Supporting Information

Doxorubicin delivered via ApoE-directed reduction-sensitive polymersomes potently inhibit orthotopic human glioblastoma xenografts in nude mice

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Materials

Trimethylene carbonate (TMC) was recrystallized over dry toluene. ApoE (ApoE, 98%, China Peptides Company, Ltd.), glutathione (GSH, 99%, Roche), and doxorubicin hydrochloride (DOX·HCl, 99%, Beijing Zhongshuo Pharmaceutical Technology Development) were used as received. Dithiolane trimethylene carbonate (DTC) was synthesized as our previous report.[1]

Preparation and characterization of ApoE-PS

PEG-P(TMC-DTC) and ApoE-PEG-P(TMC-DTC) diblock copolymers were synthesized as our previous report.[1-2] ApoE-PS was prepared from PEG-P(TMC-DTC) and ApoE-PEG-P(TMC-DTC) via the solvent exchange method. Typically, 100 μL of DMF solution of PEG-P(TMC-DTC) and ApoE-PEG-P(TMC-DTC) (10.0 mg/mL) was added to 900 μL of citrate buffer (10 mM, pH 4), and the mixed solution was placed at 37 ℃ for 1 hour. After adjusting the pH to 7.8 using Na_2HPO_4 (2M), a predetermined amount of DOX·HCl was added, and the solution was stirred for 15 min and stood at 37℃ for overnight, followed by dialysis (Spectra/Pore, MWCO 7000) against PB for 6 h. The resulting formulations were denoted as ApoEx-PS-DOX, wherein x represents the molar percentage of ApoE-PEG-P(TMC-DTC). The stability of ApoE-PS-DOX against extensive dilution or 10% FBS and reduction-sensitivity against 10 mM GSH were investigated by tracking the size changes using DLS. Transmission electron microscopy (TEM) was carried out using a Tecnai G220 TEM operated at an accelerating voltage of 200 kV. The samples were prepared by dropping 20 μL of 0.5 mg/mL polymersomes suspension on the copper grid followed by staining with 1.0 wt.% phosphotungstic acid.

Cell viability assay

The cytotoxicity of ApoE-PS and ApoE-PS-DOX was evaluated using MTT assays in LRP1, LRP2, and LDLR overexpressing U-87 MG glioma cancer cells. The U-87 MG cells

(5×10^3/well) were seeded in a 96-well plate for 24 h. DOX·HCl, Lipo-DOX, PS-DOX, and ApoE-PS-DOX with different ApoE contents (10 ~ 30 mol.%) in 10 μL of PBS were added, and the DOX concentrations ranged from 0.01 μg/mL to 20 μg/mL. The cells following 4 h treatment were incubated in fresh media for another 44 h. Then, 10 μL of MTT was added to each well. The plates were incubated at 37°C for 4 h prior to reading OD492 nm on a microplate reader (Varioskan, Thermo, USA). The cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assays. The cytotoxicity of blank ApoE-PS was assessed in U-87 MG cells following 48 h incubation at a concentration of 0.1-0.5 mg/mL.

Endocytosis analysis

U-87 MG cells were seeded in a 6-well plate (5-10 × 10^5 cells/well) overnight, followed by the treatment with ApoE-PS-DOX, DOX·HCl, or Lipo-DOX in 1.0 mL of PBS (DOX·HCl concentration: 10 μg/mL) at 37 °C for 4 h. Then, the cells were digested by 0.25% (w/v) trypsin and 0.03% (w/v) EDTA, and the fluorescence histograms were immediately recorded with a BD FACS Calibur flow cytometer (Becton Dickinson, USA) and analyzed using Cell Quest software based on 10,000 gated events.

For CLSM observation, U-87 MG cells were seeded in 24-well plate at a density of 5 × 10^4 cells/well and cultured with DMEM medium under 5 % CO_2 atmosphere at 37 °C overnight. Then the cells were incubated with ApoE20-PS-DOX or PS-DOX (DOX·HCl concentration: 10 μg/mL) at 37 °C for 4 h. The cells were further cultured in fresh medium for 4 h and fixed with 4% paraformaldehyde solution for 15 min. The cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) for 10 min. The fluorescence images were obtained using a confocal microscope (TCS SP5).

Western blot assay

Approximately 1.0-1.5×10^5 U-87 MG cells per well were plated in six-well plates, and treated with Lipo-DOX, PS-DOX, or ApoE-PS-DOX at a DOX·HCl concentration of 5.0 μg/mL for 4 h. The media were removed and replaced by fresh media. The cells were incubated for another 44 h and then harvested and lysed with RIPA buffer (Beyotime Biotechnology, Nantong, China) supplemented with protease and phosphatase inhibitors (Beyotime Biotechnology). BCA Protein Assay kit (Beyotime Biotechnology) was used to determine the concentrations of the collected proteins. Protein (30-50 μg) was separated by sodium dodecyl sulfonate (SDS)-polyacrylamide gel electrophoresis using 12% gel and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% BSA for 1 h at room temperature and probed with specific antibodies at 4 °C overnight. After washing with Tris-buffered saline/0.1% Tween-20 (TBST), the membranes were incubated with secondary antibodies, and the expression of protein was detected by electrochemiluminescence (ECL) assay (Millipore, Billerica, MA, USA).

In vivo pharmacokinetics and biodistribution

For pharmacokinetic studies, 150 μL of ApoE-PS-DOX, PS-DOX or Lipo-DOX (10 mg DOX·HCl equiv./kg) was intravenously injected via the tail vein (n = 3). At different time points, about 20 μL collected blood was dissolved in 100 μL Triton X-100 (1%) with brief sonification. DOX was extracted by using a DMF solution containing 20 mM dithiothreitol (DTT) overnight at 25 ℃. After centrifugation at 10,000 rpm for 20 min, DOX concentration in the supernatant was determined by using fluorescence measurement (λex = 480 nm, λem = 560 nm). The blood circulation half-lives ($t_{1/2,\alpha}$ and $t_{1/2,\beta}$) were obtained by second-order exponential decay fits.

For in vivo biodistribution study, orthotopic U87 tumor-bearing nude mice were randomly

grouped (n = 3), and 150 μL of ApoE-PS-DOX, PS-DOX or Lipo-DOX in PB were i.v. injected via tail veins at a DOX concentration of 10 mg equiv./kg. The mice were sacrificed at 8 h post injection. The major organs or brain tumors were collected, washed with PBS, wiped with tissues, and quickly homogenized. DMF solution that containing 20 mM DTT was used to extract DOX overnight at 25°C. Following centrifugation at 10,000 rpm for 30 min, DOX concentration in the supernatant was determined by fluorescence measurement (n = 3).

Statistical analysis

Data were presented as mean ± standard deviation. Differences between groups were assessed using one-way analysis of variance. Statistical significance was defined as $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

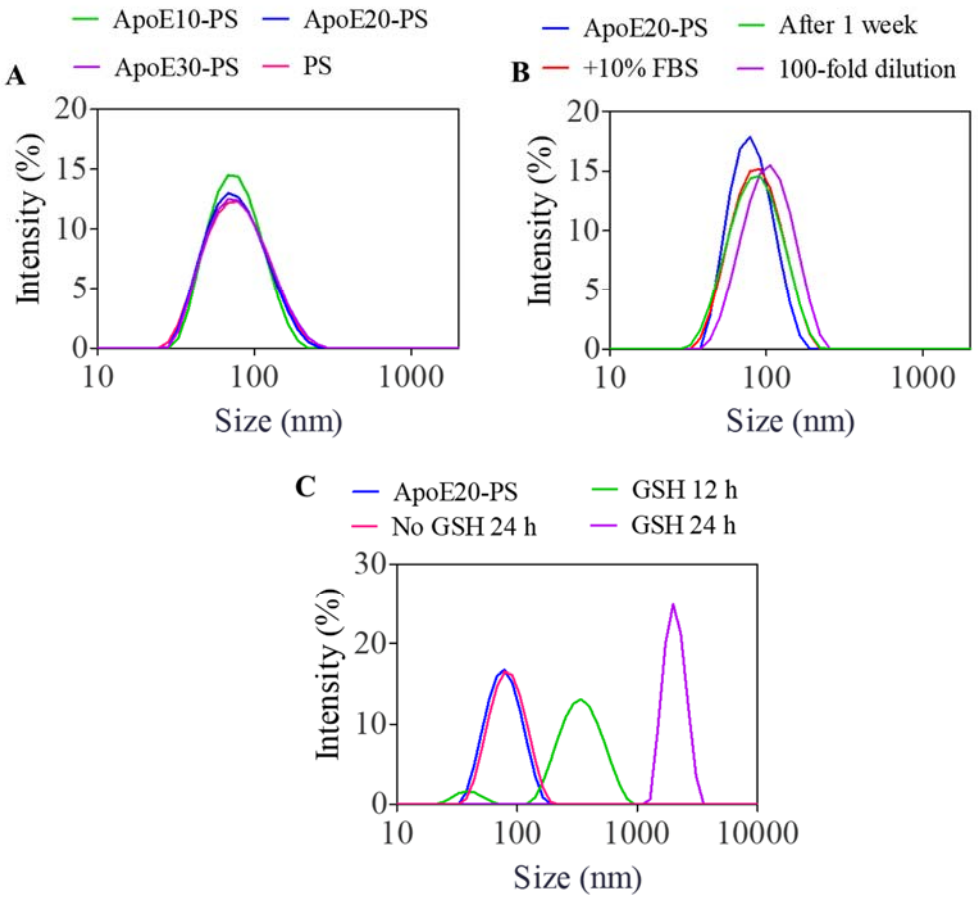

Figure S1 Characterizations of PS and ApoE-PS. (A) Size distribution determined by DLS. (B) Stability of ApoE20-PS under different conditions (1 week in PB, 10% FBS, 100-fold dilution). (C) Responsiveness of ApoE20-PS under GSH conditions.

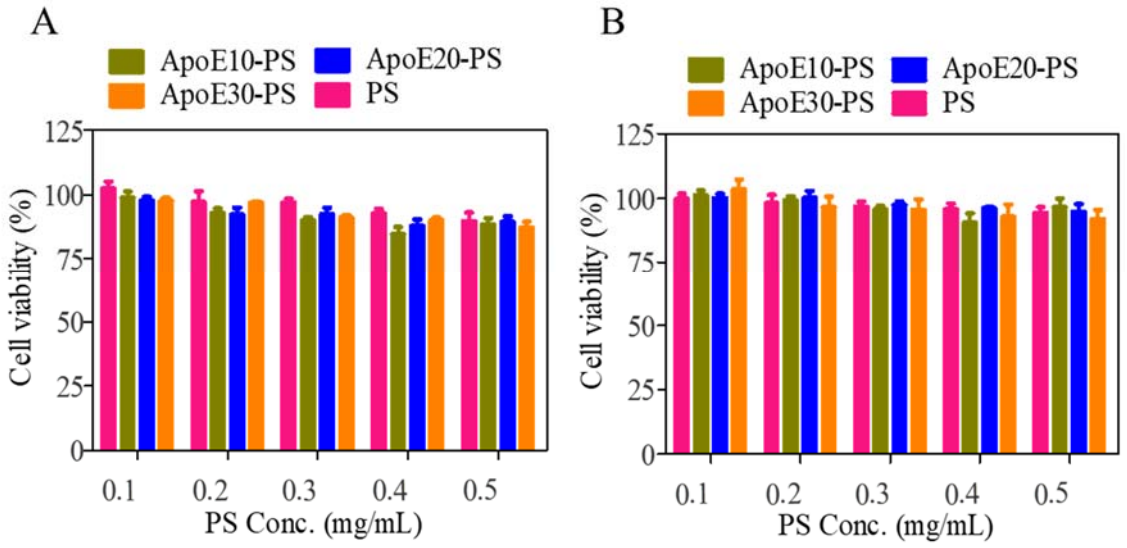

Figure S2 MTT assays of blank ApoE-PS and PS in U-87 MG cells (A) and healthy astrocyte (B).

References

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[2] Jiang Y, Zhang J, Meng F, Zhong Z. Apolipoprotein E peptide-directed chimeric polymersomes mediate an ultrahigh-efficiency targeted protein therapy for glioblastoma. *ACS Nano*. 2018;12(11):11070-11079.