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Electronic Supporting Information

A tumor microenvironment dual responsive contrast agent for contrary contrast-magnetic resonance imaging and specific chemotherapy of tumors

Yudie Lu,[a‡] Jie Feng,[b‡] Zhiyu Liang,[b] Xuanyi Lu,[a] Shuai Guo,[a] Lin Huang,[a] Wei Xiong,[b] Sijin Chen,[b] Huimin Zhou,[c] Xuehua Ma,[d] Yikai Xu,[b] Xiaozhong Qiu,[c] Aiguo Wu,[d] Xiaoyuan Chen,[e,f,g] Zheyu Shen[a,b,c*]

[a] School of Biomedical Engineering, Southern Medical University, 1023 Shatai South Road, Guangzhou, Guangdong 510515, China
[b] Medical Imaging Center, Nanfang Hospital, Southern Medical University, 1023 Shatai South Road, Guangzhou, Guangdong 510515, China
[c] Guangdong Provincial Key Laboratory of Construction and Detection in Tissue Engineering, School of Basic Medical Sciences, Southern Medical University, 1023 Shatai South Road, Guangzhou, Guangdong 510515, China
[d] Cixi Institute of Biomedical Engineering, CAS Key Laboratory of Magnetic Materials and Devices, Ningbo Institute of Materials Technology and Engineering, Chinese Academy of Sciences, 1219 Zhongguan West Road, Ningbo, Zhejiang 315201, China
[e] Departments of Diagnostic Radiology, Surgery, Chemical and Biomolecular Engineering, and Biomedical Engineering, Yong Loo Lin School of Medicine and Faculty of Engineering, National University of Singapore, Singapore, 119074, Singapore
[f] Clinical Imaging Research Centre, Centre for Translational Medicine, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 117599, Singapore
[g] Nanomedicine Translational Research Program, NUS Center for Nanomedicine, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 117597, Singapore

Corresponding Author

[*] E-mail address: sz@smu.edu.cn (Z. Shen).

Author Contributions

[‡] Y.L. and J.F. contributed equally to this work.

Experimental Methods

Synthesis of FeGdNP Nanoparticles: 160 mL of PAA (Mw = 1800) solution (4.0 mg/mL) was first purged with nitrogen (≥ 1.0 h) to remove oxygen. The polymer solution was then heated to reflux (100 °C). After that, 3.2 mL of mixture of iron precursors (500 mM $FeCl_3$ plus 250 mM $FeSO_4$) was quickly injected into the heated polymer solution, followed by addition of 48 mL of ammonia solution (28 %). The reaction was kept at 100 °C under magnetic stirring. After 30 min, 3.2 mL of $Gd(NO_3)_3$ (500 mM) and 24 mL of ammonia solution (28 %) were added into the reaction system. The reaction was continued for 60 min under magnetic stirring at 100 °C to obtain the FeGdNP. Finally, the solutions were cooled down to room temperature. The obtained FeGdNP nanoparticles were purified by membrane dialysis (Mw cut-off 6-8 kDa) against Milli-Q water for 5 days with a daily change of the water. The purified FeGdNP was concentrated by centrifugal ultrafiltration (Millipore, molecular size cutoff of 10 kDa). The Fe and Gd concentrations of the solutions were measured by inductively coupled plasma optical emission spectrometry (ICP-OES).

Synthesis of mPEG-FBA: Methoxy poly(ethylene glycol) (mPEG, Mw = 2000) was dissolved in dichloromethane with a concentration of 50 mg/mL. To 160 mL of the mPEG solution (8.0 g, 4.0 mmol), 4-Formylbenzoic acid (FBA, 1.8 g, 12 mmol), dimethylformamide (DMF, 20 mL), Dicyclohexylcarbodiimide (DCC, 2.48 g, 12 mmol), and 4-(Dimethylamino)pyridine (DMAP, 732 mg, 6.0 mmol) were added. The solution was stirred at room temperature for 24 h, and then filtered thrice to discard the precipitate dicyclohexylurea (DCU). After that, dichloromethane of the filtrate was evaporated via N2 bubbling, and the product was dispersed in MilliQ water (50 mL). The resulting mPEG-FBA was collected by freeze-drying. The obtained white powder of mPEG-FBA was stored at room temperature for further use.

Influence of Solvents on the Reaction between FBA and CA: 0.3 mL of cystamine dihydrochloride (CA) in methanol (20 mg/mL) was mixed with 37.1 μL of triethylamine (TEA). After that, 0.80 mL of FBA (5.0 mg/mL in DMF, DMSO, THF, or ethanol) was added into the

above-mentioned mixture. After vortexing for 30 s, the mixtures were kept shaking in an incubator at 37 °C for 72 h. The obtained auto-fluorescent FBA-CA1-4 were measured by a fluorescence spectrophotometer.

Synthesis of mPEG-FBA-CA: 3.0 mL of cystamine dihydrochloride (CA) in methanol (20 mg/mL) and 371 μL of triethylamine (TEA) were respectively added into 8.0 mL of methanol. 28.4 mL of mPEG-FBA in THF (20 mg/mL) were then mixed with the above-mentioned solution. After vortexing for 30 s, the mixture was kept shaking in an incubator at 45 °C for 72 h. The obtained mPEG-FBA-CA was stored for further use.

Synthesis of FeGdNP@mPEG: 0.5 mL of FeGdNP nanoparticles in pure water (Gd = 22.2 mM, Fe = 45.3 mM) was added into 3.624, 4.062, 4.281, 4.390, and 4.445 mL of DMF respectively. After that, 0.876, 0.438, 0.219, 0.110 and 0.055 mL of the above-obtained mPEG-FBA-CA (15.94 mg/mL) (mPEG-FBA-CA/Gd mass ratio = 8.0, 4.0, 2.0, 1.0 and 0.5) were respectively charged into the above-mentioned solutions with following addition of 10 μL of N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide (EDC). Although the reaction between –COOH and $–NH_2$ is faster in mild acid solutions than in neutral or alkaline solutions, EDC (but not EDC.HCl) was used in this study because the benzoic imine in mPEG-FBA-CA is an acid labile linker. The total volume of the five reaction solutions was all 5.0 mL. The obtained FeGdNP@mPEG1-5 nanoparticles (in organic solvents, i.e., DMF, THF, and methanol) were then obtained after reaction under magnetic stirring at room temperature for 16 h.

Synthesis of self-assembled FeGdNP@mPEG (SA-FeGdNP@mPEG): Milli-Q water was dropwise added into the above obtained FeGdNP@mPEG1-5 nanoparticles (volume ratio of water to nanoparticle solution = 9 : 1) under magnetic stirring. After that, the nanoparticle solutions were subjected to 3 days of dialysis (Mw cut-off 12-14 kDa) against Milli-Q water to remove the organic solvents (i.e., DMF, THF, and methanol) and un-conjugated organic molecules (i.e., mPEG-FBA, CA, TEA, mPEG-FBA-CA, and (mPEG-FBA)2-CA). After the

dialysis, the FeGdNP@mPEG1-5 nanoparticles were purified and self-assembled forming SA-FeGdNP@mPEG1-5.

Synthesis of FeGdNP-DOX@mPEG and SA-FeGdNP-DOX@mPEG: 0.5 mL of FeGdNP nanoparticles in pure water (Gd = 22.2 mM, Fe = 45.3 mM) was added into 1.269, 2.666, 3.364, 3.713, and 3.887 mL of DMF, respectively. After that, 0.438 mL of the mPEG-FBA-CA (15.94 mg/mL) (mPEG-FBA-CA/Gd mass ratio = 4.0) were charged into the above-mentioned solutions with following addition of EDC (10 μL). Five batches of FeGdNP@mPEG2 nanoparticles were then obtained after reaction under magnetic stirring at room temperature for 16 h. 1.0 mg/mL of DOX in DMF ranging from 2.793 to 0.175 mL (DOX/Gd mass ratio = 1.6, 0.8, 0.4, 0.2, or 0.1) was then added into the obtained FeGdNP@mPEG2 nanoparticles. The total volume of the five reaction solutions was all 5.0 mL. After 4.0 h of reaction under magnetic stirring at room temperature, FeGdNP-DOX1-5@mPEG2 nanoparticles in organic solvents were obtained. The SA-FeGdNP-DOX1-5@mPEG2 were finally prepared utilizing similar procedures as the above-mentioned SA-FeGdNP@mPEG.

Disassembling of the SA-FeGdNP-DOX2@mPEG2 nanoparticles: 1.0 mL of SA-FeGdNP-DOX2@mPEG2 nanoparticles (C_{Gd} = 2.0 mM, C_{DOX} = 139 μg/mL) were mixed with 1.0 mL of PBS (pH 7.4 or pH 6.8) with or without GSH (20 mM). The solutions were kept in a shaking incubator at 37 °C for 24 h, and then taken for TEM observation and MRI measurement.

Release Behavior of DOX from SA-FeGdNP-DOX2@mPEG2: The release behavior of DOX from SA-FeGdNP-DOX2@mPEG2 nanoparticles at pH 7.4 without GSH or at pH 6.8 with 10 mM of GSH was determined by a fluorescence spectrophotometer. Typically, 1.0 mL of SA-FeGdNP-DOX2@mPEG2 nanoparticles (C_{Gd} = 2.0 mM, C_{DOX} = 139 μg/mL) were mixed with 1.0 mL of PBS (pH 7.4 or pH 6.8) with or without GSH (20 mM). The solutions were kept in a shaking incubator at 37 °C. At predetermined time intervals, 0.2 mL of the solution was taken and centrifuged at 15,000 × g for 20 min. The supernatants were then measured by a fluorescence spectrophotometer (Ex: 480 nm) and the fluorescence intensity at 593 nm was

converted into the DOX concentration using a calibration curve constructed with standard DOX solutions. The DOX release behavior at different conditions was monitored *via* plot of the cumulative released DOX content (*i.e.,* the mass percentage of the released DOX to the total amount of DOX in the nanoparticles) as a function of incubation time.

Cell culture: U-87 MG (human glioblastoma cell line), and HepG2 (human hepatoma cell line) cells were cultured in the DMEM medium supplemented with 10 wt% of fetal bovine serum (FBS), 100 units/mL of penicillin and 100 μg/mL of streptomycin. All of the cells were incubated at 37 ^{o}C in a humidified atmosphere containing 5 % of CO_2.

Cellular uptake by laser scanning confocal microscopy (LSCM): Uptake of the nanoparticles by U-87 MG, or HepG2 cells was studied using LSCM. Typically, 0.5 mL of U-87 MG, or HepG2 cells in growth medium were seeded into each well of Falcon® Culture Slide (8 Well, Corning) at a density of 5.0×10^5 cells/mL and allowed to adhere at 37 ^{o}C for 24 h. The growth medium was replaced with a fresh one (0.5 mL, without FBS) containing SA-FeGdNP-DOX2@mPEG2 nanoparticles (C_{Gd} = 140 μM). Herein, before dispersing in DMEM medium (without FBS), the SA-FeGdNP-DOX2@mPEG2 nanoparticles were incubated at 37 ^{o}C for 24 h at pH 7.4 without GSH, or at pH 6.8 with 10 mM of GSH. After further 2 h incubation, the cells were washed twice with PBS. The cells were then fixed with aqueous buffered zinc formalin fixative (Z-FIX) for 30 min, permeabilized with 0.1 % Triton X-100 for 5 min, blocked with 1.0 % BSA for 30 min and treated with the mixture of Phalloidin-FITC (0.5 μg/mL) and Hoechst (5 μg/mL) for 30 min at room temperature. A small drop volume (~ 20 μL) of the antifade mounting medium (Vectashield, H-1000) was added onto each cell specimen, which was then covered using cover slips. The cover slips were then fixed onto the slides by nail polish. After that, the LSCM images of the samples were observed on a LSCM imaging system.

Cell Viability Assay: The cytotoxicity and therapeutic efficacy of the nanoparticles was assessed with U-87 MG, or HepG2 cells by using the MTT method. Typically, 100 μL of U-87 MG or HepG2 cells in complete DMEM medium were seeded into each well of a 96-well plate

at a concentration of 5×10^4 cells/mL and allowed to adhere overnight. The growth medium was replaced with a fresh one (without FBS) containing various concentrations of free DOX, SA-FeGdNP@mPEG2, or SA-FeGdNP-DOX2@mPEG2 nanoparticles. Herein, before dispersing in DMEM medium (without FBS), the SA-FeGdNP@mPEG2, and SA-FeGdNP-DOX2@mPEG2 nanoparticles were incubated at 37 °C for 24 h at pH 7.4 without GSH, or at pH 6.8 with 10 mM of GSH. After charging the fresh growth medium without FBS containing nanoparticles or free DOX, the cells were incubated for 4 h at 37 °C. After that, the growth medium containing nanoparticles or DOX was replaced with complete DMEM medium. After further incubation for 44 h, 10 μL of MTT (5.0 mg/mL in PBS) was added to each well of the 96-well plate. After an additional 4.0 h of incubation, the growth medium was removed and the resulted formazan crystals in each well were dissolved with 100 μL of dimethyl sulfoxide (DMSO). The absorbance was recorded at a wavelength of 570 nm using a multi-mode microplate reader.

Tumor Model: All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Southern Medical University and approved by the Animal Ethics Committee of Southern Medical University. The U-87 MG tumor-bearing nude mice were prepared by inoculating U-87 MG cells (4×10^6 cells in 100 μL PBS) into the right shoulder of each mouse (female, 5 weeks) under anesthesia. The tumor size was measured *via* a caliper at predetermined times. The tumor volume was calculated through $ab^2/2$, where a and b are respectively the length and width of a tumor. The relative tumor volume (V/V_0) was the ratio of tumor volume after treatment (V) to that before treatment (V_0).

CC-MRI of tumors: The U-87 MG tumor-bearing nude mice were anaesthetized by isoflurane (1.0-2.0%) in oxygen, and placed in an animal-specific body coil for MRI data acquisition. Mice were kept warm by circulating warm water (37 °C), and were placed in a stretched prone position with a respiratory sensor during the experiments. T_1-weighted images were acquired at pre-injection and post injection (intravenously) of commercial Dotarem® (C_{Gd} = 5.0 mg / kg),

or SA-FeGdNP-DOX2@mPEG2 nanoparticles (C_{Gd} = 5.0 mg / kg). Multi-slice multi-echo sequence was employed to acquire images using parameters as follows: repetition time (TR) = 1000 ms, echo time (TE) = 30 ms, flip angle = 180°, matrix size = 256 × 256, field of view = 40 × 40 mm^2, slices = 16, slice thickness = 1 mm. MR images were analyzed by measuring signal intensity with the software Image J. The signal-to-noise ratio (SNR) and SNR ratio (*i.e.,* signal enhancement) were calculated according to the following equations (1) and (2).

$$SNR = SI_{mean} / SD_{noise} \quad (1)$$

$$\Delta SNR = (SNR_{post} - SNR_{pre}) / SNR_{pre} \times 100\ \% \quad (2)$$

High-performance chemotherapy: When the tumor volume was around 150 mm^3, 100 μL of saline, SA-FeGdNP@mPEG2, free DOX, SA-FeGdNP-DOX2@mPEG2 were injected intravenously into the mice under anesthesia (n = 5/group). The DOX dosage was 5 mg/kg or 10 mg/kg. After injection, dimensions of the tumors and body weights of the mice were monitored every other day.

Statistical analysis: Statistical significance was determined by applying Student's *t*-test or by a one-way ANOVA followed by Student-Newman-Keuls test using Sigma Stat version 3.5. The significance level was fixed as $P < 0.05$.

Table S1. Synthesis conditions and characterization results of SA-FeGdNP@mPEG.

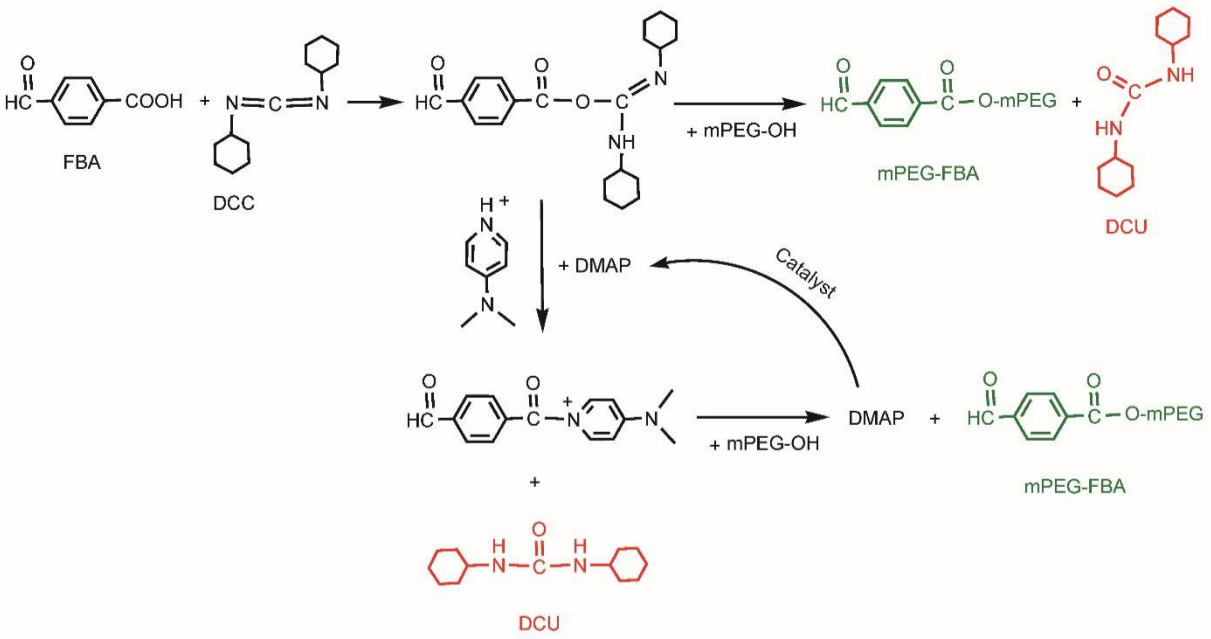
Sample Nomenclature	FeGdNP [a] (mL)	mPEG-FBA-CA [b] (mL)	mPEG-FBA-CA/Gd Mass Ratio [c]	EDC (μL)	DMF (mL)	Gd Recovery [d] (%)
SA-FeGdNP@mPEG1	0.5	0.876	8.0	10	3.624	83
SA-FeGdNP@mPEG2	0.5	0.438	4.0	10	4.062	84
SA-FeGdNP@mPEG3	0.5	0.219	2.0	10	4.281	82
SA-FeGdNP@mPEG4	0.5	0.110	1.0	10	4.390	78
SA-FeGdNP@mPEG5	0.5	0.055	0.5	10	4.445	76

[a] The feeding FeGdNP was dispersed in pure water with 22.2 mM of C_{Gd} and 45.3 mM of C_{Fe}.
[b] The concentration of feeding mPEG-FBA-CA is 15.94 mg/mL.
[c] Calculated from the mass ratio of feeding mPEG-FBA-CA to Gd in FeGdNP.
[d] Calculated from the molar ratio of Gd in SA-FeGdNP@mPEG to that in the feeding FeGdNP.

Table S2. Synthesis conditions and characterization results of SA-FeGdNP-DOX@mPEG.

Sample Nomenclature	FeGdNP [a] (mL)	mPEG-FBA-CA [b] (mL)	DOX/Gd Mass Ratio [c]	DOX (mL)	EDC (μL)	DMF (mL)	DOX Loading Efficiency [d] (%)	DOX Loading Content [e] (%)	r_1 [f] (mM^{-1} s^{-1})	r_2 [f] (mM^{-1} s^{-1})	r_2/r_1
SA-FeGdNP-DOX1@mPEG2	0.5	0.438	1.6	2.793	10	1.269	28.8	21.4	-	-	-
SA-FeGdNP-DOX2@mPEG2	0.5	0.438	0.8	1.396	10	2.666	55.4	20.6	18.37±0.35	336.9±7.5	18.4±0.7
SA-FeGdNP-DOX3@mPEG2	0.5	0.438	0.4	0.698	10	3.364	71.2	13.2	18.82	303.3	16.1
SA-FeGdNP-DOX4@mPEG2	0.5	0.438	0.2	0.349	10	3.713	76.0	7.1	18.78	280.2	14.9
SA-FeGdNP-DOX5@mPEG2	0.5	0.438	0.1	0.175	10	3.887	76.5	3.6	20.06	258.8	12.9
FeGdNP	-	-	-		-	-	-		20.32	146.9	7.2

[a] The feeding FeGdNP was dispersed in pure water with 22.2 mM of C_{Gd} and 45.3 mM of C_{Fe}.
[b] The concentration of feeding mPEG-FBA-CA is 15.94 mg/mL.
[c] Calculated from the mass ratio of feeding DOX to Gd in FeGdNP.
[d] Calculated from the mass percentage of loaded DOX to the feeding DOX.
[e] Calculated from the mass percentage of loaded DOX to the SA-FeGdNP-DOX@mPEG nanoparticles.
[f] The r_1 and r_2 were measured on a MRI scanner system (7.0 T, Bruker, B-C 70/16 US) (mean ± SD, n = 3).



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196 **Fig. S1.** Schematic illustration for the synthesis of mPEG-FBA.

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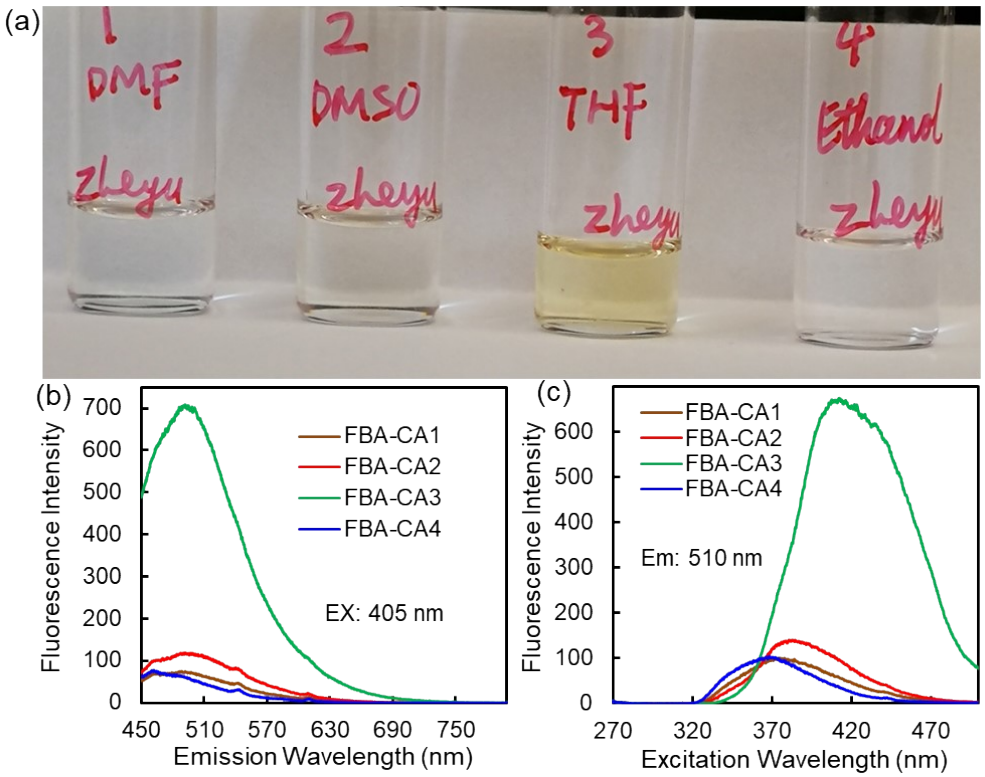

198

199 **Fig. S2.** Influence of formylbenzoic acid (FBA) solvents (DMF, DMSO, THF, and ethanol) on
200 the reaction between FBA and cystamine dihydrochloride (CA) in the presence of triethylamine
201 (TEA). CA solvent is methanol. Reaction temperature: 37 °C. Reaction time: 72 h. (a): Photo
202 of the resulting FBA-CA1-4 solutions reacted in DMF, DMSO, THF, or ethanol. Yellow color
203 of FBA-CA3 solution indicates much higher recovery in THF than other solvents. (b, c):
204 Fluorescence spectra of the auto-fluorescent FBA-CA1-4. (b): Emission curves at the excitation
205 of 405 nm. (c): Excitation curves at the emission of 510 nm. The maximum excitation, and
206 emission of FBA-CA3 is respectively measured to be 412, and 492 nm.

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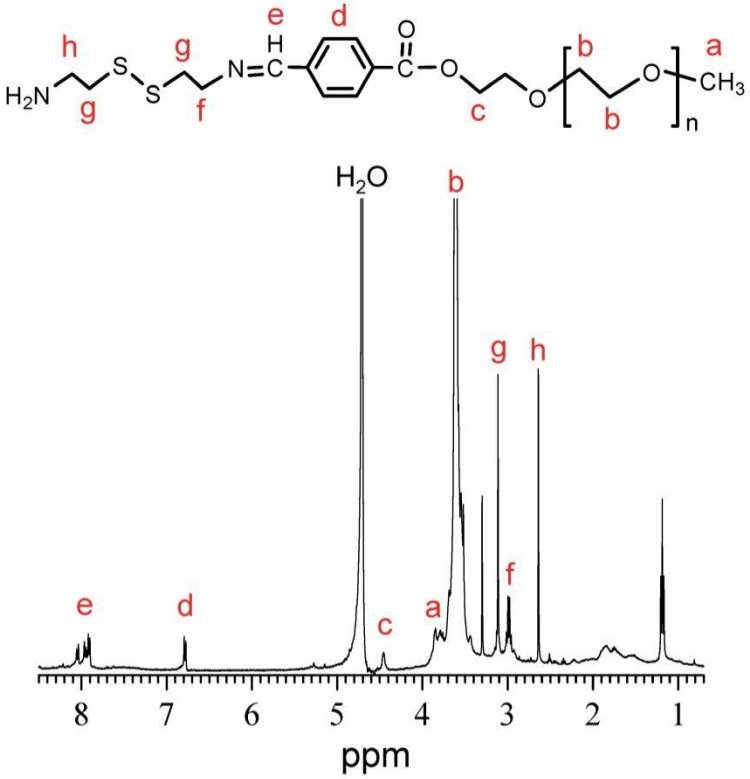

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210 **Fig. S3.** The ^{1}H NMR spectrum of mPEG-FBA-CA.

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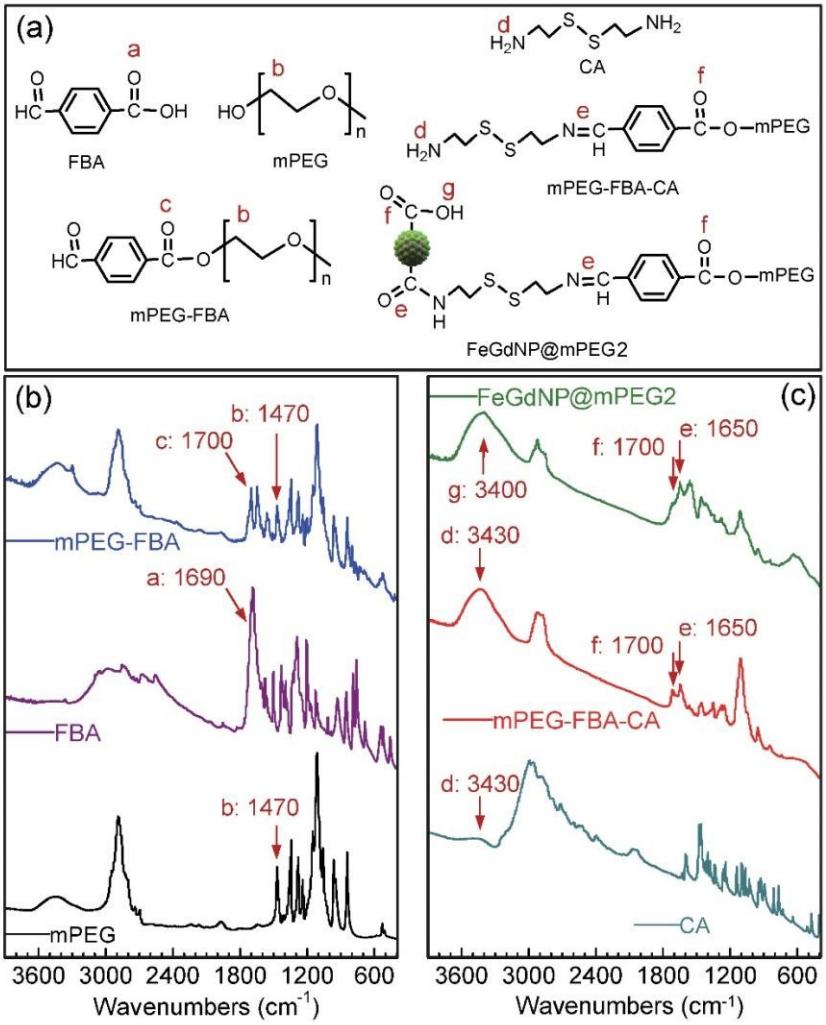

212

213 **Fig. S4.** (a): Molecular structures of the compounds. (b): FT-IR spectra of mPEG, FBA, and
214 mPEG-FBA. (c): FT-IR spectra of CA, mPEG-FBA-CA, and FeGdNP@mPEG2. FBA has a
215 characteristic peak at 1690 cm^{-1} (C=O stretching vibration in carboxyl group). mPEG has a
216 characteristic peak at 1470 cm^{-1} (C-H stretching vibration in methylene). Both the characteristic
217 peak at 1700 cm^{-1} (C=O stretching vibration in ester) and that at 1470 cm^{-1} demonstrate the
218 successful synthesis of mPEG-FBA. In addition, CA has a characteristic peak at 3430 cm^{-1}
219 (N-H stretching vibration in primary amine). Both the characteristic peak at 3430 cm^{-1} and that
220 at 1650 cm^{-1} (C=N stretching vibration) demonstrate the successful formation of mPEG-FBA-
221 CA. The peaks at 1650 cm^{-1} (C=N stretching vibration) and 3400 cm^{-1} (O-H stretching vibration
222 in carboxyl group) indicate the conjugation of mPEG-FBA-CA on the surface of FeGdNP
223 generating FeGdNP@mPEG2.

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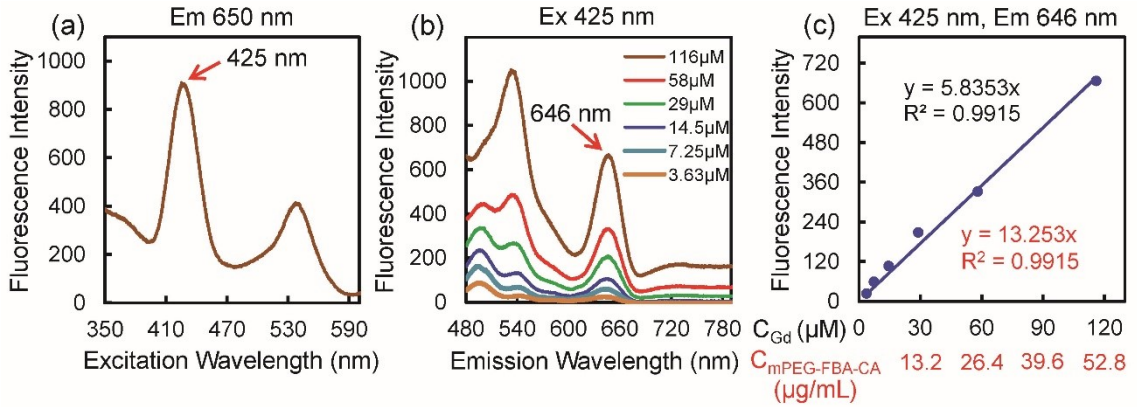

225

226 **Fig. S5.** Fluorescence spectra and standard curve of SA-FeGdNP@mPEG2 nanoparticles. (a):

227 Fluorescence spectrum of SA-FeGdNP@mPEG2 (Em = 650 nm). (b): Fluorescence spectra of

228 SA-FeGdNP@mPEG2 (Ex = 425 nm) with various Gd concentrations from 3.63 to 116 μM.

229 (c): Plot of fluorescence intensity at 646 nm (Ex = 425 nm) as a function of C_{Gd} (μM) or C_{mPEG-}

230 $_{FBA-CA}$ (μg/mL).

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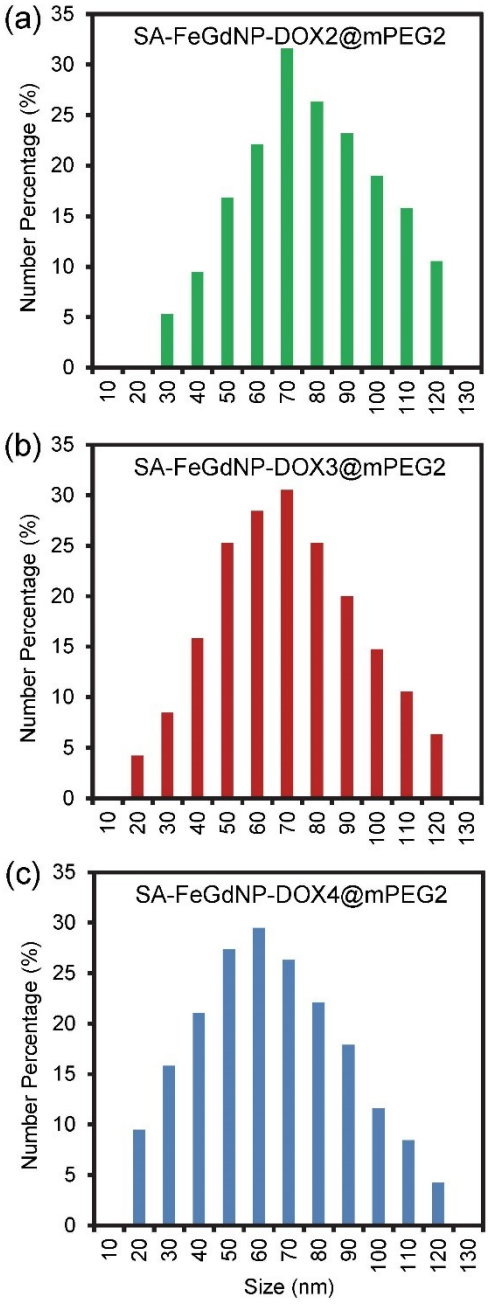

232

233 **Fig. S6.** Size distribution of SA-FeGdNP-DOX2-4@mPEG2 measured from the TEM images.

234 The average particle sizes were respectively measured to be 77.8, 71.3, and 66.6 for SA-

235 FeGdNP-DOX2-4@mPEG2.

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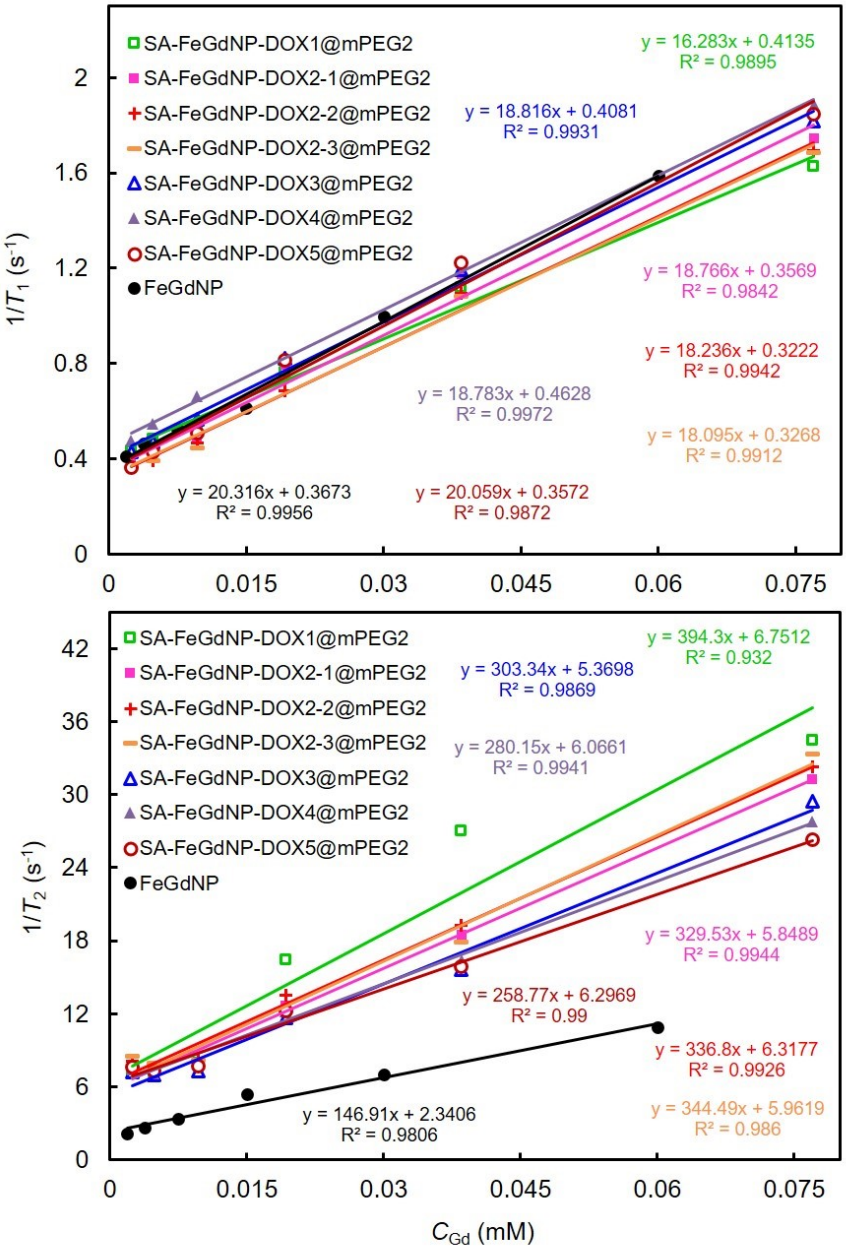

237

238 **Fig. S7.** T_1 relaxation rate ($1/T_1$) (a) or T_2 relaxation rate ($1/T_2$) (b) plotted as a function of C_{Gd}

239 for SA-FeGdNP-DOX1-5@mPEG2. For T_1 relaxation rates: TE = 10 ms, TR = 100 ~ 4000 ms.

240 For T_2 relaxation rates: TR = 2000 ms, TE = 10 ~160 ms. SA-FeGdNP-DOX2-1@mPEG2, SA-

241 FeGdNP-DOX2-2@mPEG2, and SA-FeGdNP-DOX2-3@mPEG2 were synthesized from 3

242 different batches at same conditions. There is no linear relationship between $1/T_2$ and C_{Gd} for

243 SA-FeGdNP-DOX1@mPEG2 (R^2 = 0.932) because 1.6 of DOX/Gd mass ratio is too high

244 resulting in unstable aggregates.

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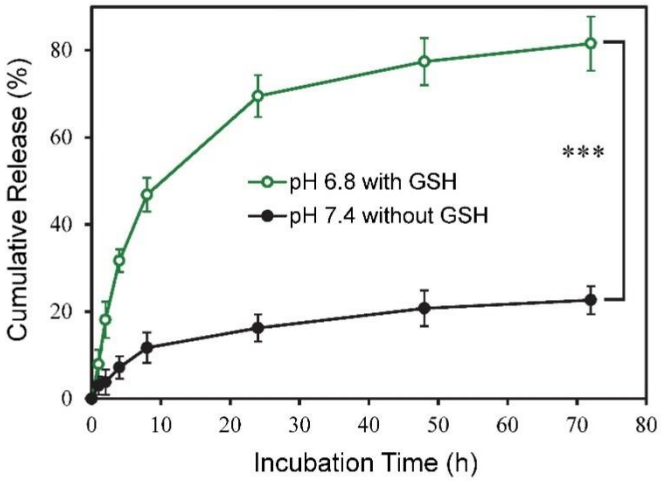

246

247 **Fig. S8.** Release behaviors of DOX from SA-FeGdNP-DOX2@mPEG2 nanoparticles at pH

248 7.4 without GSH or at pH 6.8 with 10 mM of GSH. *** $P < 0.001$.

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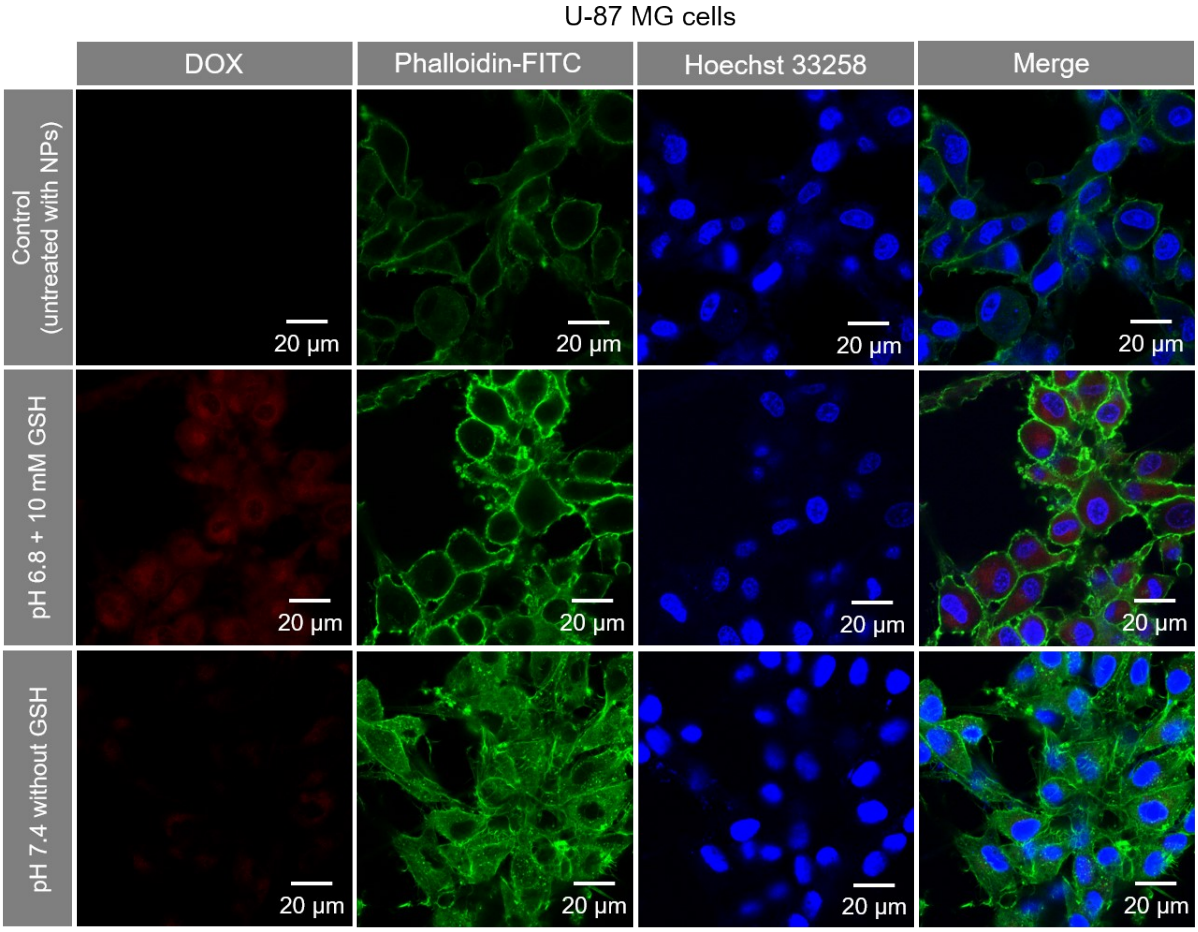


Fig. S9. LSCM images of U-87 MG cells incubated with SA-FeGdNP-DOX2@mPEG2 nanoparticles. Before incubation with cells, the nanoparticles were incubated at 37 °C for 24 h at pH 6.8 with 10 mM of GSH, or at pH 7.4 without GSH. The cells untreated with nanoparticles are used as the control. DOX is red. The cytoskeleton stained with phalloidin-FITC is green. The nucleus stained with Hoechst 33258 is blue.

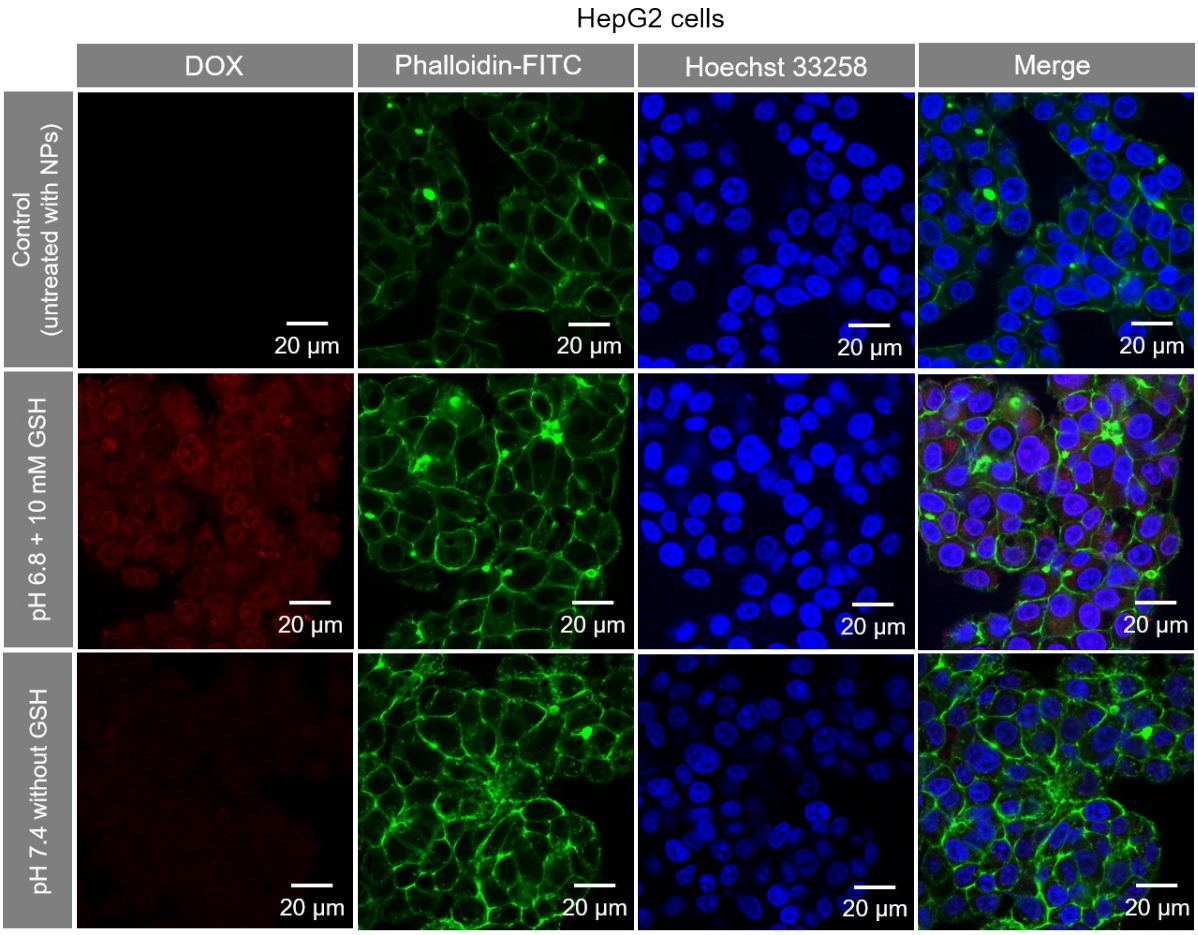


Fig. S10. LSCM images of HepG2 cells incubated with SA-FeGdNP-DOX2@mPEG2 nanoparticles. Before incubation with cells, the nanoparticles were incubated at 37 °C for 24 h at pH 6.8 with 10 mM of GSH, or at pH 7.4 without GSH. The cells untreated with nanoparticles are used as the control. DOX is red. The cytoskeleton stained with phalloidin-FITC is green. The nucleus stained with Hoechst 33258 is blue.

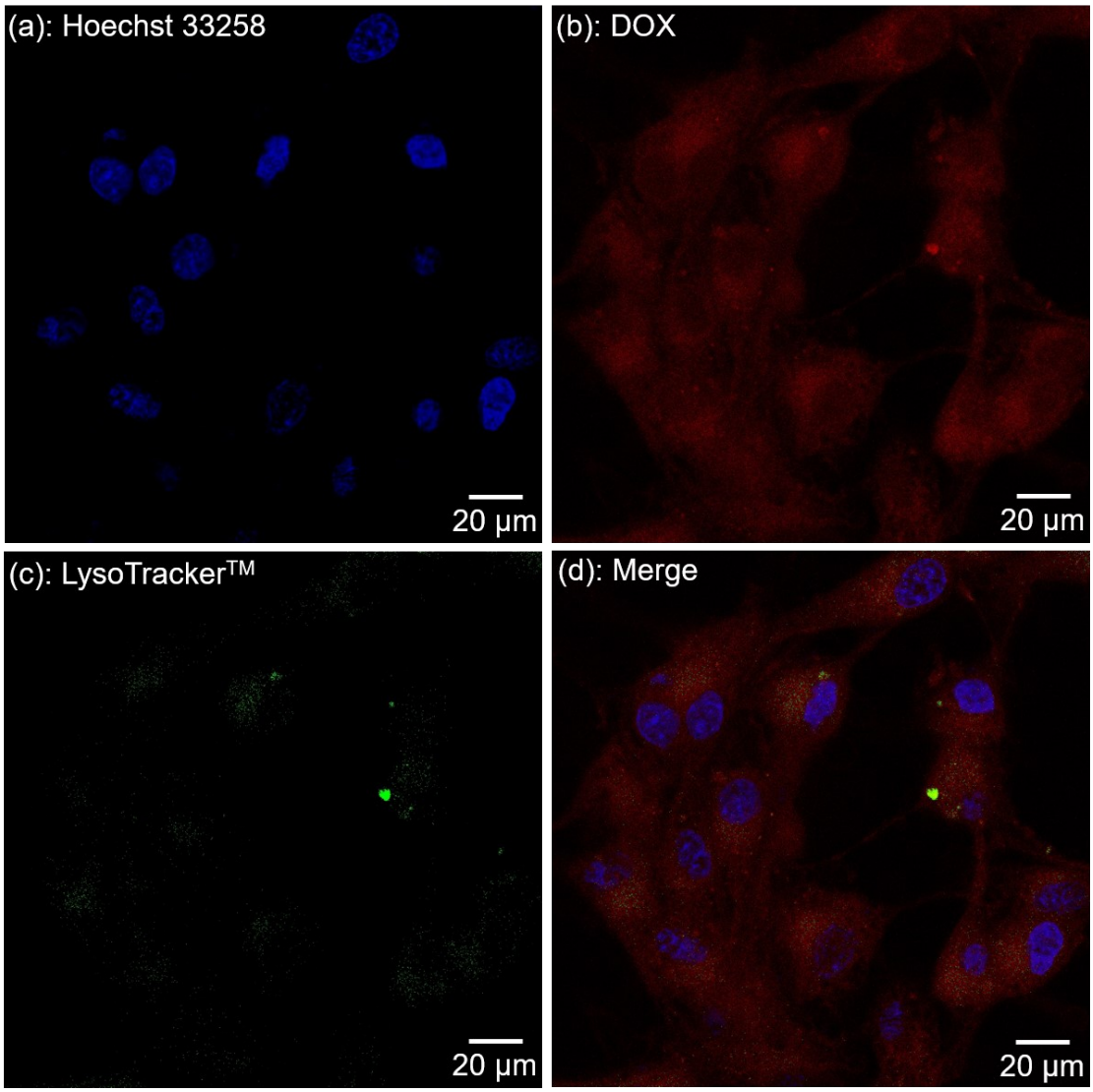


Fig. S11. LSCM images of U-87 MG cells incubated with SA-FeGdNP-DOX2@mPEG2 nanoparticles. Before incubation with cells, the nanoparticles were incubated at 37 °C for 24 h at pH 6.8 with 10 mM of GSH. The nucleus stained with Hoechst 33258 is blue (a, d). The DOX is red (b, d). The endosome and lysosome stained with LysoTracker™ is green (c, d). The red signal overlaps with green and blue, which demonstrates that the nanoparticles were internalized into endosomes *via* endocytosis mechanism, and some of them escaped from the endosome and lysosome and entered into the nucleus.

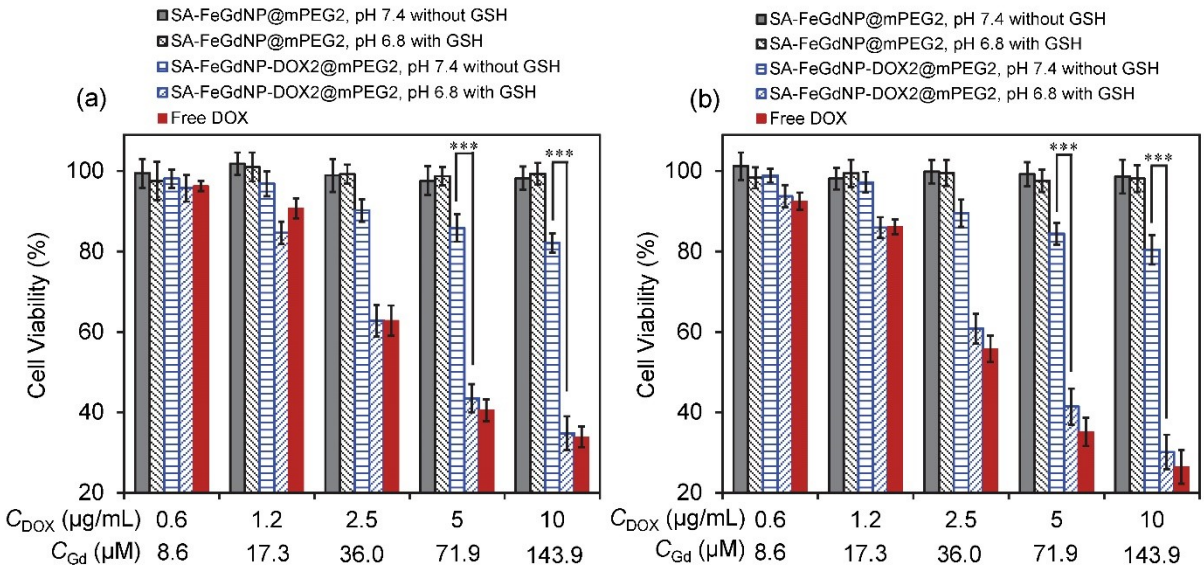
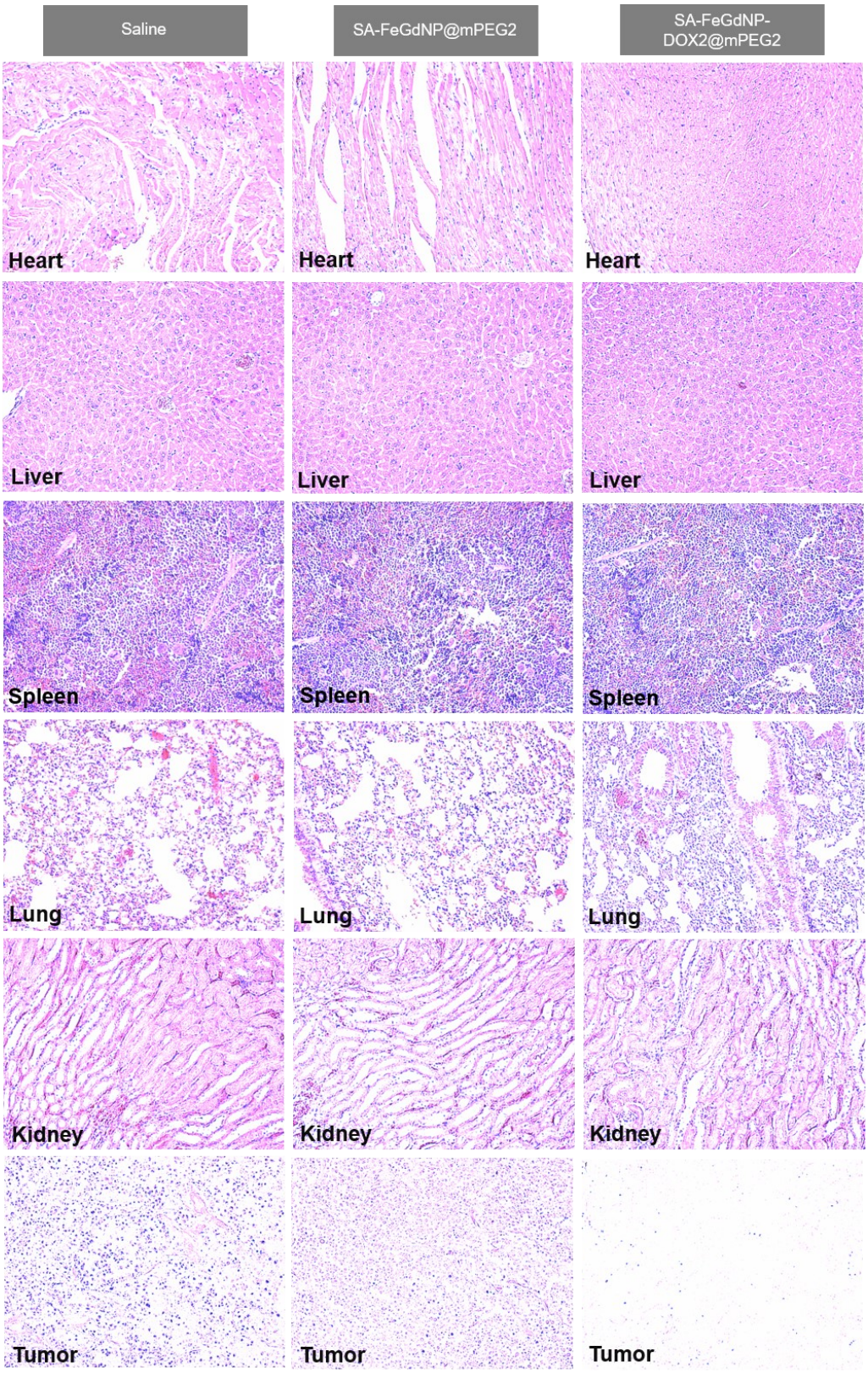


Fig. S12. Cell viabilities of SA-FeGdNP@mPEG2 nanoparticles, SA-FeGdNP-DOX2@mPEG2 nanoparticles, or free DOX on U-87 MG cells (a), or HepG2 cells (b). Before incubation with cells, the nanoparticles were incubated at 37 °C for 24 h at pH 7.4 without GSH, or at pH 6.8 with 10 mM of GSH. The incubation time of the nanoparticles or free DOX with cells was 48 h. ***P < 0.001.



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281 **Fig. S13.** Histological analyses (H&E staining) of main organs and tumors from the
282 subcutaneous U-87 MG tumor-bearing nude mice at day 2 post-injection of saline, SA-
283 FeGdNP@mPEG2 or SA-FeGdNP-DOX2@mPEG2 nanoparticle.