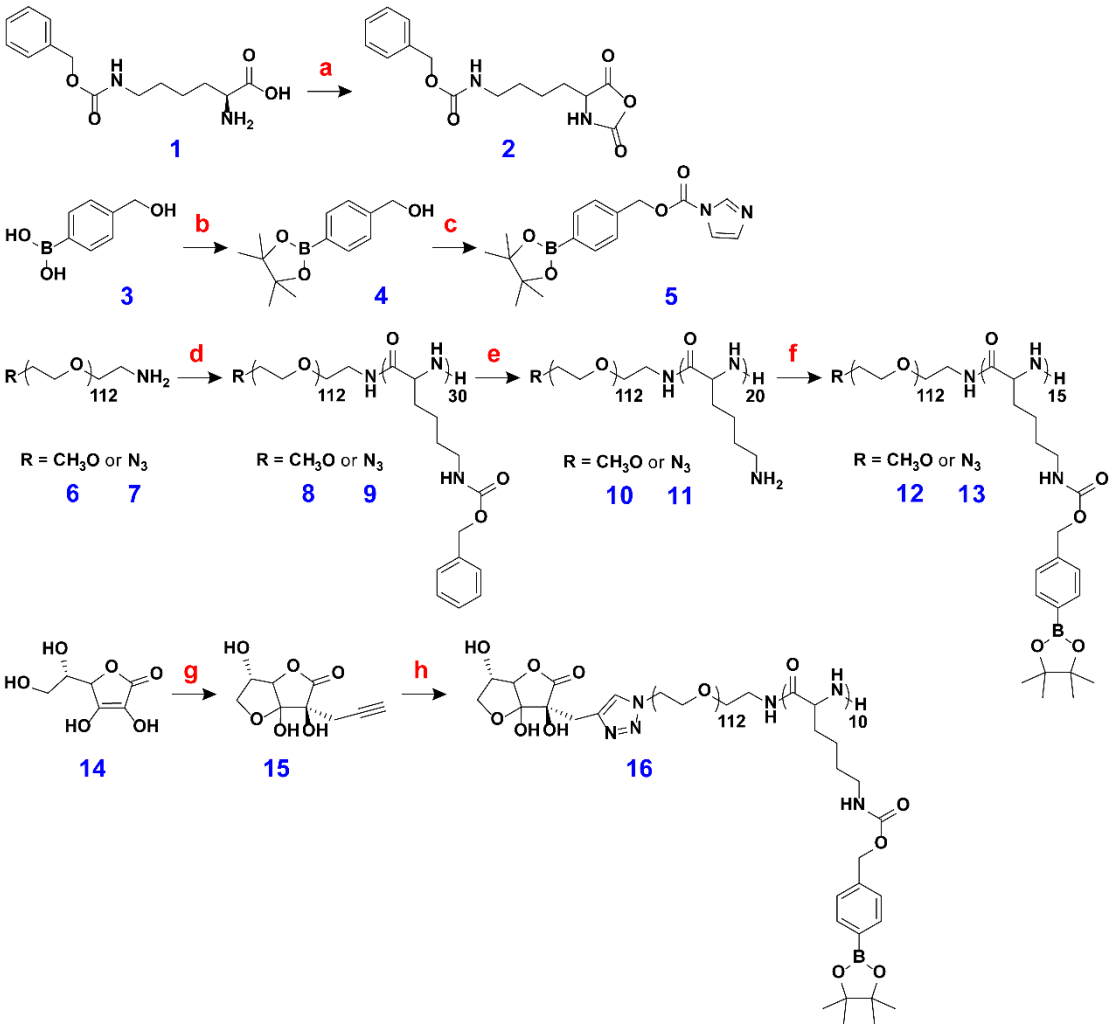
*Corresponding author. Tel.: +86 021 51980079

E-mail address: jiangchen@shmu.edu.cn (Chen Jiang).

1. Reagents

CH_3O-PEG_{5000}-NH_2 and N_3-PEG_{5000}-NH_2 were purchased from Seebio Biotechnology (Shanghai, China). *Nε*-Carbobenzoxy-L-lysine, trifluoroacetic acid (TFA), edetate disodium (EDTA), *N*,*N*,*N*′,*N*′,*N*″-pentamethyldiethylenetriamine (PMDETA), copper iodide (CuI), pilocarpine hydrochloride, coumarin 6 and colchicine were purchased from Aladdin (Shanghai, China). 4-Hydroxybenzyl alcohol, dimethyl sulfoxide-d_6 (DMSO-δ6), 4-(hydroxymethyl)phenylboronic acid, *N*,*N*′-carbonyldiimidazole (CDI), pinacol, propargyl bromide and filipin were purchased from Energy Chemical (Shanghai, China). Anhydrous solvents, lamotrigine, 4-dimethylaminopyridine (DMAP) and hydrobromic acid were purchased from J&K scientific (Beijing, China). Triphosgene was purchased from TCI Shanghai (Shanghai, China). Triethylamine and phenylarsine oxide were purchased from Tansoole (Shanghai, China). (–)-Scopolamine hydrobromide trihydrate was purchased from Macklin (Shanghai, China). All other solvents or reagents were purchased from Sinopharm Chemical Reagent (Shanghai, China).

2. Synthesis, preparation and characterization of micelles



Scheme S1 Synthetic steps and structures of mPEG-poly-LysB (PLB, compound **12**) and DHAA-PEG-poly-LysB (DPLB, compound **16**) polymer.

Detailed steps are shown as below:

Step **a**: *Nε*-Carbobenzoxy-L-lysine (compound **1**, 1.0 g, 3.57 mmol) and triphosgene (423.4 mg, 1.43 mmol) were distributed in anhydrous tetrahydrofuran (THF, 30 mL) under Ar. The reaction was heated to 50 °C for 6 h and then left to cool down. The mixture was precipitated in *n*-hexane (200 mL) to give compound **2** (754.5 mg, 90.3%) as white powder. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.11 (d, J = 5.0 Hz, 1H), 7.34 (q, J = 6.7, 6.2 Hz, 5H), 5.00 (d, J = 5.0 Hz, 2H), 4.43 (q, J = 5.6 Hz, 1H), 2.98 (t, J = 6.1 Hz, 2H), 1.79 – 1.60 (m, 2H), 1.37 (tt, J = 22.3, 11.1 Hz, 4H).

Step **b**: 4-(Hydroxymethyl)phenylboronic acid (compound **3**, 1.0 g, 6.58 mmol), pinacol (1.1666 g, 9.87 mmol) and Na_2SO_4 (10 g) were dispersed in anhydrous THF (30 mL). The mixture was stirred overnight under Ar at room temperature (r.t.) and then filtered, concentrated and redissolved in ethyl acetate (50 mL). The organics were washed with water (3×20 mL) and brine (20 mL), and dried over Na_2SO_4. Ethyl acetate was removed by rotary evaporation to give compound **4** (1.1323 g, 73.5%) as buff oil. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.62 (td, J = 7.7, 2.0 Hz, 2H), 7.39 – 7.26 (m, 2H), 5.31 – 5.18 (m, 1H), 4.50 (dd, J = 8.6, 5.7 Hz, 2H), 1.32 – 1.24 (m, 12H).

Step **c**: Compound **4** (1.0 g, 4.27 mmol) and CDI (1.3853 g, 8.54 mmol) were dissolved in anhydrous dichloromethane (30 mL) and the reaction was stirred for 2 hours under Ar at r.t.. The mixture was then concentrated and redissolved in ethyl acetate (50 mL). The organics were washed with water (3×20 mL) and brine (20 mL), and dried over Na_2SO_4. Ethyl acetate was removed by rotary evaporation to give compound **5** (1.1323 g, 73.5%) as white solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.31 (d, J = 4.0 Hz, 1H), 7.76 – 7.68 (m, 2H), 7.63 (d, J = 4.2 Hz, 1H), 7.50 (d, J = 7.2 Hz, 2H), 7.09 (d, J = 3.9 Hz, 1H), 5.47 (d, J = 3.9 Hz, 2H), 1.29 (d, J = 4.7 Hz, 12H).

Step **d**: CH_3O-PEG_{5000}-NH_2 (compound **6**, 200 mg, 0.04 mmol) or N_3-PEG_{5000}-NH_2 (compound **7**, 200 mg, 0.04 mmol) and compound **2** (280.92 mg, 1.2 mmol) was dissolved in anhydrous *N*,*N*-dimethylformamide (DMF, 30 mL). The reaction, also called the ring opening polymerization (ROP), was stirred at 50 °C for 2 days under Ar. The mixture was then dialyzed (SnakeSkin^{TM} Dialysis Tubing, 3.5K MWCO, Thermo Scientific, U.S.A.) against deionized water (3×2 L) for 12 h and further lyophilized to give compound **8** or **9** as white powder. ^{1}H NMR (400 MHz, DMSO-d_6) of **8**: δ 7.40 – 7.17 (m, 161H), 4.97 (d, J = 9.0 Hz, 57H), 4.11 (d, J = 66.3 Hz, 26H), 3.49 (dd, J = 8.2, 3.5 Hz, 448H), 3.01 – 2.87 (m, 60H), 1.42 (dd, J = 97.7, 51.2 Hz,

179H). ^{1}H NMR (400 MHz, DMSO-d_6) of **9**: δ 7.32 (p, J = 8.5, 8.0 Hz, 152H), 4.99 (s, 60H), 4.41 – 3.83 (m, 28H), 2.95 (q, J = 6.6 Hz, 62H), 1.89 – 1.08 (m, 182H).

Step **e**: The deprotection of lysine was critical. Compound **8** (400 mg) or **9** (400 mg) was dissolved in TFA (10 mL) and hydrobromic acid (33 % *w*/*w* in acetic acid, 0.5 mL), and the mixture was stirred at r.t. for 3 h, followed by dialysis (3.5K) and lyophilization to give compound **10** or **11** as white powder. ^{1}H NMR (400 MHz, DMSO-d_6) of **10**: δ 4.22 (s, 18H), 3.23 (s, 3H), 2.76 (d, J = 9.3 Hz, 40H), 1.84 – 1.18 (m, 121H). ^{1}H NMR (400 MHz, DMSO-d_6) of **11**: δ 4.19 (d, J = 46.7 Hz, 20H), 2.75 (d, J = 12.1 Hz, 41H), 1.44 (d, J = 83.2 Hz, 127H).

Step **f**: Compound **10** (300 mg, 0.04 mmol) or **11** (300 mg, 0.04 mmol), compound **5** (262.5 mg, 0.8 mmol), DMAP (97.7 mg, 0.8 mmol) and triethylamine (81.0 mg, 0.8 mmol) were dissolved in anhydrous DMF (30 mL). The mixture was stirred under Ar at r.t. for 2 days, and then dialyzed (3.5K MWCO, SnakeSkin™ Dialysis Tubing, Thermo Fisher Scientific, U.S.A.) against DMF for 12 h and deionized water for another 12 h before lyophilization to give compound **12** or **13** as white powder. ^{1}H NMR (400 MHz, DMSO-d_6) of **12**: δ 7.64 – 6.90 (m, 60H), 4.98 (s, 31H), 4.09 (d, J = 93.4 Hz, 13H), 3.50 (d, J = 3.8 Hz, 448H), 3.00 – 2.88 (m, 32H), 1.32 (d, J = 43.7 Hz, 154H). ^{1}H NMR (400 MHz, DMSO-d_6) of **13**: δ 7.27 (s, 61H), 4.98 (s, 32H), 4.20 (s, 11H), 3.50 (t, J = 4.7 Hz, 448H), 2.94 (s, 36H), 2.08 – 0.53 (m, 262H).

Step **g**: KOH (0.2389 g, 4.26 mmol) was dissolved in H_2O (15 mL) under Ar and left to cool down. L(+)-ascorbic acid (compound **14**, 0.75 g, 4.26 mmol) was added to the solution that was then stirred for 1 h at r.t., followed by adding propargyl bromide (1.0132 g, 8.52 mmol) dissolved in acetone (30 mL). The mixture was stirred at 40 °C for 2 days and then cooled to r.t.. Acetone was removed by rotary evaporation and the residue was extracted by ethyl acetate (3×30 mL). The organics were combined,

washed with brine (20 mL) and dried over Na_2SO_4. Ethyl acetate was removed by rotary evaporation to give compound **15** (DHAA, 0.5628 g, 61.7%) as yellow oil. MS-ESI Calc. for [**15** + H]$^+$ 215.18, found, 215.0; Calc. for [**15** + Na]$^+$ 237.16, found, 237.0; Calc. for [**15** – H]$^-$ 213.17, found, 213.0; Calc. for [**15** + ^{35}Cl]$^-$ 249.14, found, 249; Calc. for [**15** + ^{37}Cl]$^-$ 251.14, found, 251.0.

Step **h**: The copper(I)-catalyzed azide-alkyne cycloaddition was the last reaction. Compound **13** (200 mg, 0.018 mmol), compound **15** (7.9 mg, 0.037 mmol), CuI (1.8 mg, 0.009 mmol), L(+)-ascorbic acid (32.5 mg, 0.18 mmol) and PMDETA (3.2 mg, 0.018 mmol) were dissolved in anhydrous DMF (30 mL) under Ar. The mixture was stirred overnight at r.t. in dark and then dialyzed (3.5K MWCO) against 10 mmol/L EDTA (pH 7.0) for 12 h and deionized water for another 12 h before lyophilization to give compound **16** as buff powder. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.10 (s, 1H), 7.76 (d, J = 7.3 Hz, 19H), 7.28 (d, J = 7.5 Hz, 20H), 4.92 (d, J = 57.6 Hz, 18H), 4.20 (s, 10H), 3.51 (s, 448H), 2.95 (s, 21H), 2.06 – 0.78 (m, 75H).

All synthesized products were characterized by ^{1}H NMR spectra or mass spectra as below:

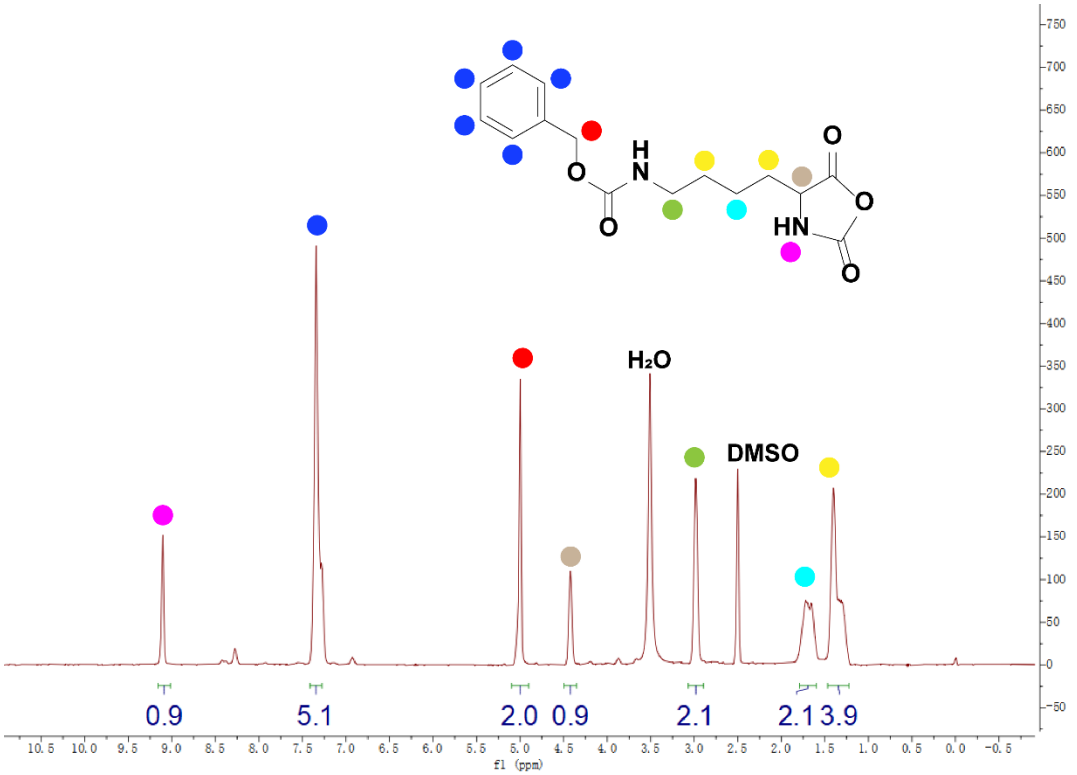


Figure S1 The ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm) spectrum of compound **2**.

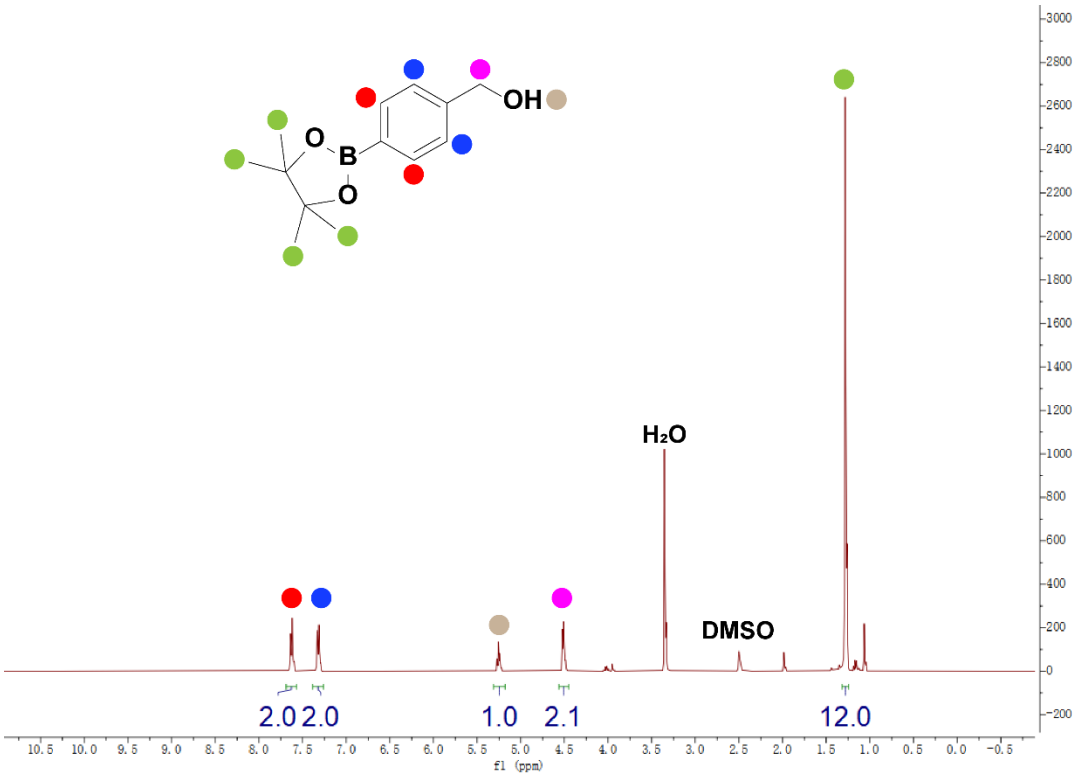


Figure S2 The ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm) spectrum of compound **4**.

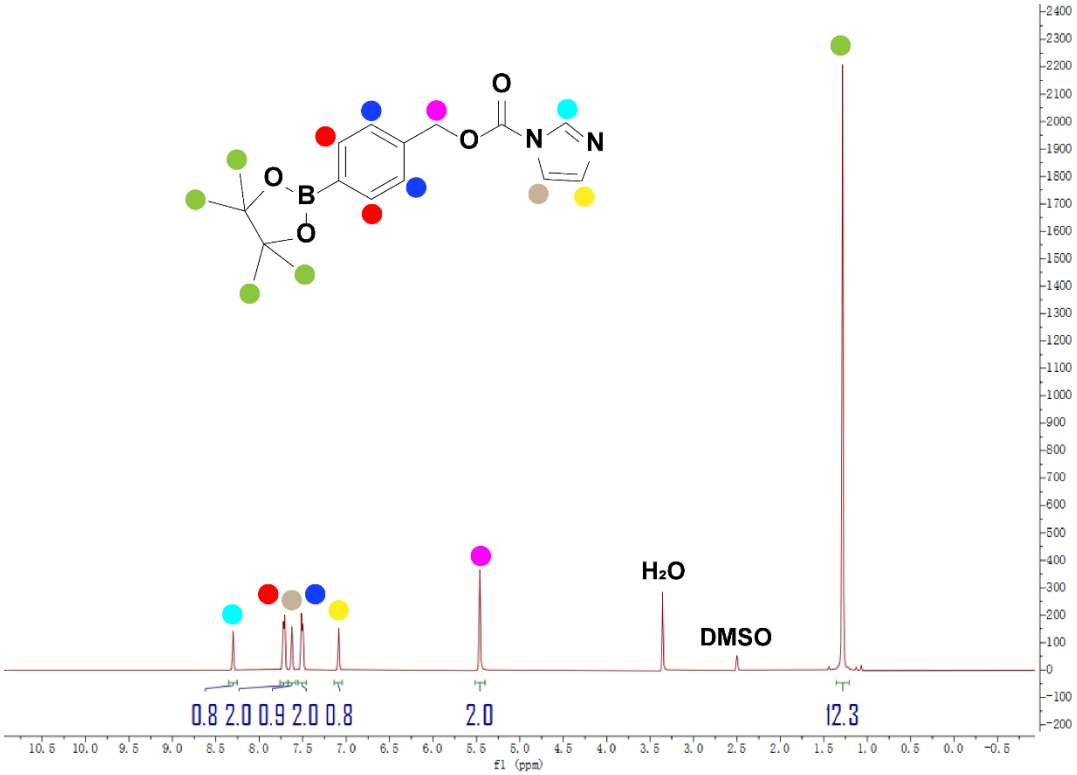


Figure S3 The ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm) spectrum of compound **5**.

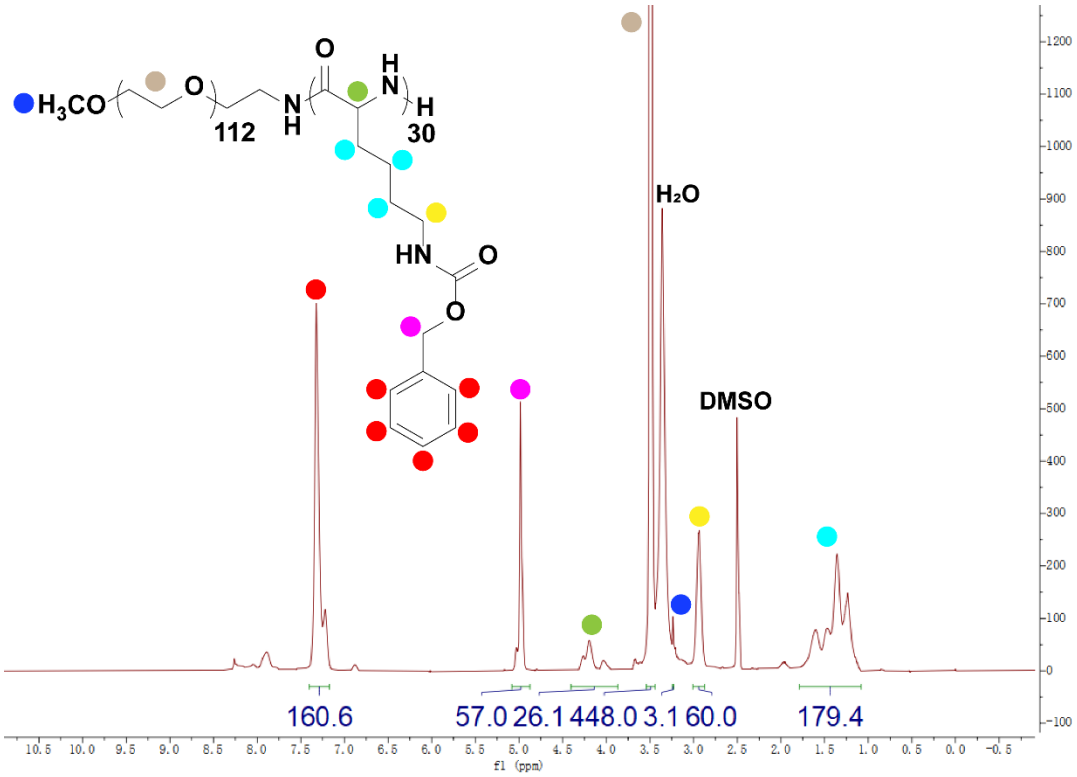


Figure S4 The ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm) spectrum of compound **8**.

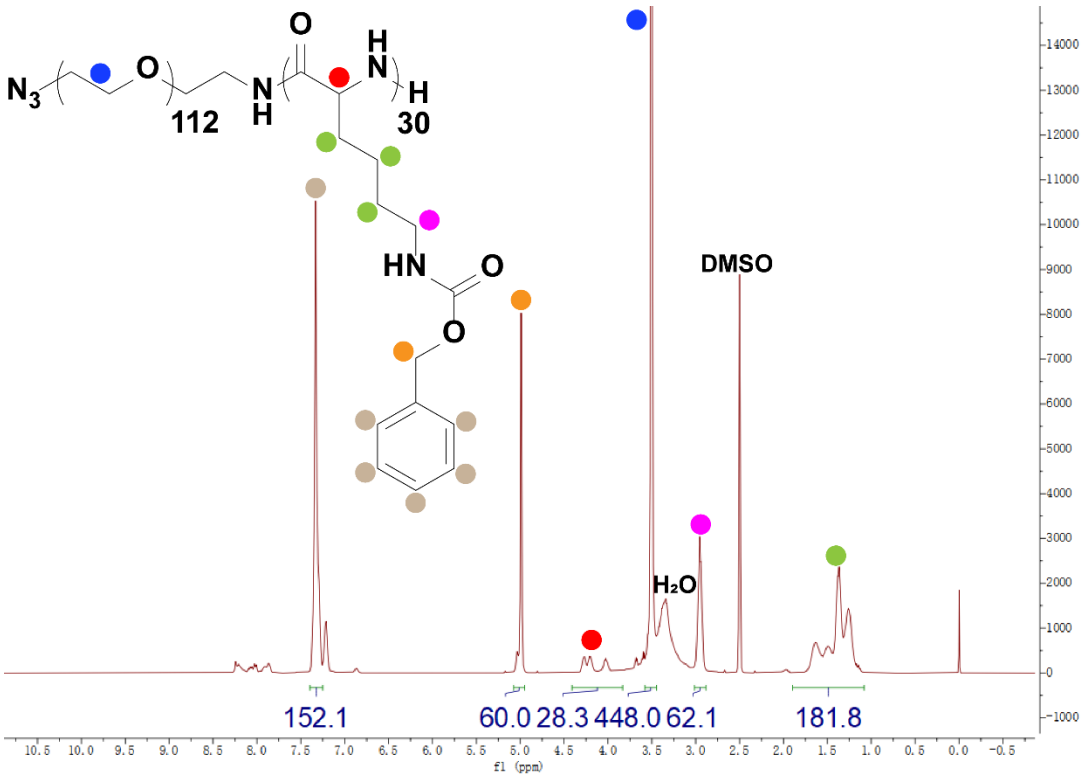


Figure S5 The ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm) spectrum of compound **9**.

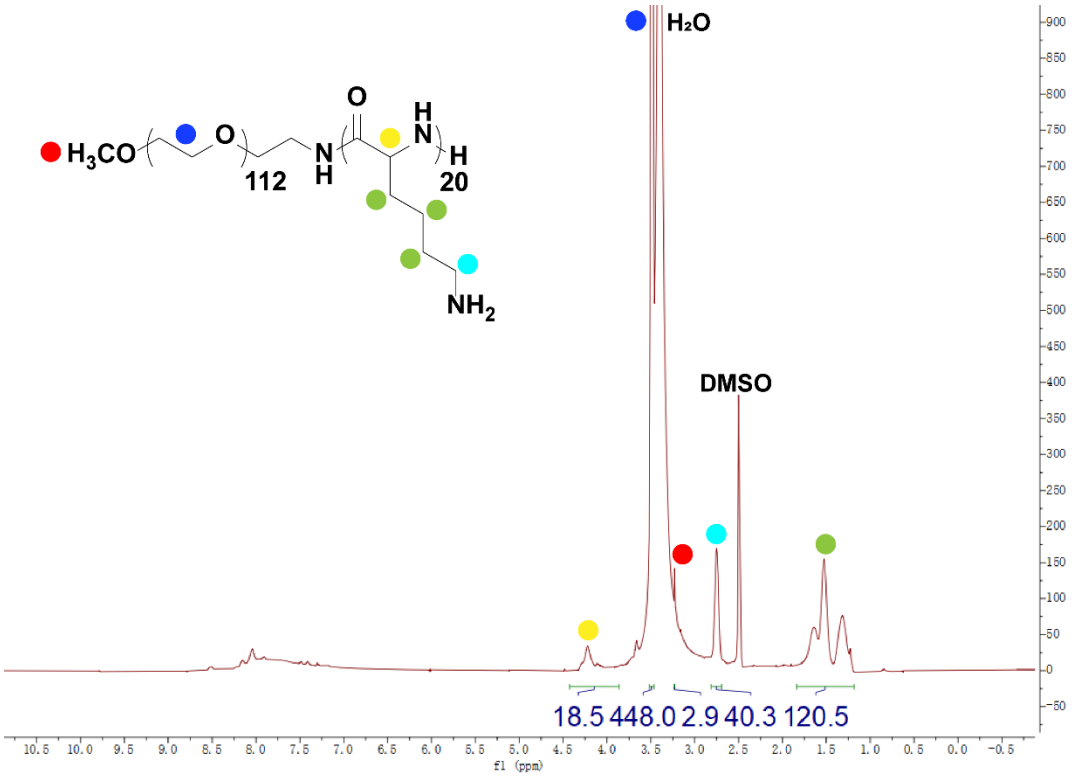


Figure S6 The ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm) spectrum of compound **10**.

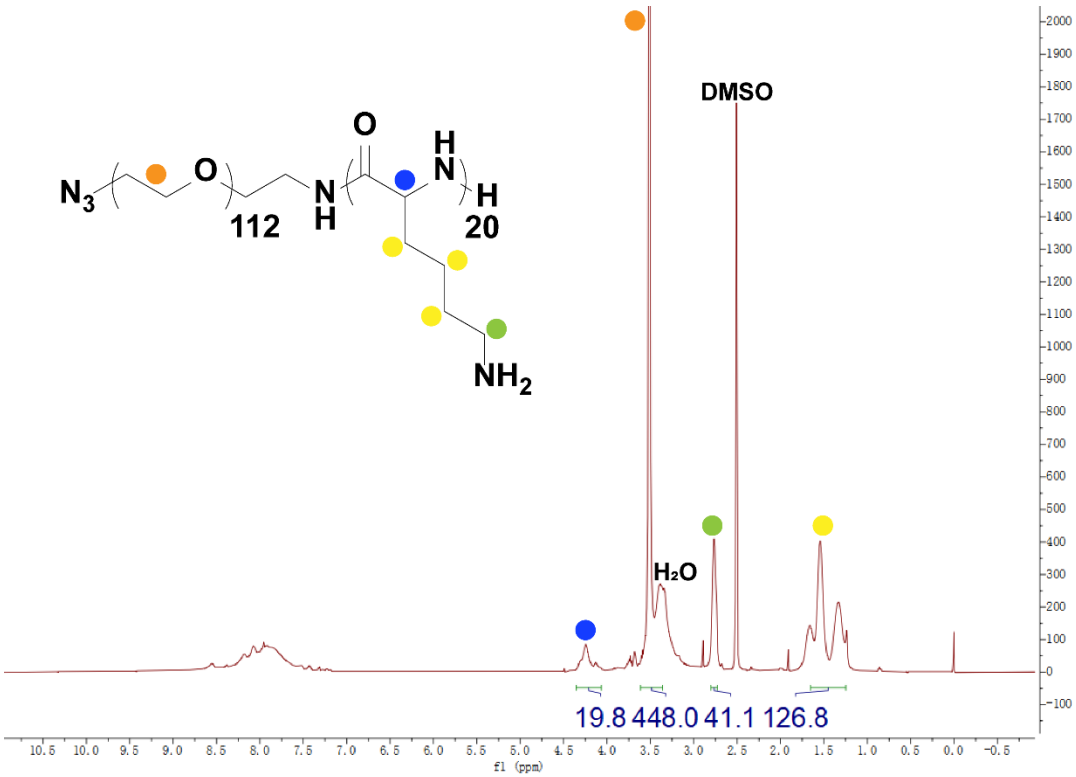


Figure S7 The ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm) spectrum of compound **11**.

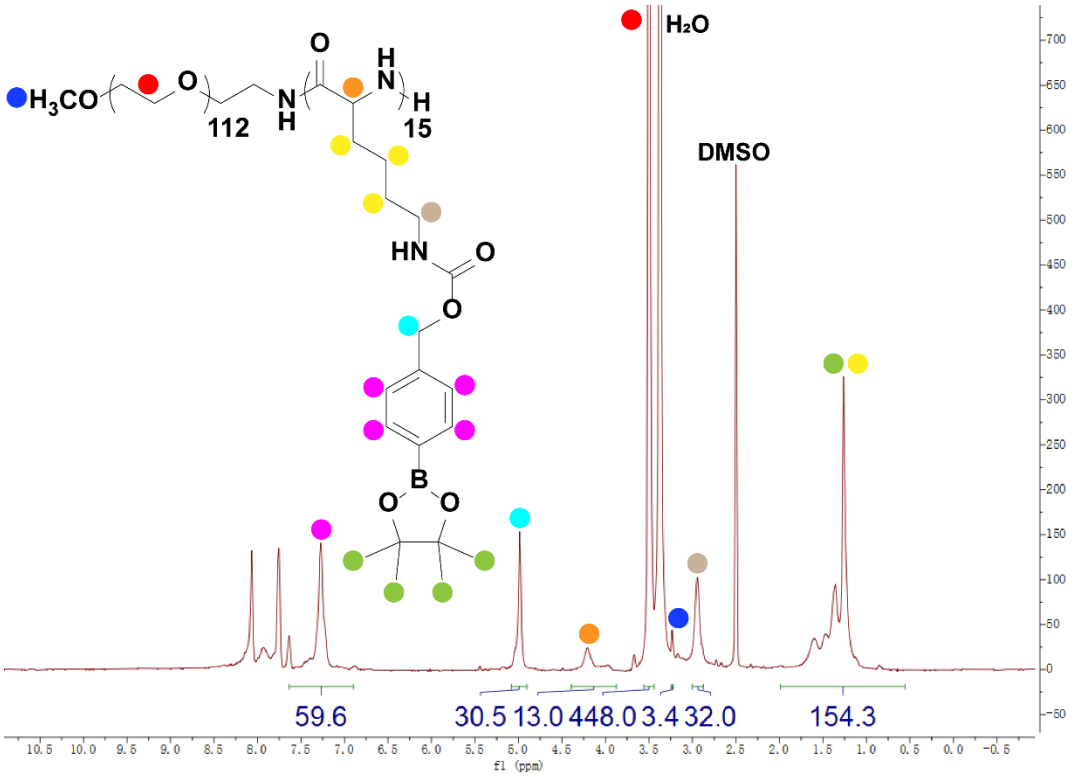


Figure S8 The ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm) spectrum of compound **12**.

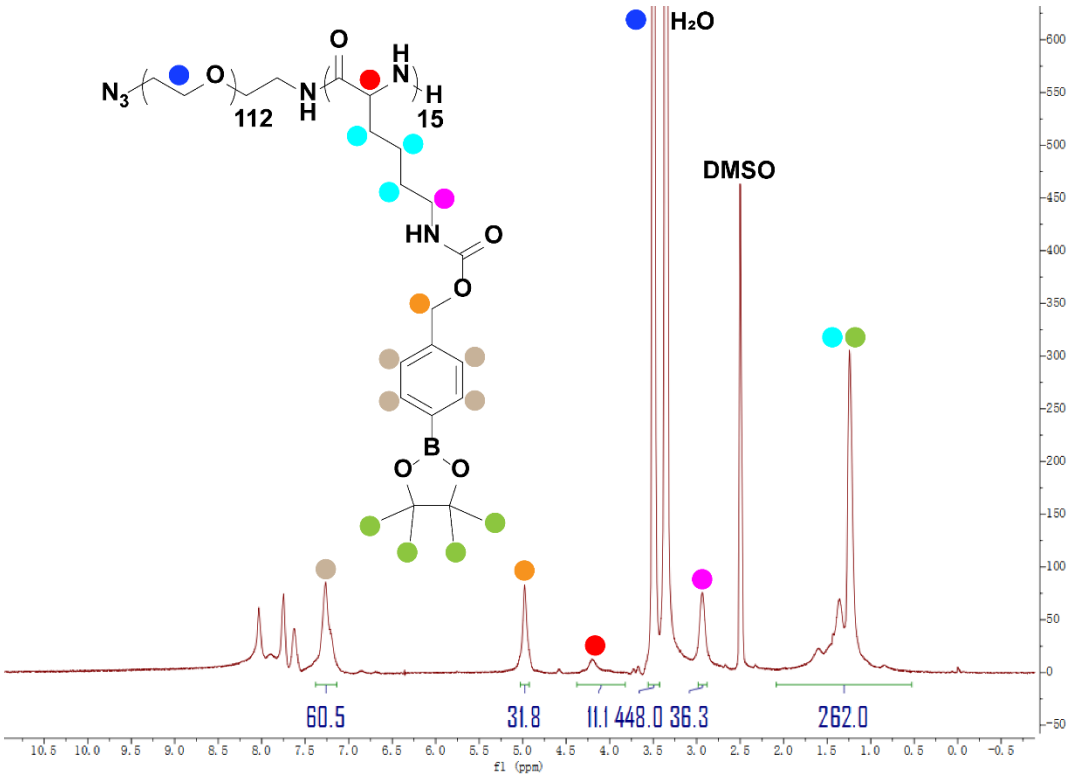
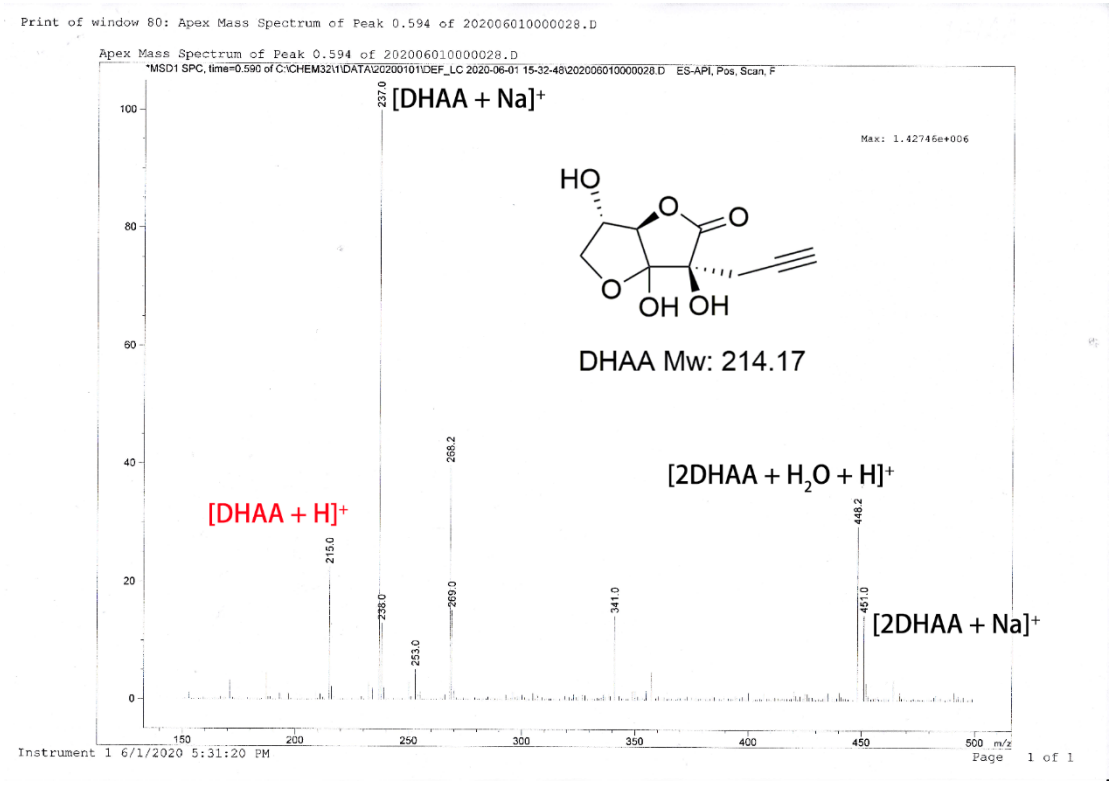


Figure S9 The ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm) spectrum of compound **13**.



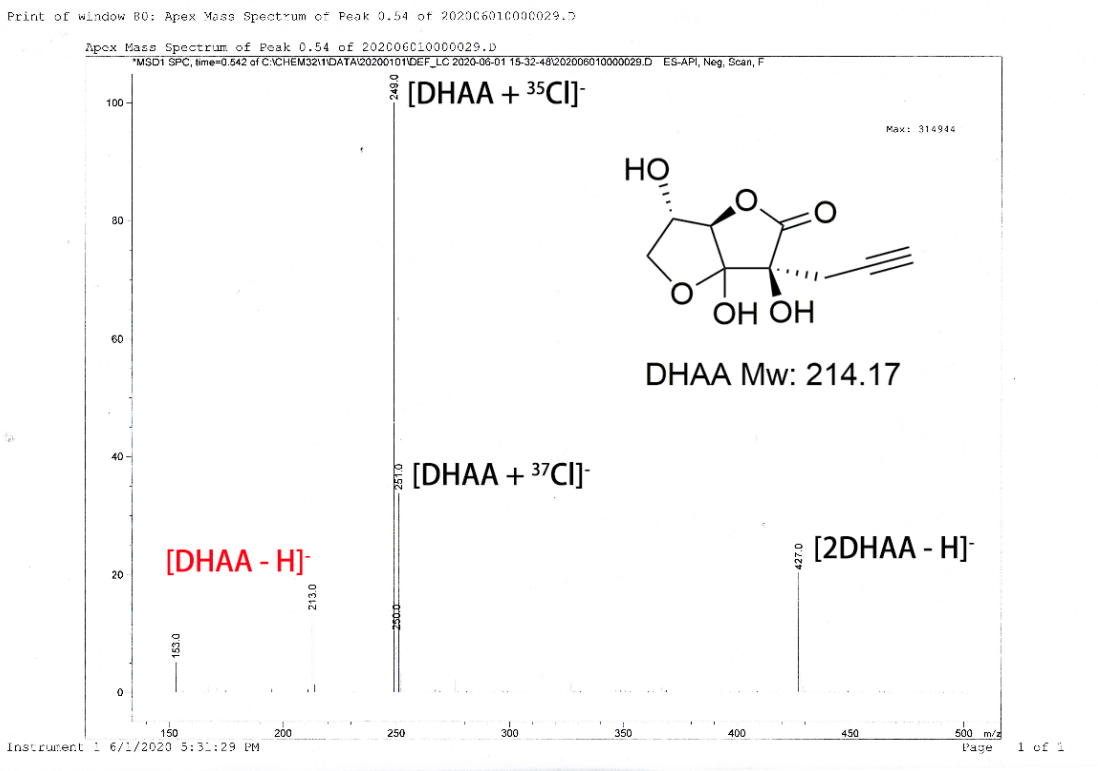


Figure S10 The MS-ESI spectrum of compound **15**.

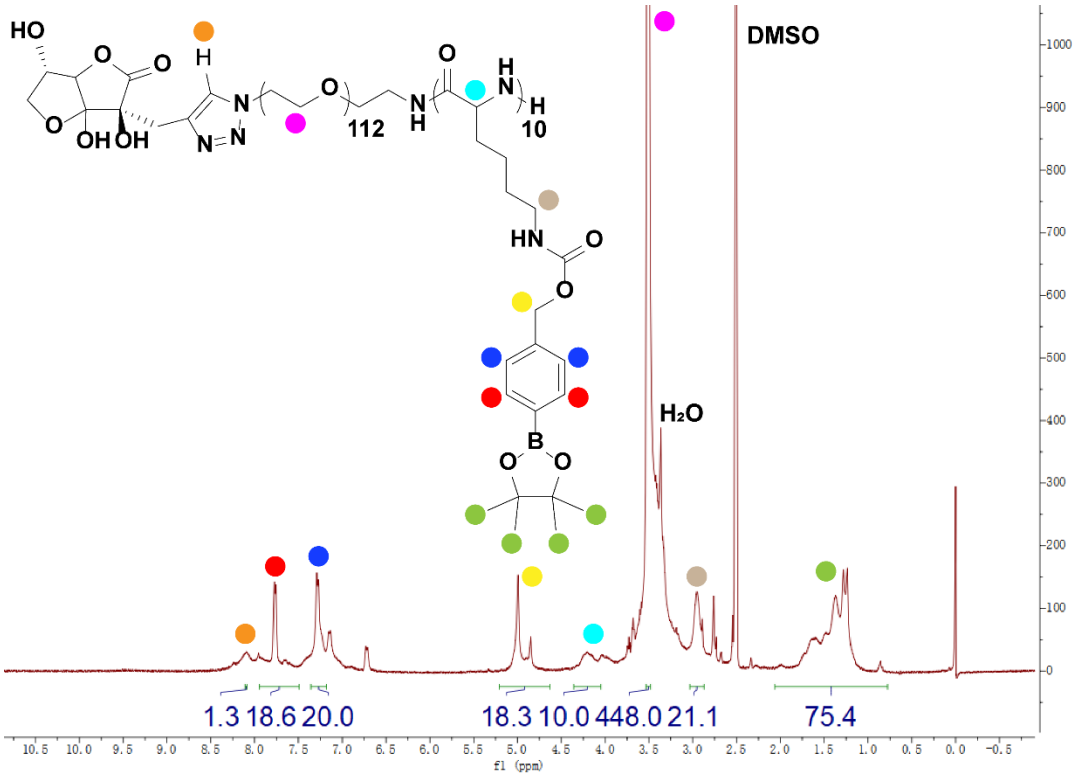
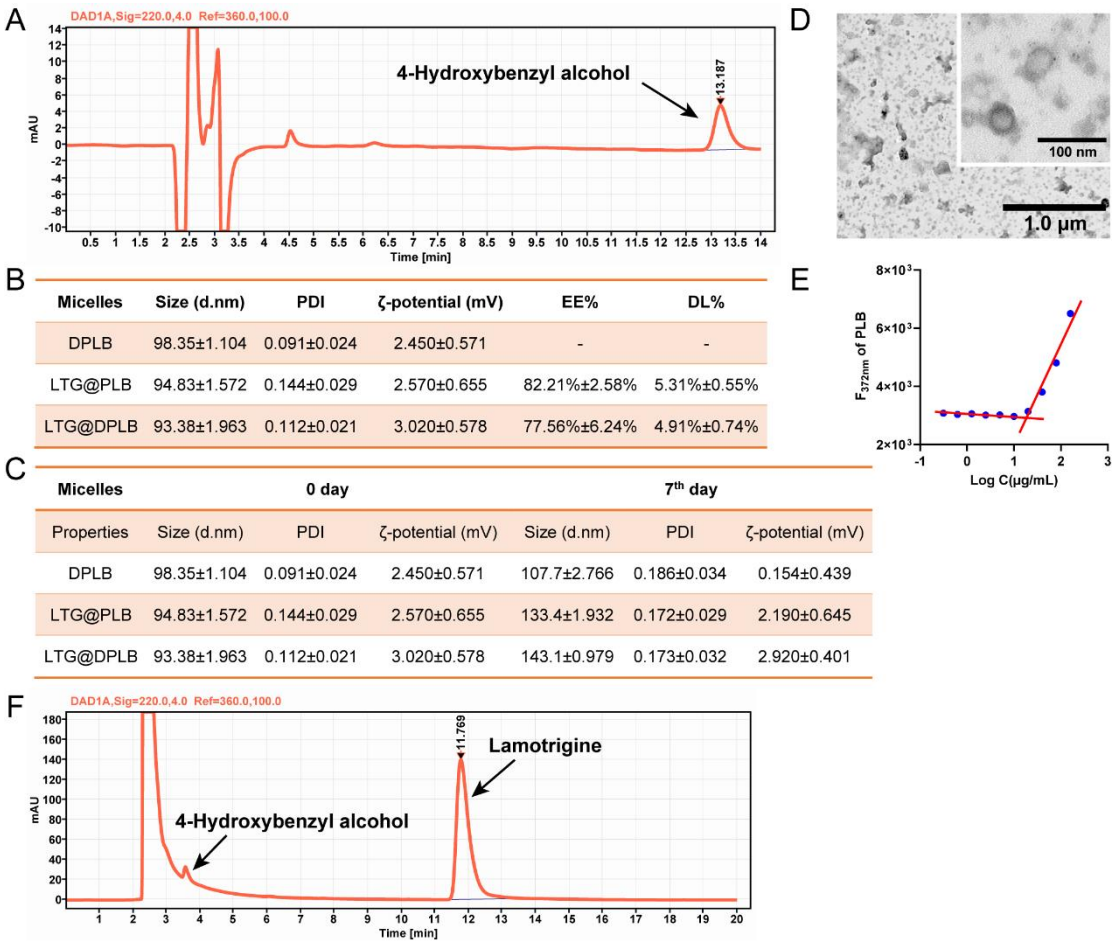


Figure S11 The ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm) spectrum of compound **16**.



B

Micelles	Size (d.nm)	PDI	ζ-potential (mV)	EE%	DL%
DPLB	98.35±1.104	0.091±0.024	2.450±0.571	-	-
LTG@PLB	94.83±1.572	0.144±0.029	2.570±0.655	82.21%±2.58%	5.31%±0.55%
LTG@DPLB	93.38±1.963	0.112±0.021	3.020±0.578	77.56%±6.24%	4.91%±0.74%

C

Micelles	0 day			7th day		
Properties	Size (d.nm)	PDI	ζ-potential (mV)	Size (d.nm)	PDI	ζ-potential (mV)
DPLB	98.35±1.104	0.091±0.024	2.450±0.571	107.7±2.766	0.186±0.034	0.154±0.439
LTG@PLB	94.83±1.572	0.144±0.029	2.570±0.655	133.4±1.932	0.172±0.029	2.190±0.645
LTG@DPLB	93.38±1.963	0.112±0.021	3.020±0.578	143.1±0.979	0.173±0.032	2.920±0.401

Figure S12 (A) HPLC result of degraded polymeric monomers after incubation with 100 μmol/L H_2O_2. The peak at the 13th minute directed at the reaction product, 4-hydroxybenzyl alcohol; (B) Particle size, PDI and ζ-potential of three kinds of micelles (also including EE% and DL% for LTG-loaded micelles); (C) Particle size, PDI and ζ-potential of three kinds of micelles after 7-day standing at r.t.; (D) Morphology of DPLB micelles measured by TEM (scale bar: 1.0 μm; inset scale bar: 100 nm); (E) Critical micelle concentration (CMC) measurement of PLB. The CMC value was determined as the point of intersection of two exponential lines shown in red lines; (F) HPLC result of degraded micelles after incubation with 100 μmol/L H_2O_2. The peak at the 12th minute directed at the model drug, lamotrigine.

3. Cellular uptake and protective effects of micelles

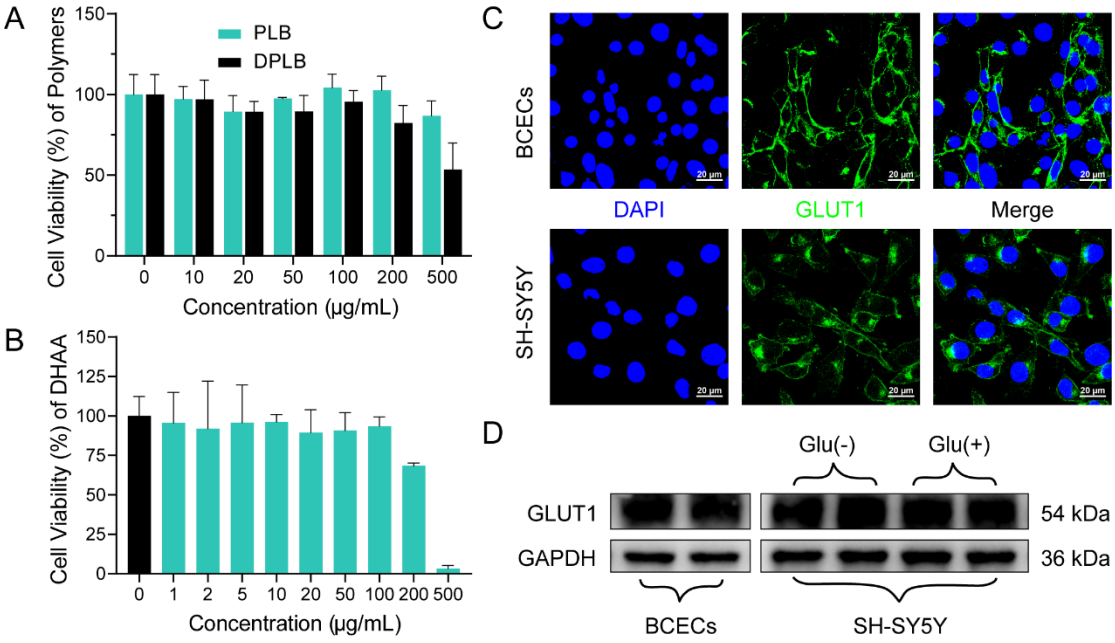


Figure S13 (A) *In vitro* study of the biocompatibility of PLB and DPLB. The cell *via*bility referred to the *via*bility of SH-SY5Y cells that were incubated with different concentrations of polymeric materials. Results are presented as mean ± SD (n = 3); (B) *In vitro* study of the biocompatibility of DHAA. The cell viability referred to the viability of SH-SY5Y cells that were incubated with different concentrations of DHAA materials. Results are presented as mean ± SD (n = 3). (C) Immunofluorescence staining of GLUT1 (a typical membrane protein) of BCECs and SH-SY5Y cells. (scale bar: 20 μm; blue signal: DAPI; green signal: GLUT1); (D) Measurement of GLUT1 of BCECs, SH-SY5Y (marked as Glu(–)) and glutamate-stimulated SH-SY5Y (marked as Glu(+)) cells by western blot (GAPDH served as the inner parameter).

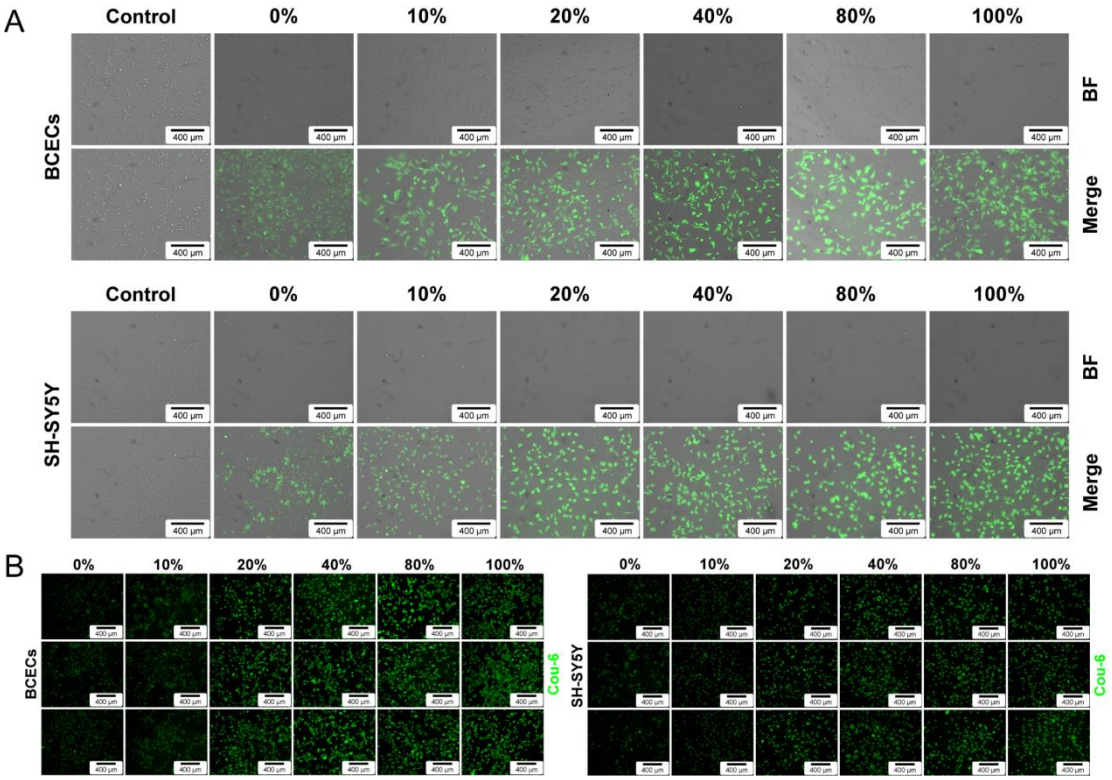


Figure S14 (A) Bright field and merged images of **Fig. 2B** as a supplement; (B) More fluorescent images of cellular uptake of Cou-6-loaded micelles with different DHAA-modification ratios in BCECs and SH-SY5Y cells.

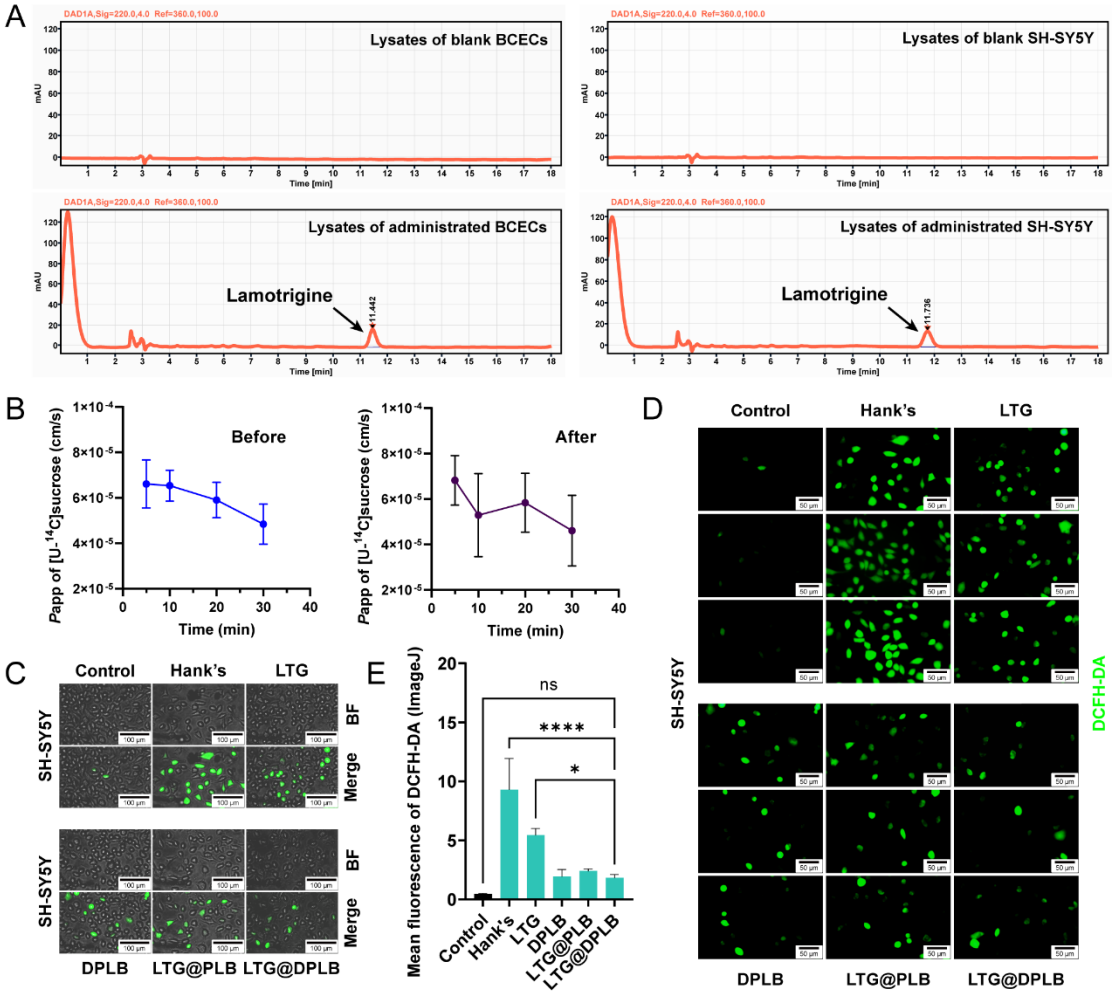


Figure S15 (A) HPLC results of cellular lysates after incubation with or without LTG-loaded micelles in BCECs and SH-SY5Y cells. The peak at the 12th minute directed at the model drug, lamotrigine; (B) The permeability of ^{14}C-sucrose before and after the experiment to verify the integrity of BBB model *in vitro*. Results are presented as mean ± SD (n = 3); (C) Bright field and merged images for **Fig. 3F** as a supplement; (D) More fluorescent images for ROS-clearance of micelles against 50 mmol/L glutamate-induced excitotoxic injury in SH-SY5Y cells; (E) Semi-quantification of ROS-staining fluorescent signals analyzed by ImageJ. Results are presented as mean ± SD (n = 3, *P < 0.05, ****P < 0.0001).

4. *In vivo* distribution and brain-targeted efficiency of micelles

Stage 1 of Racine's scale.gif

Stage 2 of Racine's scale.gif

Stage 3 of Racine's scale.gif

Stage 4 of Racine's scale.gif

Stage 5 of Racine's scale.gif

Figure S16 Different stages of acute seizures according to Racine's scale. Please refer to the animation as 5 GIF files in the appendix.

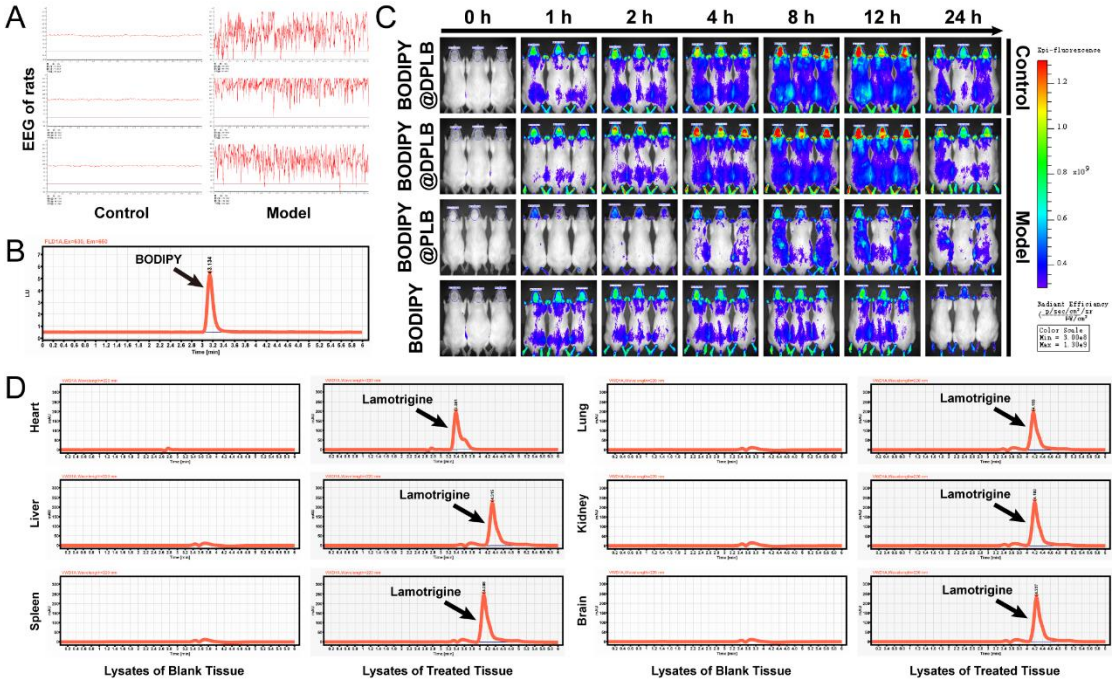


Figure S17 (A) EEG signals of epilepsy induced by lithium and pilocarpine in SD rats; (B) HPLC result of BODIPY-containing samples (1 μg/mL). The peak at the 3rd minute directed at the probe, BODIPY; (C) Detailed IVIS images of BODIPY-marked micelles' distribution in epileptic model rats for **Fig. 4A** as a supplement; (D) HPLC results of blank tissue lysates from model rats and LTG-treated tissue lysates. The peak at the 4th minute directed at the model drug, lamotrigine.

5. *In vivo* anti-epileptic efficacy of micelles

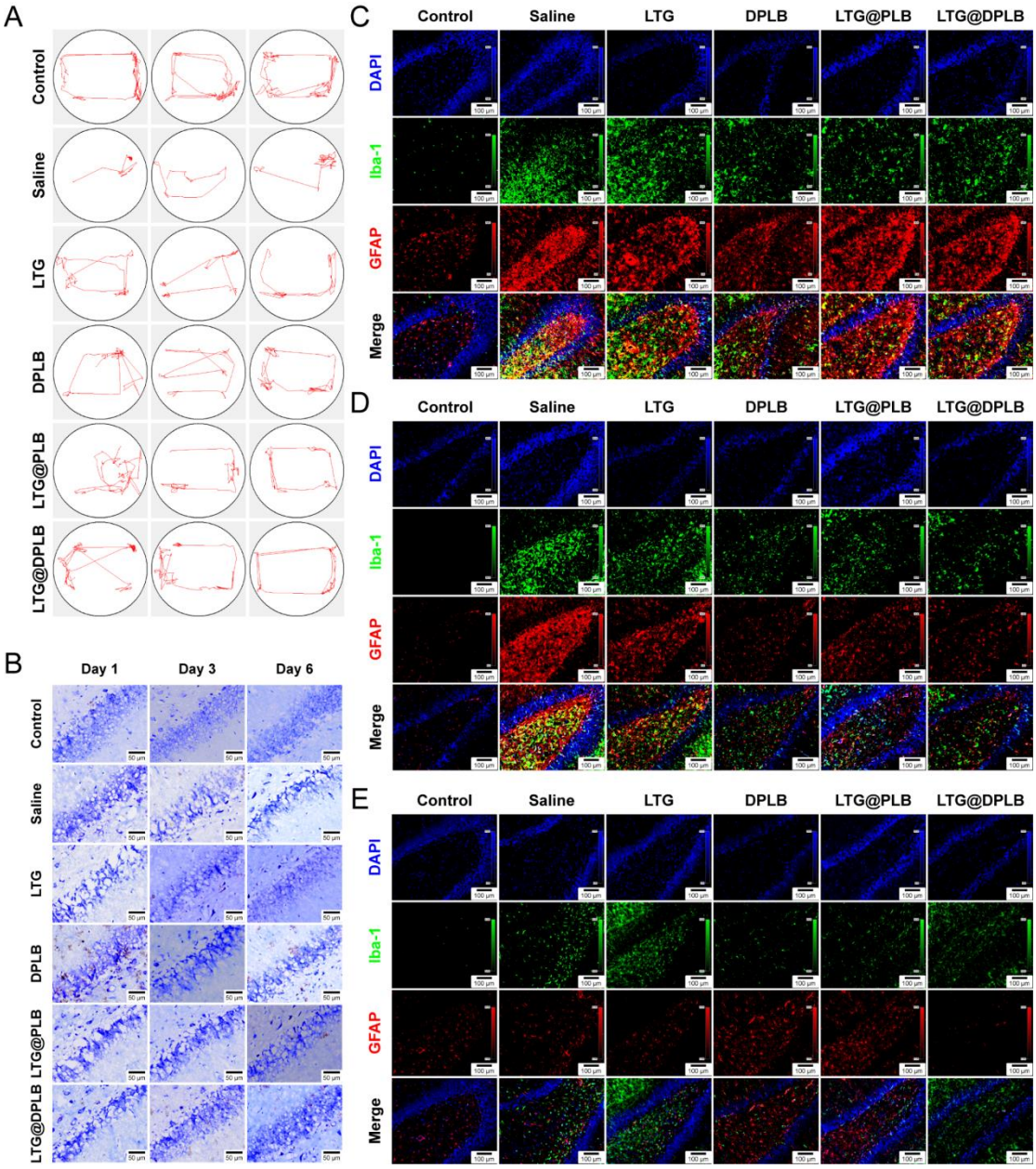


Figure S18 (A) More results of open-field tests for **Fig. 5C** as a supplement; (B) Nissl's staining images of hippocampus CA3 sections (20×) after administration of Days 1, 3 and 6 as a supplement to **Fig. 5E**; (C) Immunofluorescence staining images of hippocampus CA3 sections (10×) after administration of Day 1; (D) Immunofluorescence staining images of CA3 hippocampus sections (10×) after administration of Day 3; (E) Immunofluorescence staining images of hippocampus CA3 sections (10×) after administration of Day 6.

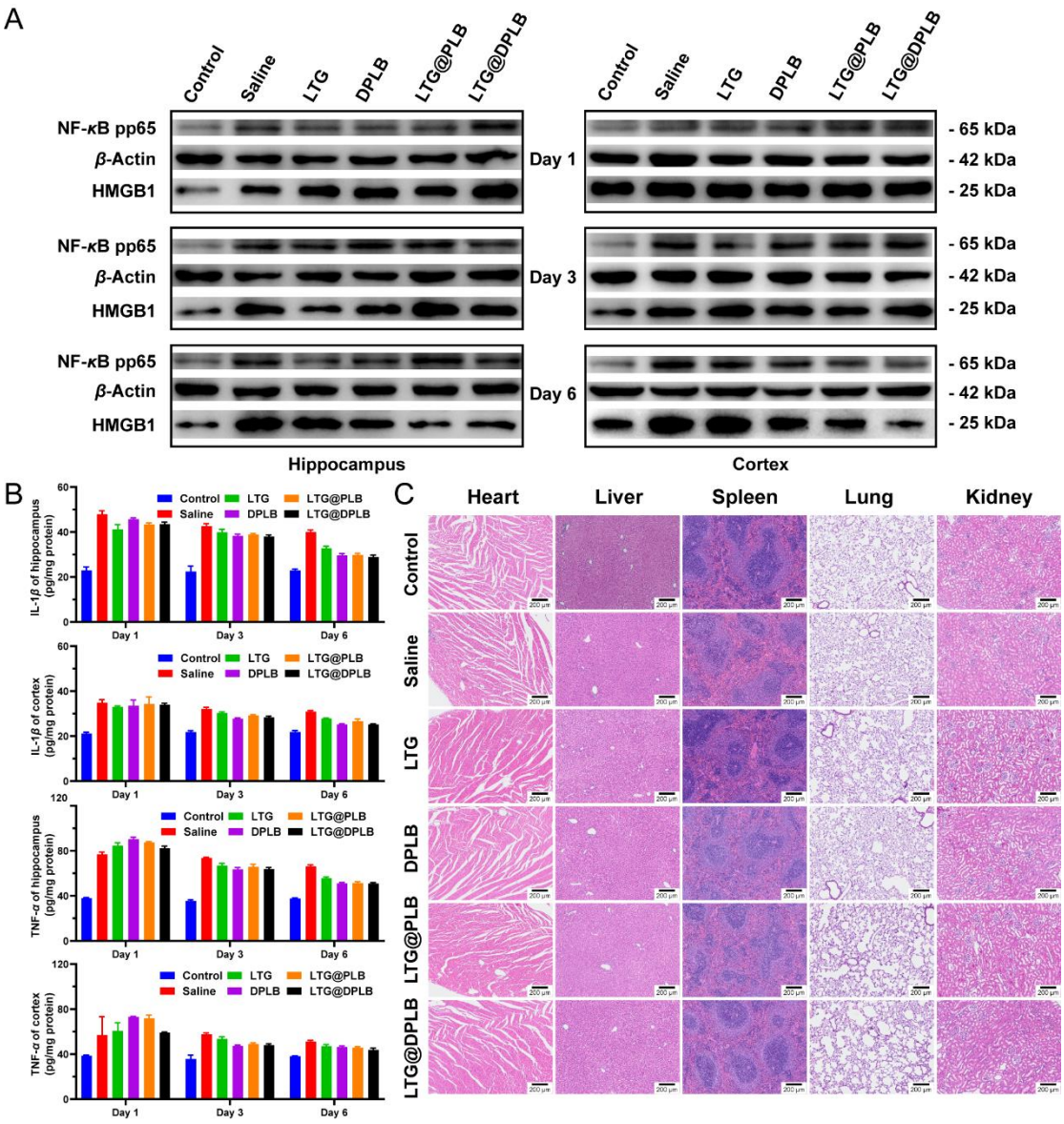


Figure S19 (A) Measurement of inflammatory cytokines from hippocampus and cortex lysates of model rats by Western blot after administration of Days 1, 3 and 6; (B) Measurement of inflammatory cytokines from hippocampus and cortex lysates of model rats by ELISA after administration of Days 1, 3 and 6; (C) Immunohistological staining images of the major organs from model rats after administration of Day 10.