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Supplementary material for "CD44 Targeted Redox-Triggered Self-assembly with Magnetic Enhanced EPR Effect for Efficacy Amplification of Gambogic Acid to Triple-Negative Breast Cancer"

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Materials, instrument, Cell Lines, and Animals.

Chitosan oligosaccharide (CSO, the average molecular weight = 5 kDa, degree of acetylation >80%) was purchased from the Dibai Chemical Reagent Co., Ltd. (Shanghai, China). 1-Hexadecanol (≥99.0%) and N,N'-dicyclohexylcarbodiimide (DCC, ≥95%) was obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China); 3,3'-dithiodipropionic acid (DTDPA, 99%), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI, 98%), N-hydroxysuccinimide (NHS, 98%), 4-dimethylaminopyridine (DMAP, 99%) and fluorescein isothiocyanate (FITC, 95%), 2,4,6-trinitro-benzenesulfonicacid (TNBS), 1,10-phenanthroline (99%), glutathione (Reduced, 95%), Trimethylamine, triphosgene and N-Ethylmaleimide (NEM) were purchased from the Aladdin Industrial Corporation (Shanghai, China). Methoxypolyethylene glycols（MEO-PEG-OH，the average molecular weight = 2 kDa）was obtained from TCI development Co. , Ltd (Shanghai, China). Hyaluronic acid (HA, average molecular weight =14600) was obtained from Zhenjiang Dong Yuan Biotech Co., Ltd. (Zhenjiang, China). Gambogic acid (GA) (GA, >97%) was prepared by the laboratory of China Pharmaceutical University (Department of Natural Medicinal Chemistry). Iron (III) acetylacetonate (Fe $(acac)_3$) (≥97%), oleylamine (80~90%), and 1,2-dodecanediol (≥90%) were obtained from Energy Chemical (Shanghai, China).

Probe-type ultrasonicator (VCX-500; SONICS&MATERIALS, INC, USA); HPLC system (Hitachi Primaide System, Japan); Transmission electron microscopy (TEM, Tecnai-12, Philips Company, Holland); Dynamic light scattering (Nano-ZS90, Malvern Instruments, U.K); Vibrating sample magnetometer (VSM, VSM-175, China); Laser scanning confocal microscopy (Carl Zeiss LSM700, Carl Zeiss AG, Germany); Microplate reader (Thermo, USA); Reflected fluorescence microscope (OLYMPUS IX53, Japan); ^{1}H NMR spectroscopy (Bruker Avance-Ⅲ 400 spectrometer, USA).

In addition, 3-(4,5-dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium bromide (MTT) and Annexin V-FITC Apoptosis Detection Kit (#C1062S) was ordered from the Beyotime Institute of Biotechnology (Haimen, China). Dulbecco's modified Eagle's medium

(DMEM), 10% fetal bovine serum (FBS) and trypsin containing EDTA were purchased from the Keygen Biotech Corp., Ltd, (Nanjing, China). Cell Counting Kit-8 (CCK-8, HY-K0301) was bought from Med. Chem. Express (USA). Iron Assay Kit (# 3100865) were purchased from Sigma (USA). Anti-CD44 (ab157107) purchased from AbCam (Cambridge, UK). All organic solvents used were of chromatographic grade or analytical grade.

The mouse mammary breast tumor cell line (4T1), human breast cancer cell line (MCF-7), human breast cancer cell line (MDA-MB-231) and mouse monocyte macrophage leukemic cell line (RAW 264.7) were obtained from the Shanghai Institutes for Biological Sciences (shanghai, China) and cultured in DMEM medium supplemented with 10% FBS at 37 ℃ in 5% CO_2 incubator. Female BALB/c mice (18 ± 2 g) and Sprague Dawley male rats (180-200 g) bought by Qinglongshan animal breeding farm (Nanjing, China). All animals are Specific Pathogen Free (SPF) and feeding at least one week before the experiment.

Mono-Aminated Poly (Ethylene Glycol) Grafted Hyaluronic Acid (mPEG-HA) Synthesis and Characterization

The synthesis of mPEG-HA was according to the **Figure S1A**.[1] Firstly, the synthesis of mono-aminated poly (ethylene glycol) (PEG-NH_2, MWCO = 2 kDa): MEO-PEG-OH (average MWCO = 2 kDa, 2 g, 1mmol) was dissolved in 20 mL anhydrous toluene and azeotropically distilled under a nitrogen atmosphere at 140℃ for 3h. After cooling to room temperature, the flask was placed in an ice bath and 4 mL anhydrous dichloromethane was gradually added until the solution became clear. Trimethylamine (0.208 mL, 1.5 mmol) was added dropwise with stirring, followed by the dropwise addition of mesyl chloride (0.116 mL, 1.5 mmol). After 18 h, the reaction solution was filtered to remove the insoluble white trimethylamine hydrochloride salt, followed by precipitation into excess diethyl ether. The off-white product was isolated by filtration and dried under vacuum. Then 20 mL aqueous ammonia solution (25%) adding to the off-white product, and the reaction solution was stirred for four days at 20℃. The ammonia was allowed to evaporate slowly over three days at the back of a fume hood.

NaOH (5 M) was added dropwise to the solution until the pH reached 13 and the polymer was extracted into dichloromethane. The combined organics were washed with brine and dried over anhydrous magnesium sulfate subsequently. After concentrating under vacuum, the crude PEG-NH_2 product was precipitated into excess diethyl ether and dried under vacuum to produce PEG-NH_2.

Secondly, the synthesis of mono-aminated poly (ethylene glycol) grafted hyaluronic acid (mPEG-HA)[2, 3] (10, 20, 30 mg) was dissolved in 3 mL pH 7.4 PBS solution, after which EDC (25.65, 51.30, 76.95 mg) and NHS (15.40, 30.8, 46.2 mg) were added and stirred for 2 h at room temperature. mPEG–NH_2 (7.98 mg) in 3 mL PBS solution was added to the reaction mixture drop by drop and stirred for another 24 h.[4] The crude product was then purified by dialysis (MWCO 8 kDa) against distilled water for 48 h with 3 changes a day and characterized by ^{1}H NMR spectroscopy operated at 500 MHz using D_2O as solvent.

mPEG-HA/CSO-SS-Hex/SPION/GA Preparation and Characterization

The method of the preparation of CSO-SS-Hex micelles and SPION loaded CSO-SS-Hex micelles was as previously reported [5]. The dialysis technique was used to entrap GA into the magnetic self-assembly CSO-SS-Hex/SPION [6]. Briefly, 1.5 mL anhydrous ethanol, contains 15 mg GA was added to the solution of magnetic self-assembly drop-by-drop under vigorous stirring for 1 h at room temperature. The mixture was ultra-sonicated for 15 min with an ice-bath using a probe-type ultrasonicator at 150 W. Then dialysised against DDW using a dialysis bag (MWCO = 1 kDa) for 24 h and obtained the crude product solution of CSO-SS-Hex/SPION/GA. The mPEG-HA decorated complex self-assembly (mPEG-HA/CSO-SS-Hex/SPION/GA) was prepared by slowly dropping 3 mL mPEG-HA water solution (1 mg/mL) into the solution of CSO-SS-Hex/SPION/GA. Then dialysis against DDW for 24 h using a dialysis bag (MWCO = 1 kDa). Afterwards, the mPEG-HA/CSO-SS-Hex/SPION/GA solution were removed from the dialysis bag and filtered through a 0.45 μm membrane to remove large aggregates and unloaded GA.

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2. X. Q. Quan, L. Kang, X. Z. Yin, Z. H. Jin and Z. G. Gao, *Chinese Chemical Letters*, 2015, **26**, 695-699.
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Table S1. Properties of different substitution degrees of mPEG to HA prepared magnetic complex self-assembles.

mPEG-HA in micelles	Drug contents (%, w/w)	PEG substitution (%)	dn (nm)	ξ(mV)
1-mPEG-HA	30	18.6 ± 0.3	175.9 ± 5.6	15.6 ± 0.6
2-mPEG-HA	20	15.1 ± 0.5	188.2 ± 8.7	17.5 ± 0.9
3-mPEG-HA	10	8.8 ± 0.2	212.7 ± 3.4	19.8 ± 1.1

Notes: dn: Number average diameter. ξ: Zeta potential. Data represent mean ± standard deviation (n = 3).

Table S2. Properties of GA loaded complex self-assembly. (n=3).

Drug	Sample	DL (%)	EE (%)
GA	CSO-SS-Hex/SPION/GA	22.26 ± 2.25	89.11 ± 3.28
GA	HA/CSO-SS-Hex/SPION/GA	25.17 ± 1.58	86.58 ± 2.44
GA	mPEG-HA/CSO-SS-Hex/SPION/GA	23.71 ± 1.15	85.14 ± 1.38

Table S3. Half maximal inhibitory concentration (IC50) of different types of cell lines. (MTT method, n=3).

Cell lines	GA(μM)	CSO-SS-Hex/SPION/GA(μM)	HA/CSO-SS-Hex/SPION/GA(μM)	mPEG-HA/CSO-SS-Hex/SPION/GA(μM)
MCF-7	9.00 ± 0.21	6.35 ± 0.32	3.95 ± 0.13	0.96 ± 0.28
MDA-	10.01 ± 0.06	7.13 ± 0.17	5.63 ± 0.24	1.54 ± 0.08

MB-231				
4T1	6.21 ± 0.15	4.81 ± 0.22	3.17 ± 0.18	1.37 ± 0.38

Table S4. Half maximal inhibitory concentration (IC50) of different types of cell lines. (CCK-8 method, n=3).

Cell lines (μM)	GA	CSO-SS-Hex	CSO-SS-Hex/SPION/GA	HA/CSO-SS-Hex/SPION/GA	mPEG-HA/CSO-SS-Hex/SPION/GA
MCF-7	10.13 ± 0.13	25.92 ± 0.41	5.96 ± 0.23	2.95 ± 0.06	1.28 ± 0.14
MDA-MB-231	11.61 ± 0.15	28.22 ± 0.18	6.44 ± 0.13	4.66 ± 0.18	1.64 ± 0.10
4T1	7.44 ± 0.09	30.28 ± 0.12	4.12 ± 0.18	2.37 ± 0.09	1.21 ± 0.07

Table S5. Pharmacokinetic parameters of mPEG-HA/CSO-SS-Hex/SPION/GA self-assembly and GA. (n=6).

Parameter	Units	mPEG-HA/CSO-SS-Hex/SPION/GA	GA
C_{max}	μg/ml	4.19 ± 2.18	1.28 ± 1.11
$t_{1/2}$	h	2.35 ± 0.11	0.51 ± 0.00
CL	(mg/kg)/(μg/ml)/h	6.19 ± 2.35	22.9 ± 5.52
AUC_{0-t}	μg/ml*h	0.96 ± 0.52	0.26 ± 0.12
$AUC_{0-\infty}$	μg/ml*h	0.97 ± 0.33	0.27 ± 0.10
MRT	h	0.35 ± 0.12	0.13 ± 0.05

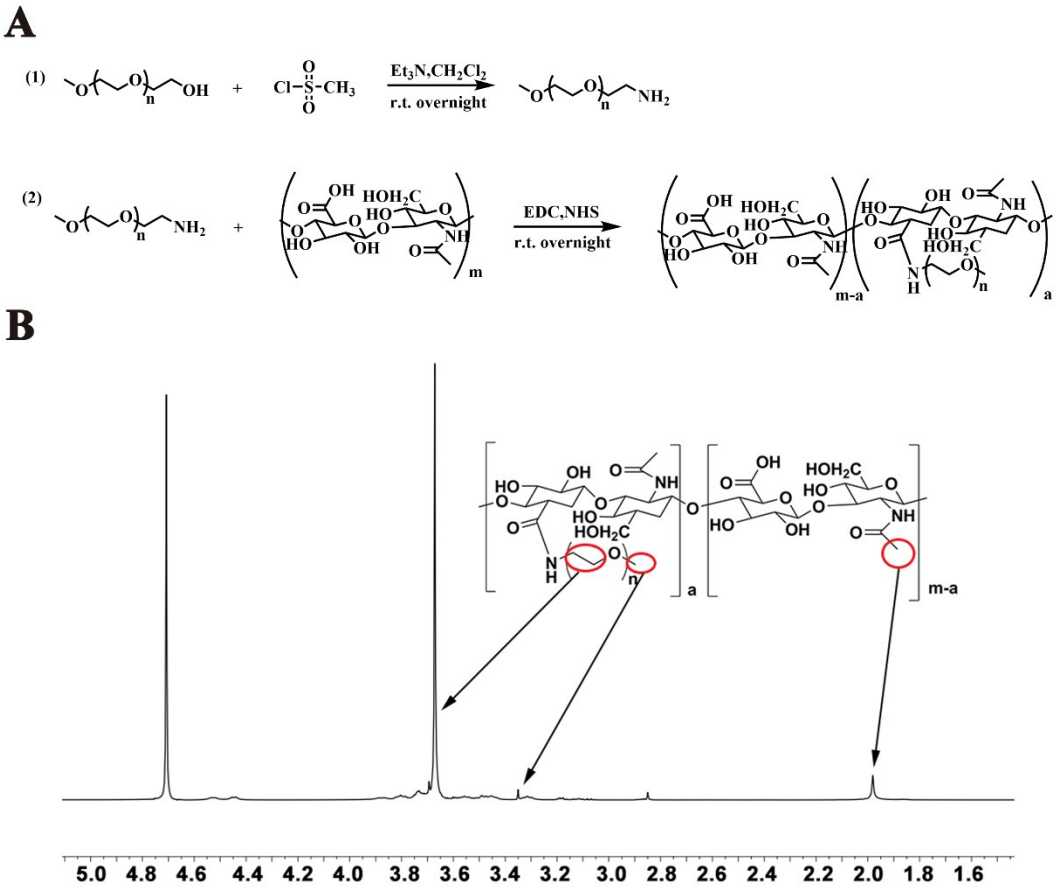

Figure S1. Synthesis route (A) and ^{1}H NMR spectra (B) of mPEG-HA copolymer.

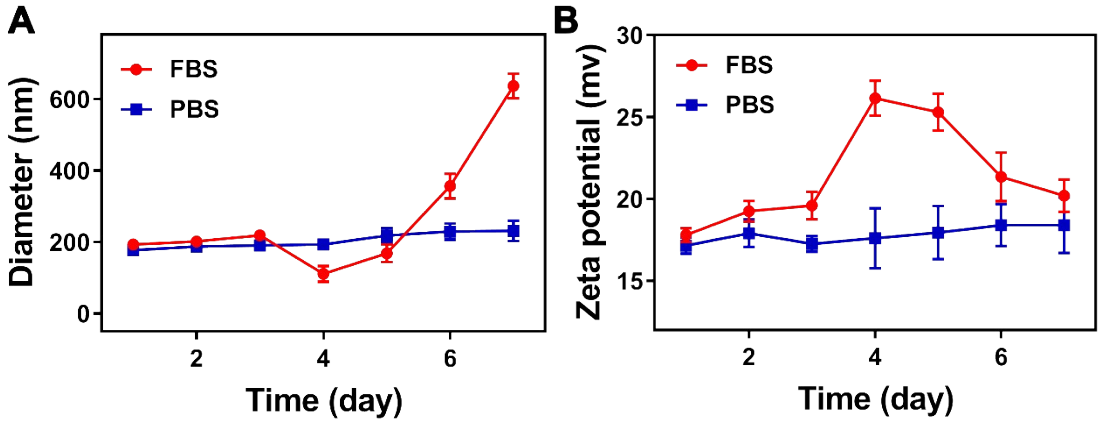

Figure S2. Particle diameter (A) and zeta potential (B) changes of mPEG-HA/CSO-SS-Hex/SPION/GA self-assembly in FBS and PBS for 7 days. (n=3).

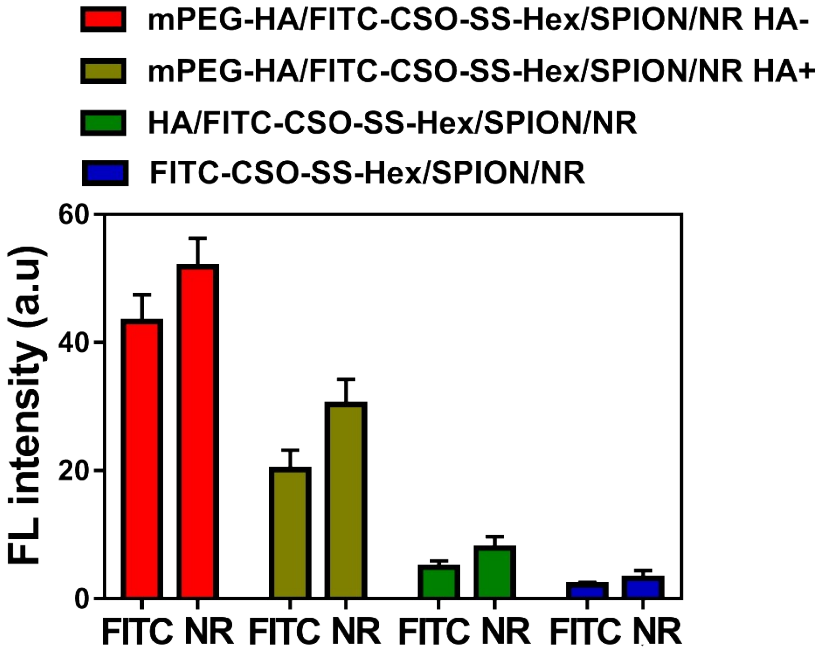

Figure S3. The fluorescence intensity change of 4T1 cells were incubated with mPEG-HA/FITC-CSO-SS-Hex/SPION/NR, HA/FITC-CSO-SS-Hex/SPION/NR and FITC-CSO-SS-Hex/SPION/NR at different condition for 3 h. (n=3).

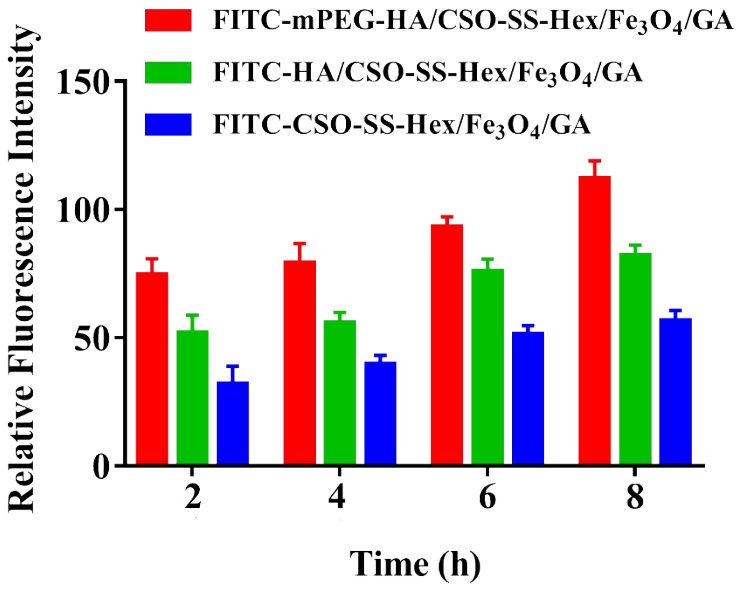

Figure S4. Quantitative determination of FITC by flow cytometry after 4T1 cells were incubated with FITC-mPEG-HA/CSO-SS-Hex/SPION/GA, FITC-HA/CSO-SS-Hex/SPION/GA and FITC-CSO-SS-Hex/SPION/GA micelles for 2, 4, 6, 8 h. (n=3).

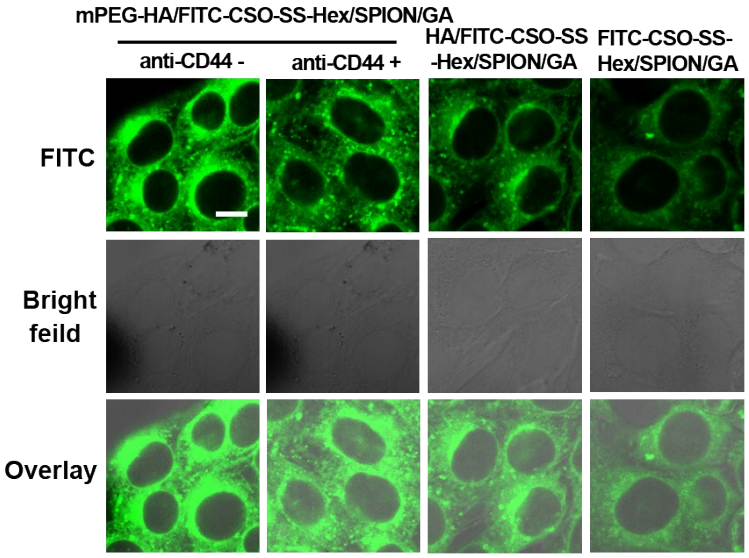

Figure S5. 4T1 cells were incubated with mPEG-HA/FITC-CSO-SS-Hex/SPION/NR pretreated with anti-CD44 (anti-CD44+) or no anti-CD44 (anti-CD44-), HA/FITC-CSO-SS-Hex/SPION/NR and FITC-CSO-SS-Hex/SPION/NR for 12 h, and labeled with DAPI (blue) to identify cell nuclei. (scale bar: 5 μm).

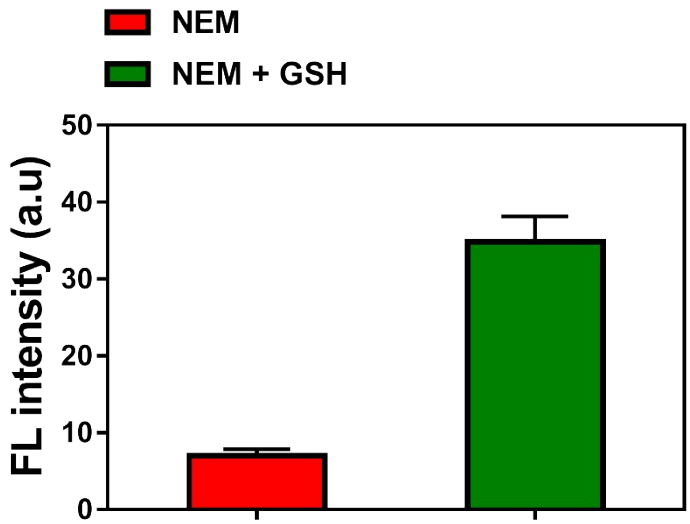

Figure S6. The fluorescence intensity change of 4T1 cells were incubated with NR loaded mPEG-HA/CSO-SS-Hex/SPION for 3 h after pretreated with NEM (1mM) and NEM (1mM) + GSH (10mM). (n=3).

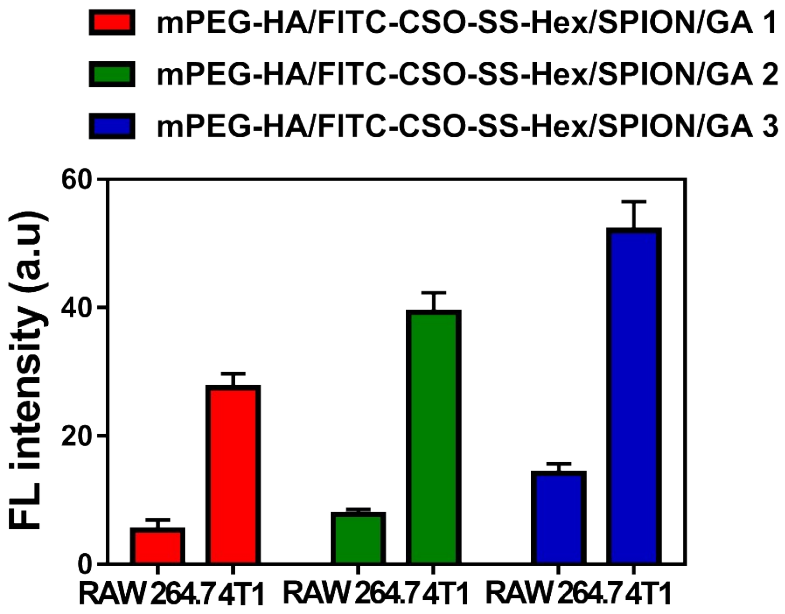

Figure S7. The fluorescence intensity changes of 4T1 and raw 264.7 cells were incubated with FITC labeled mPEG-HA/CSO-SS-Hex/SPION/GA with different substitution degrees (as table S1) for 3 h. (n=3).

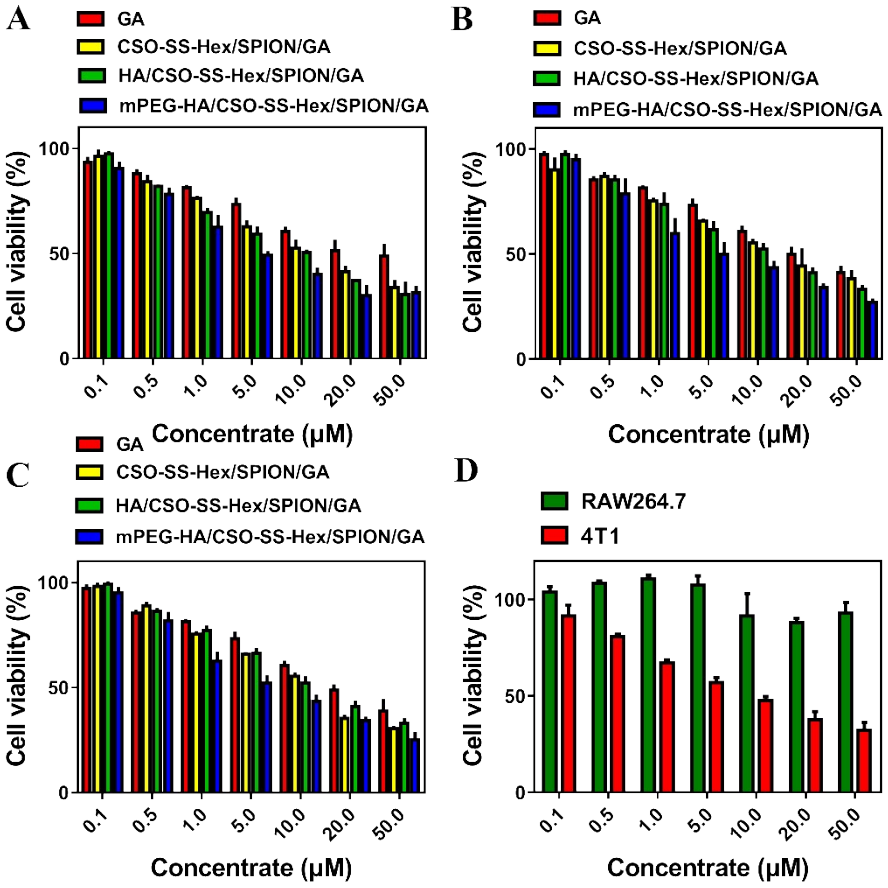

Figure S8. *In vitro* pharmacodynamics evaluation of mPEG-HA/CSO-SS-Hex/SPION/GA complex self-assembly. (A-C) Cell viability of 4T1 cells, MCF-7 cells

and MDA-MB-231 cells treated with GA and different self-assembles (GA, CSO-SS-Hex/SPION/GA, HA/CSO-SS-Hex/SPION/GA, mPEG-HA/CSO-SS-Hex/SPION/GA) for 48 h. (n = 3). (D) Cell viability of 4T1 cells and RAW 264.7 treated with mPEG-HA/CSO-SS-Hex/SPION/GA for 48 h. (n = 3).

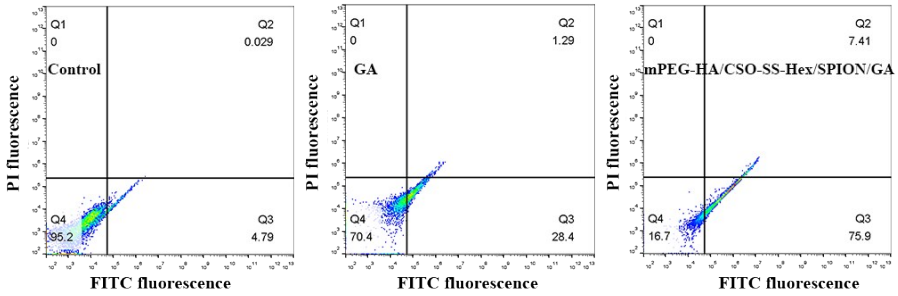

Figure S9. Apoptosis of 4T1 cells incubated with DMEM, GA and mPEG-HA/CSO-SS-HEX/SPION/GA for 24 h.

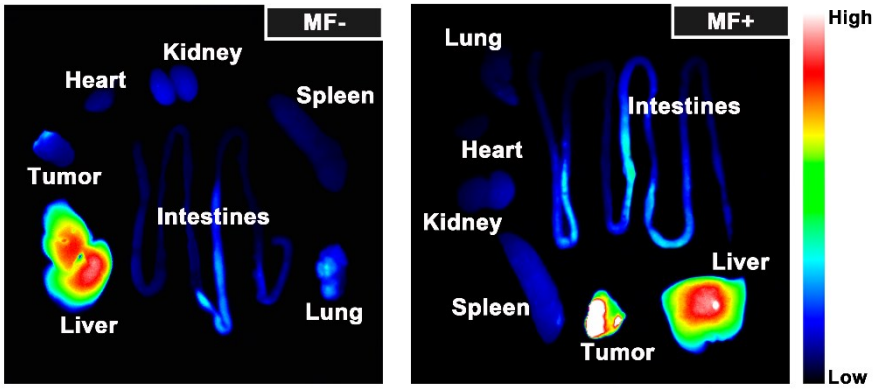

Figure S10. *In vivo* fluorescence images of organ distribution.