Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.
This journal is © The Royal Society of Chemistry 2023

Supporting Information

Dual-modality probe nanodrug delivery system with ROS-sensitive for atherosclerosis diagnosis and therapy

1. Experimental

1.1. Materials

Polyethylene glycol monomethyl ether 1000 ($mPEG_{1000}$, 99%) was purchased from Macklin Chemistry Co., Ltd. (Shanghai, China). Glycidyl methacrylate (GMA, 96%), cuprous (I) chloride (CuCl, 98%), oxalyl chloride (OC, 98%), simvastatin (Sim, 98%), paraformaldehyde (PFH, 98%), anhydrous citric acid (99.5%), N- (β-aminoethyl-γ-aminopropyl) methyldimethoxysilane (AEAPMS, 99%), Fe_3O_4@SiO_2 aqueous solution, (3-aminopropyl) triethoxysilane (APTES, 98%), ethyl orthosilicate (98%), 4-dimethylaminopyridine (DMAP, 98%), N, N'-dicyclohexylcarboximide (DCC, 99%), N-hydroxysuccinimide (NHS, 98%) and propidium iodide (PI) were purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China). 2,2- bipyridyl (99%), ammonia water (28%), ethylenediamine (EDA, 98%), triethylamine (TEA, 99%), N, N-dimethylformamide (DMF, 99.9%), dichloromethane (DCM, 99.9%), hydrogen peroxide (30%) and dimethyl sulfoxide (DMSO, 99%) were ordered from Beijing Chemical Co. (Beijing, China). Ethyl 2-bromoisobutyrate (99%) were obtained from J&K Scientific Ltd. (Shanghai, China). $ApoE^{-/-}$ mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (8-10 weeks old, 20 ± 5 g). New Zealand white female rabbits purchased from Liaoning Changsheng Biotechnology Co., Ltd. (2-2.5 kg). All the animal experimental protocols were performed with approval from the Animal Ethical and Experimental Committee of Jilin University (SYXK (JI) 2014-0013).

1.2. The Synthesis of Bimodal Probe Fe_3O_4@SiO_2-CDs (FC) Nanoparticles

The CDs were prepared by a simple one-step hydrothermal method[27] and FC was prepared using the improved Stöber method[28]. 0.5 g citric acid, 10 mL AEAPMS and 10 mL deionized water were sealed in a polytetrafluoroethylene stainless steel autoclave, and placed in a drying oven for hydrothermal treatment at 180 °C for 12 h. After the reaction, the light brown crude product was extracted and purified with ethyl acetate and petroleum ether for several times, and the pure CDs was obtained by rotary evaporation. 100 mg CDs was added to a mixed solution with 20 mL anhydrous ethanol and 200 μL APTES, and 0.5 mL Fe_3O_4@SiO_2 (5 mg/mL) nanoparticles and 1 mL ethyl orthosilicate ester were added to a mixed solution with 20 mL deionized water, 80 mL anhydrous ethanol, and 1 mL ammonia water, and add. The above two solutions were mixed uniformly Fe_3O_4@SiO_2 under mechanical stirring and reacted in the dark for 6 h. Then, the crude product was separated by a strong magnetic field, and washed with absolute ethanol and deionized water for several times to harvest pure FC after vacuum-dried for 48 h. Fe_3O_4@SiO_2. The synthesized FC nanoparticles were characterized by FT-IR, fluorescence and magnetic properties to verify whether the nanoparticles were synthesized successfully.

1.3. The Synthesis of Amphiphilic Polymers PGMA-PEG (PP)

PGMA was synthesized via atom transfer radical polymerization (ATRP) as previously described[29, 30]. Briefly, 6 mL DMF, 6 mL GMA and 0.1 mL ethyl 2-bromoisobutyrate were added after 0.03 g 2,2-bipyridyl and 0.03 g CuCl were successfully complexed in anhydrous and oxygen-free atmosphere, and the reaction was carried out at 50 °C for 4 h. Subsequently, the reaction was terminated in an ice bath, and the catalyst CuCl was removed by passing the solution through the

Al_2O_3 neutral column after the reaction solution was dissolved in DCM. The crude product was precipitated in methanol after rotary evaporation to remove most of the DCM, and the precipitate was dehydrated in a vacuum desiccator to obtain 4.5 g PGMA.

10 g $mPEG_{1000}$ was dissolved in 20 mL anhydrous DCM, and it was slowly dropped into 5 mL anhydrous DCM containing 5.02 g oxalyl chloride under N_2 and ice bath conditions, and then 0.2 mL EDA was added to react for 2 h. After the reaction was completed, the solvent and excess oxalyl chloride were removed by rotary evaporation, and 11.55 g OC-PEG was obtained by vacuum drying.

To obtain PP, the epoxy group of PGMA was first opened[30]. 80 mL EDA was dropped into 60 mL DMSO that containing 4 g PGMA, and the reaction was carried out at 80 °C for 4 h. After that, 5.277 g white solid product PGMA-EDA was obtained via dialysis and lyophilization. An amidation reaction was then carried out to introduce PEG. Under the protection of N_2, 30 mL DMSO containing 1.839 g OC-PEG was slowly dropped into 50 mL DMSO containing 2.5 g PGMA-EDA, followed by 0.3 mL triethylamine, and the reaction was carried out at room temperature for 12 h. After that, 3.503 g PP was obtained by dialysis and lyophilization.

1.4. The Synthesis of PGMA-PEG-Sim (PPS)

The synthesis steps of OC-Sim are the same as the synthesis process of OC-PEG. Under the protection of N_2, 20 mL DMSO containing 0.54 g OC-Sim was slowly dropped into 50 mL DMSO containing 1.0 g PP, then 0.3 mL EDA was added dropwise, and the reaction was carried out at room temperature for 24 h. After the reaction was completed, 1.32 g white solid PPS was obtained by dialysis and lyophilization.

1.5. The Synthesis of PGMA-PEG-ISO-1-Sim (PPIS)

The target molecule tautomerase activity inhibitor ISO-1 was synthesized by the laboratory according to the method of reference[31].

PGMA-PEG-ISO-1 (PPI) was synthesized first. 0.35 g ISO-1, 0.05 g EDC and 0.05 g NHS were dissolved in a mixed solution of 10 mL deionized water and 5 mL DMSO and activated for 0.5 h, which were then dropped into 50 mL deionized water containing 1.5 g PGMA-EDA-OC-PEG, and the reaction was carried out at room temperature for 24 h. After that, the 1.73 g white solid PPI were obtained by dialysis and lyophilization.

1.0 g PPI was dissolved into 50 mL DMSO, and 20 mL DMSO that containing 0.86 g OC-Sim was slowly dropped into it under the N_2 protection, then 0.3 mL EDA was added to react at room temperature for 24 h. After the reaction was completed, 1.53 g white solid PPIS was obtained by dialysis and lyophilization.

1.6. The Synthesis of PPS@FC and PPIS@FC

8 mg PPS or PPIS and 2 mg FC were dissolved in 5 mL DMSO, which was dropped into 30 mL deionized water under sonication, and continued to sonicate for 1h. DMSO was removed by dialysis using a dialysis bag (MWCO 2000) for 24 h to obtain FC-coated micelles PPS@FC or PPIS@FC.

1.7. Characterization of PPS@FC and PPIS@FC

The size and morphology of PPS@FC and PPIS@FC were observed by dynamic light scattering (DLS, ZetaPlus, Brookhaven Co., Ltd., USA) and transmission electron microscopy (TEM, JEM-2100F, JEOL Ltd., Japan), and their stability was judged by the change of micelle size with time. To evaluate the hydrogen peroxide sensitivity of the delivery system, the fluorescence intensity

of the probes within the micelles was measured using a fluorescence spectrophotometer (PE, LS-55, PerkinElmer Ltd., US). The saturation magnetization of PPS@FC and PPIS@FC was measured using a vibrating sample magnetometer (VSM, Lake Shore 7404, Lake Shore Ltd., US), and the MRI detection capability of the nanomicelle probe was determined by measuring the hysteresis of the micelles. The chemical structure of the FC, OC-Sim, ISO-1 and polymers were determined by ^1H NMR spectrum (Bruker, Germany) on a spectrometer operating at 300 MHz and fourier transform infrared spectrometer (FT-IR, Nicolet iS10, Thermo Scientific Co. Ltd., USA). The molecular weight of PPS was determined using GPC (Shimadzu LC-20AD, Japan) and the Sim mass percentage was calculated using $(m_{PPS}-m_{PP})/m_{PPS}$.

1.8. Study on Drug Release Properties of PPIS@FC

Simvastatin-loaded PPIS@FC was transferred to different concentrations of H_2O_2 for dialysis, and the experiments were performed in the dark with stirring at 37 °C. At predetermined intervals, 3 mL samples were collected and replaced with fresh solution. The release of simvastatin was determined by UV (UV-2450, SHIMADZU Ltd., Japan). All tests were repeated three times in the dark and the results were averaged.

1.9. Hemolysis Experiment

Hemolysis experiments of PPS@FC and PPIS@FC were performed using fresh mouse blood. When the hemolysis rate is greater than 5%, it can be judged that the material has a hemolysis effect and is not suitable for in vivo treatment[30]. Different concentrations of PPS@FC and PPIS@FC were incubated with blood at 37 °C for 1 h, and the supernatant was collected by centrifugation. The absorbance was measured at a wavelength of 540 nm using the UV, and the hemolysis rate of the sample was calculated according to the hemolysis rate formula as follows: Hemolysis rate = (sample absorbance-negative control absorbance) / (positive control absorbance-negative control absorbance) × 100%

1.10. In Vitro Cytotoxicity Study

The mouse microvascular endothelial cells RBMEC or mouse macrophage RAW264.7 were seeded in 96-well plates, cultured at 37 °C and 5% CO_2 for 24 h, and then incubated with the PPS@FC or PPIS@FC at concentrations of 0, 50, 100, 150, and 200 μg/mL for another 24 h. Finally, 10 μL MTT solution was added to continue incubation for 4 h. The absorbance of the solution in each well at 492 nm was determined using an enzyme-linked immunosorbent assay reader (Tecan Spark, Switzerland) and performed five parallel experiments per group. The cell viability was calculated by the following formula: Cell viability (%) = (sample absorbance-blank absorbance) / (control absorbance-blank absorbance) × 100%

We further detected live/dead cells with calcein-AM and PI staining. After cells were cultured with 200 μg/mL PPS@FC or PPIS@FC for 24 h, each well was stained with 2 μmol/L calcein-AM and 2 μmol/L PI for 15 min and observed with CLSM. The ImageJ software was used for cell counting, fluorescence volume, and area calculations.

1.11. In Vitro Cell Imaging Experiment

Mice RBMEC cells or RAW264.7 were seeded in confocal dishes and cultured at 37 °C in an incubator containing 5% CO_2 for 24 h. FC, PPS@FC or PPIS@FC was added at an identical FC dosage of 200 μg/mL and co-cultured with the cells for another 4 h. Then the cells were washed twice with pre-warmed PBS buffer at 37 °C to remove the PPS@FC or PPIS@FC and the 4% paraformaldehyde was added to fix at room temperature for 15 min. Subsequently, the nucleus was

stained with propidium iodide (PI) for 5 min and examined with a confocal laser microscope (CLSM, Leica TCS SP8, Germany). The steps for co-culturing RBMEC cells with RAW264.7 were the same as above, with five parallel experiments per set. Cells not co-cultured with FC, PPS@FC and PPIS@FC as control.

1.12 Intracellular ROS Response Experiment

Due to the low ROS content in normal cells, it is difficult to observe changes in the ROS response of PPIS@FC or PPS@FC, so the LPS stimulated RAW 264.7 model was established. Briefly, RAW 264.7 was inoculated in a dish at a density of 1×105 /well and treated with LPS (4 μg/mL) for 36 h. Cells were then washed three times with PBS buffer and the PPIS@FC or PPS@FC was added at 0 h, 1 h or 2 h for incubated 1 h. After discarding the medium, the RAW 264.7 was incubated with 10 μM DCFH-DA for 30 min, then remove the fluorescent probe solution and the fluorescence imaging was obtained using CLSM. Cells that not treated with FC, PPIS@FC, PPS@FC and LPS were used as controls.

1.13. Establishment of Atherosclerosis Model

8-week-old ApoE$^{-/-}$ mice were fed a high-fat diet containing 1% cholesterol, 15% lard, 0.25% sodium cholate, 5% egg yolk powder, 2.5% sugar and a basic diet for 20 weeks. Mice were subjected to a light / dark cycle for 12 h at 22 ± 2 °C, 55 ± 5% relative humidity.

1.14. In Vivo Magnetic Imaging Experiment

Prior to in vivo MRI in atherosclerotic mice, in vitro MRI scans were first performed on different concentrations of FC to ensure that FC nanoparticles could be used as T2 contrast agents for MRI.

Mice were anesthetized with 10% sodium pentobarbital, and the physiological saline solution of PPS@FC or PPIS@FC was injected into ApoE$^{-/-}$ mice through the tail vein at a dose of 16 mg/kg. The mice abdominal aorta were scanned for T2 weighted imaging using MRI (PHILTPS-OA7MF61, Netherlands) before sample injection and at 0, 2 and 4 h after injection. The specific imaging parameters were repetition time 3742.8 ms, echo time 40 ms, layer thickness 2 mm, bandwidth 167 Hz/Px, field of view 80×80 mm. The ApoE$^{-/-}$ mice that uninjected FC, PPS@FC and PPIS@FC as control.

1.15. In Vivo Fluorescence Imaging and Bio-distribution

The targeting ability and ROS responsiveness of PPS@FC and PPIS@FC were measured by in vivo fluorescence imaging. After atherosclerotic mice were injected with FC, PPS@FC or PPIS@FC via the tail vein at an identical FC dosage of 16 mg/kg, we euthanized the mice and isolated the major organs (aortic arch, heart, liver, spleen, lungs, and kidneys) at specific time. The fluorescence intensity of PPS@FC and PPIS@FC was detected using the small animal living imaging system (IVIS Lumina XR, Caliper Life Sciences, USA) at an excitation wavelength of 360 nm and an emission wavelength of 452 nm. The atherosclerotic mice that uninjected FC, PPS@FC and PPIS@FC as control.

1.16. Rabbit Carotid Artery Thrombosis Models

The carotid atherosclerotic plaque model was established in New Zealand white rabbits, and the state of the vessel wall and the blood flow in the carotid artery were observed by color Doppler ultrasound (PHILTPS EPIQ7C, Netherlands) to evaluate the therapeutic effects of PPS@FC and PPIS@FC. The specific operation was as follows: 8-week-old female New Zealand white rabbits were selected for the experiment. After the rabbit was anesthetized with 30% sodium pentobarbital,

the left common carotid artery of the rabbit was isolated, and 4 mL normal saline, 0.25 mg/mL Sim, 1 mg/mL PPS@FC or 1 mg/mL PPIS@FC was injected into rabbits through the marginal ear vein, respectively. Doppler ultrasound was used to observe the blood flow velocity in the middle of the common carotid artery, and 30% $FeCl_3$ saline solution was applied to the middle of the carotid artery for 3 min at the beginning of the observation at 5 and 15 min to establish an atherosclerosis model. The carotid artery status and blood flow velocity were then observed at 10 min, 20 min and 30 min, respectively. Finally, the rabbits were euthanized, and the carotid arteries of the rabbits were isolated, placed in 4% PFH solution, and the plaque morphology was observed by H&E staining. In order to compare the length and weight of thrombus between different groups, the above experimental process was repeated and the common carotid artery of rabbits were collected to place them in 4% PFH solution for fixation for 48 h, and the carotid artery thrombus was dried and separated to analyze its length and weight. Healthy New Zealand white rabbits injected with normal saline were used as control.

1.17. The Treatment of ApoE$^{-/-}$ mice

To compare the therapeutic effects of PPS@FC and PPIS@FC on atherosclerotic mice, 0.2 mL normal saline, 0.25 mg/mL Sim, 1 mg/mL PPS@FC or 1 mg/mL PPIS@FC were passed through tail vein injection into mice, which was given twice a week for 3 weeks. After that, the mice were euthanized, and the abdominal aorta was isolated and stained with Oil Red O to observe the plaque state. The atherosclerotic mice injected with normal saline were used as control.

1.18. In Vivo Safety Analysis

To evaluate the in vivo safety of PPIS@FC, 0.2 mL normal saline, 0.25 mg/mL Sim, 1 mg/mL PPS@FC or 1 mg/mL PPIS@FC were passed through tail vein injection into the healthy KM mice, and collected blood and major organs (heart, liver, spleen, lung, and kidney) after treated twice a week for 3 weeks. The major organs were performed blood routine, coagulation time, liver and kidney function indexes, and H&E staining, respectively. Healthy KM mice injected with normal saline were used as control.

1.19. Data Analysis

Each experiment was repeated at least three times, and all data were expressed as mean ±standard deviation (SD). Statistical analysis was performed using one-way analysis of variance or T-test. Significance was reported as*$p<0.05$, **$p<0.01$, or ***$p<0.001$.

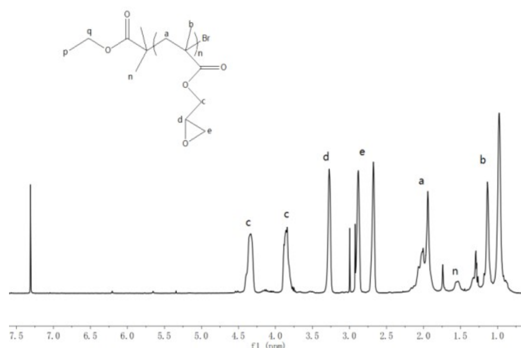


Fig. S1. ^1H NMR spectra of PGMA ($CDCl_3$, 25 °C).

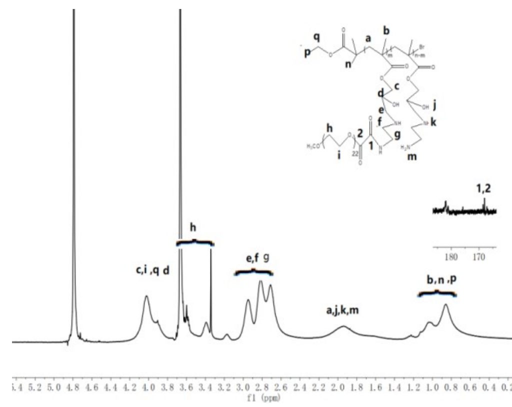


Fig. S2. ^{1}H NMR and ^{13}C NMR spectra of PP (H_2D, 25 °C).

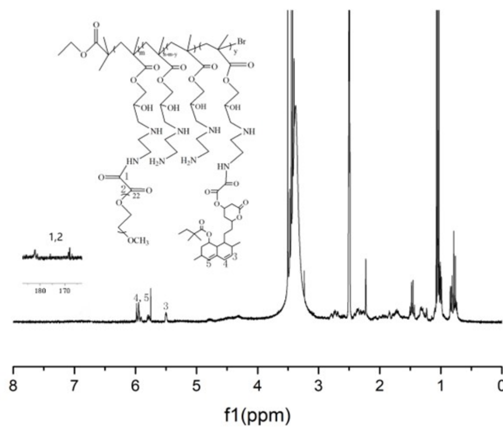


Fig. S3. ^{1}H NMR and ^{13}C NMR spectra of PPS (DMSO, 25 °C).

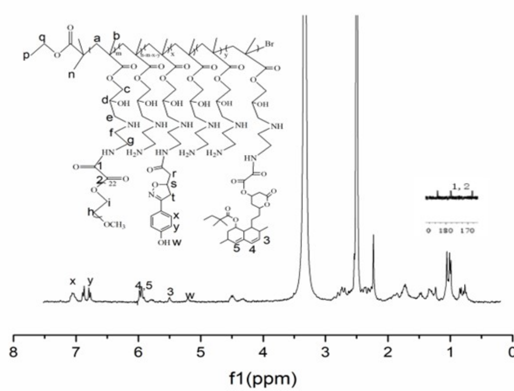


Fig. S4. ^{1}H NMR and ^{13}C NMR spectra of PPIS (DMSO, 25 °C).

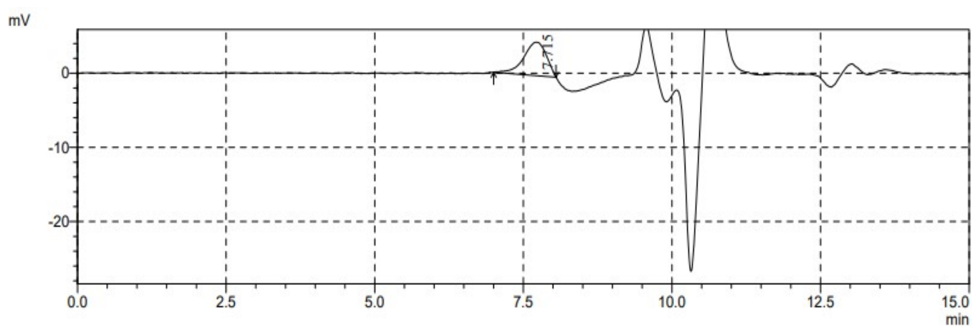


Fig. S5. The GPC image of PPS (DMF, 25 °C).

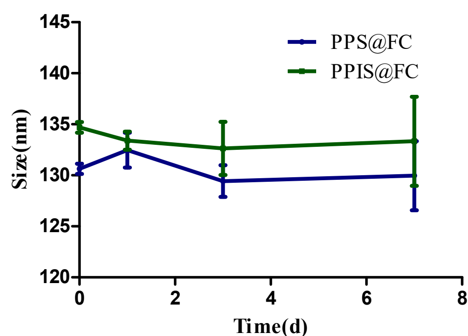
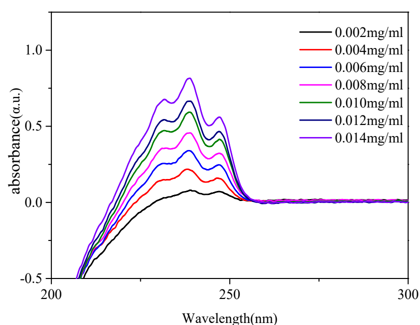


Fig. S6. The stability of PPS@FC and PPIS@FC over a 7-day period.

(a)



(b)

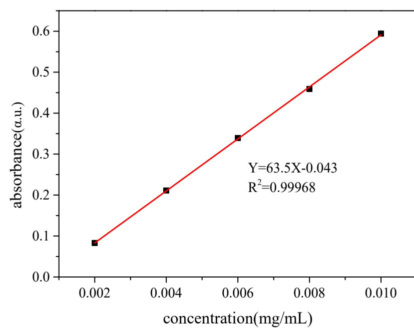


Fig. S7. (a) UV absorption profiles of simvastatin at different concentrations. (b) The standard curve of simvastatin as a function of its UV absorbance and concentrations.

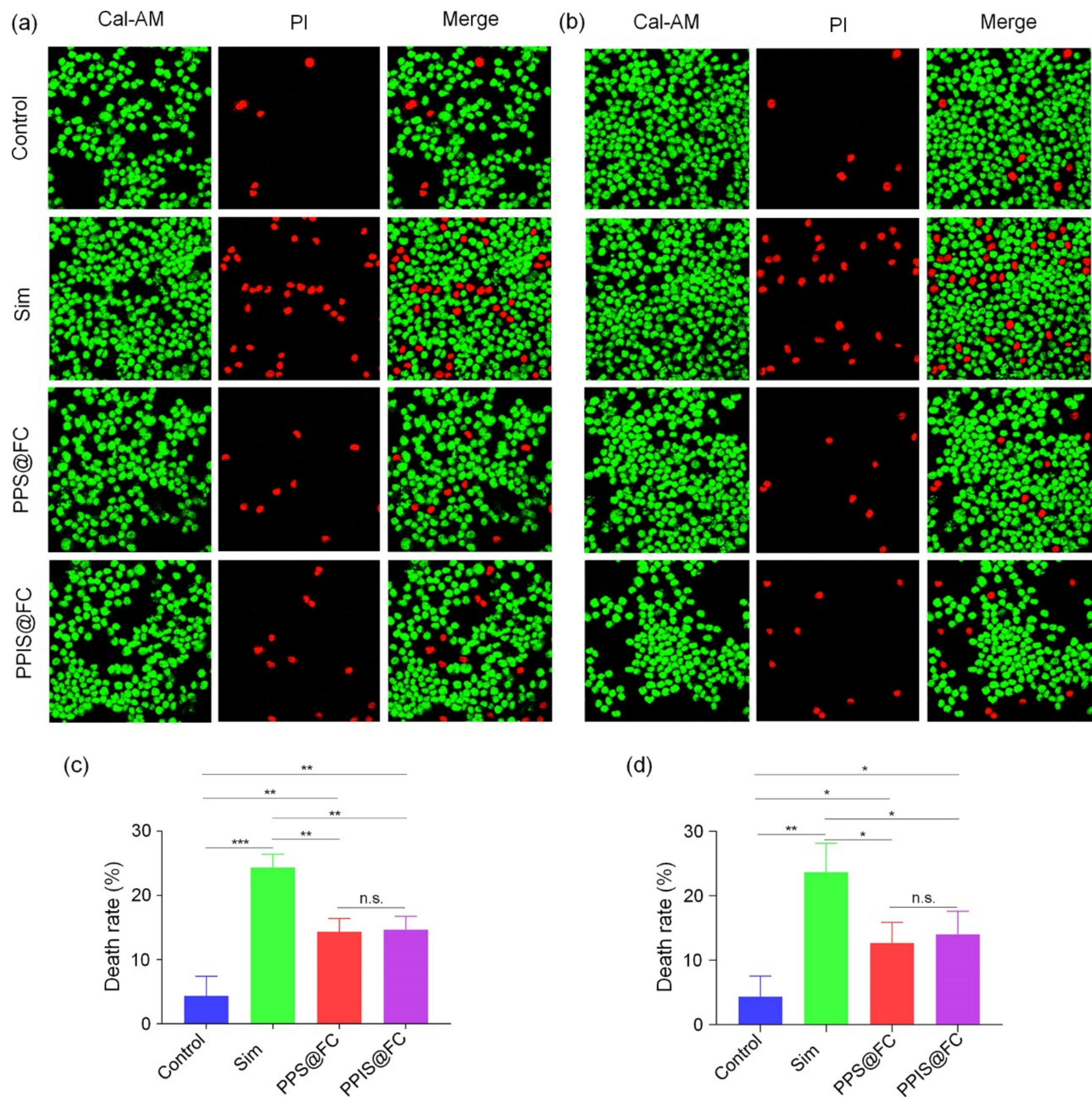


Fig. S8. In vitro fluorescence imaging of calcein AM and PI co-stained with RAW 264.7 and RBMEC. (a-b) The CLSM plot of PPS@FC or PPIS@FC co-incubated with RAW 264.7(a) and RBMEC (b). (c-d) The PI fluorescence intensity quantification of RAW 264.7(c) and RBMEC(d).

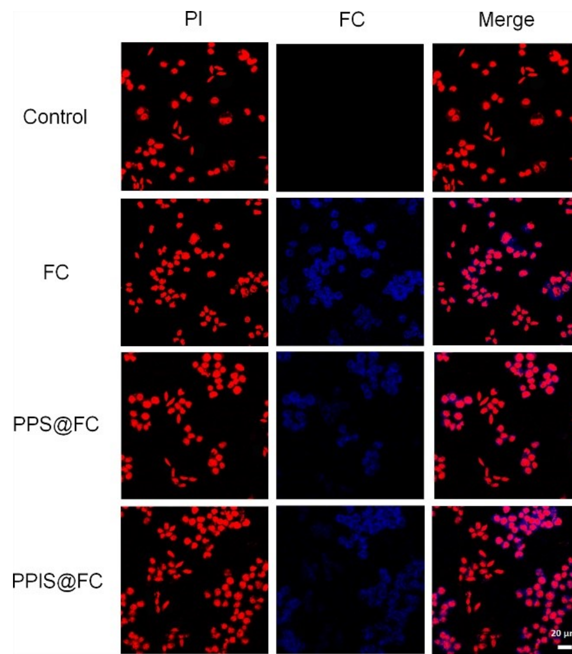


Fig. S9. The CLSM images of RAW 264.7 co-cultured with RBMEC that incubation with 200 μg/mL PPS@FC and PPIS@FC for 4 h.

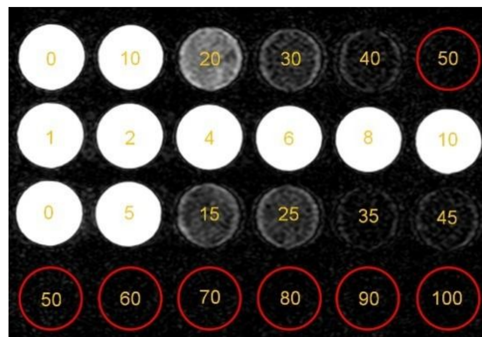


Fig. S10. The comparison of T2 signal intensity at different concentrations of FC.