Electronic Supplementary Information

Sequential Catalytic Nanomedicine Augments Synergistic Chemodrug and Chemodynamic Cancer Therapy

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A: Experimental Section

1. Chemicals

Ferric acetylacetonate ($Fe(acac)_3$, 98 %) was purchased from J&K Chemical Reagent Co., Ltd, 1,2-dodecanediol (90 %), glucose oxidase (GOD), 3,3',5,5'-tetramethyl-benzidine (TMB, 95 %), methylene blue (MB, 25 % in water), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC, 98 %), and N-Hydroxysuccinimide (NHS, 98 %) were purchased from Sigma-Aldrich Chemical Company Co., Ltd. Oleic acid (medicinal reagent), β-D-glucose and benzyl ether (97 %) were purchased from Shanghai Macklin Biochemical Co., Ltd. 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino-(polyethylene glycol)$_{2000}$] (DSPE-PEG_{2000}-NH_2, 98%) was purchased from Yarebio Co., Ltd. Mitomycin C (98 %) was purchased from Shanghai Raise Chemical Technology Co., Ltd ,and oleylamine (80 %) was purchased from Aladdin Biotechnology Co., Ltd. All the chemical agents were used directly with no further purification.

2. Synthesis of iron oxide nanoparticles (SPION)

12 nm-sized superparamagnetic iron oxide nanoparticles were synthesized by high-temperature pyrolysis protocol. Typically, $Fe(acac)_3$ (2 mmol), 1,2-dodecanediol (10 mmol), oleic acid (6 mmol) and oleylamine (6 mmol) were dispersed in benzyl ether (10 mL) at room temperature. The mixture was magnetically stirred and then heated to 200 ℃ for 2 h under argon atmosphere, afterwards it was further heated to reflux (about 300 ℃) for 1 h. After cooled down to room temperature by removing the heat source, the acquired mixture was dispersed in ethanol for precipitation. After collected by centrifugation (9000 rpm, 10 min), hydrophobic SPIONs were then washed for three times with ethanol and chloroform, and re-dispersed into chloroform for further use.

3. PEGylation of iron oxide nanoparticles

The as-prepared hydrophobic iron oxide nanoparticles could not be used for in vivo cancer therapy. To achieve high stability in physiological solution for further in vivo evaluation, DSPE-PEG_{2000}-NH_2 was applied to modify the surface of SPIONs. Typically, DSPE-PEG_{2000}-NH_2 (50 mg) was dissolved into chloroform (9 mL). After the addition of chloroform solution (1 mL) containing SPIONs (10 mg), the mixture was sonicated for 10 min and then incubated at 60 ℃ in a rotary evaporator for 1 h under vacuum. Finally, deionized water (1 mL) was added into the flask and then sonicated until uniform solution was formed. The as-prepared solution was kept at 4 ℃ for further use.

4. Synthesis of PEG modified MMC-loaded iron oxide nanoparticles (SPION-MMC@PEG NPs)

The synthesis of SPION-MMC@PEG NPs was similar to that of SPION@PEG NPs. Typically, DSPE-PEG_{2000}-NH_2 (50 mg) was dissolved into chloroform (8 mL). After the addition of chloroform solution (1 mL) containing SPIONs (10 mg) and the tetrahydrofuran (THF) solution of MMC (1 mL, 5 mg mL^{-1}), the mixture was then sonicated for homogenization. Following the experimental protocols as-mentioned above, PEGylated MMC-loaded iron oxide nanoparticles were produced.

5. Loading of GOD onto SPION@PEG and SPION-MMC@PEG

The loading of GOD onto SPION@PEG NPs and SPION-MMC@PEG NPs was achieved via amide reaction. Typically, EDC (76 mg), NHS (114 mg) and GOD (5 mg) were dissolved into deionized water (2 mL). The mixture was then magnetically stirred for 3 h to active the carboxyl groups of GOD. Afterwards, PEGylated SPIONs with or without MMC were added, respectively. After 24 h reaction, the as-synthesized samples were collected by high speed centrifugation (17000 rpm, 8 min) and washed with deionized water for several times.

6. Characterization

X-ray diffraction (XRD) was fulfilled on a Rigaku D/MAX-2200 PC XRD system with Cu Kα radiation (λ = 1.54 Å) at 40 kV and 40 mA. X-ray photoelectron spectroscopy (XPS) spectrum was gathered by ESCAlab250 (Thermal Scientific). Hydrodynamic particle size and zeta potential measurements were determined on Zetasizer Nanoseries (Nano ZS90, Malvern Instrument Ltd.). The Fe_3O_4 concentration was determined by inductively coupled plasma optical emission spectrometry (ICP-OES, Agilent Technologies, US). Electron spin resonance (ESR) spectra of SMG nanocatalysts were performed by Bruker EMX1598 spectrometer. UV-vis-NIR absorption spectra were recorded by UV-3600 Shimadzu UV-vis-NIR spectrometer with QS-grade quartz cuvettes at room temperature. Transmission electron microscopy (TEM) and electron energy loss spectrum (EELS) images were obtained for morphology and elemental analysis on a JEM-2100F electron microscope operated at 200 kV. The confocal laser scanning microscopy (CLSM) images were obtained in FV1000 (Olympus Company, Japan). The optical absorbance spectra were determined on Molecular Device SpectraMax M2. Thermogravimetric analysis (TGA) and differential thermal analysis (DTA) were performed in STA449C (NETZSCH Company, Germany).

7. In vitro dissolved oxygen analysis

Dissolved oxygen assays were conducted by a dissolved oxygen meter in PBS buffer with the addition of varied concentration of β-D-glucose (10 mM, 20 mM, 40 mM, and 80 mM). Herein, the corresponding concentration β-D-glucose was dispersed into PBS buffer solution (pH = 6.5, 20 mM, 10 mL) under magnetic stirring, followed with the addition of SPION@PEG-GOD NPs. The electrode probe of dissolved oxygen meter was promptly immersed in the solution for dissolved oxygen level assessment.

To estimate the impacts of pH values on the catalytic performance of SPION@PEG-GOD NPs, the concentration of β-D-glucose was fixed to 40 mM while the pH values of PBS buffer were varied to 4.5, 5.5 and 6.5. Then, the corresponding data were obtained following the afore-mentioned procedure.

8. Michaelis-Menten kinetic assay

To visualize the chromogenic reaction (λ = 650 nm) between SPION@PEG-GOD NPs (10 mg mL^{-1}, 75 μL) and β-D-glucose in a series of concentration (0.375 mM, 0.75 mM, 1.5 mM, and 3 mM), TMB (1 mL, 3.2 mM) was dissolved in NaAc buffer solution (pH = 6.5, 0.2 M) with the final volume fixed to 3 mL. By protracting the respective initial velocities to β-D-glucose concentrations, the Michaelis-Menten kinetic curves of SPION@PEG-GOD were obtained. Finally, the corresponding Michaelis-Menten constant (K_m) and maximal velocity (V_{max}) were figured out.

9. Methylene blue depigmentation assay

Methylene blue (MB, λ = 664 nm) was applied for hydroxyl radical detection. Typically, MB test solution (2.5 mg L^{-1}) was diluted with PBS buffer (pH = 6.5, 0.02 M) containing glucose (10 mM, 200 μL) and SPION@PEG-GOD NPs (10 mg mL^{-1}, 20 μL). The general volume was fixed to 3 mL and afterwards the absorbance was recorded by a microplate reader at corresponding time points. As to control group one (glucose and free GOD) and control group two (glucose only), the above-mentioned SPION@PEG-GOD NPs were replaced with free GOD at the equal dose or PBS buffer for comparison.

10. Cellular experiments

10.1 Intracellular uptake evaluation

The mammary cancer line 4T1 cells (noted as 4T1 cells) were purchased from Shanghai Institute of Cells, Chinese Academy of Sciences and cultured in Dulbecco's Modified Eagle's Medium (DMEM, high glucose, GIBCO, Invitrogen) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were cultured at 37 °C in a humidified atmosphere

containing 5% CO_2.

For CLSM observation, SPION@PEG NPs was labeled by FITC (fluorescein isothiocyanate), and 4T1 cells were seeded onto CLSM-exclusive culture dishes at the density of 1×10^5 cells/dish in DMEM medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and incubated at 37 ℃ for 24 h. After being co-incubated with 1 mg mL^{-1} of FITC-labeled SPION@PEG NPs for 2 h, cells were washed for three times with PBS and counterstained with 4',6-diamidino-2-phenylindole (DAPI) to label nuclei. Then, the cells were visualized at interval time with CLSM to observe the intracellular uptake of SPION@PEG NPs.

10.2 In vitro cytotoxicity assessment

The cytotoxicity of SMG nanocatalysts was assessed by a standard CCK-8 viability assay (Cell Counting Kit-8, 7Sea Pharmatech Co., Ltd, Shanghai, China) of 4T1 cells. The 4T1 cells were seeded in 96-well plates at a density of 1×10^4 cells/well for 24 h. The culture medium was then replaced by medium containing various concentrations of SPION@PEG NPs, SPION-MMC@PEG NPs, SPION@PEG-GOD NPs and SMG nanocatalysts. After 24 h co-incubation, the medium was replaced with the CCK-8 solution (10% volume in serum free DMEM medium) for 2 h. Then, the cell viabilities were evaluated and compared to the control group. The hypoxic and normoxic cytotoxicity of free MMC was evaluated with 4T1 mammary cancer cells cultured at 37 °C in a humidified atmosphere containing 5% CO_2, 21% O_2 and 5% CO_2, 1% O_2, respectively.

10.3 Live/dead cell viability test

4T1 cells were seeded into CLSM-exclusive culture dishes at the density of 1×10^5 cells/dish in DMEM medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and incubated at 37 ℃ for 24 h to attach. Afterwards, the medium was replaced with or without nanoparticles dispersed in DMEM medium at the concentration of 2.54 μg mL^{-1}, and the cells were co-incubated for 24 h. To visualize the live cells and dead cells, the Calcein-AM/PI staining reagents were chosen for staining the viable cells as green fluorescence (λ_{Ex} = 490 nm and λ_{Em} = 515 nm), and dead cells as red fluorescence (λ_{Ex} = 535 nm and λ_{Em} = 617 nm). Briefly, Calcein-AM solution (200 μL of 10 mM) and PI solution (200 μL of 10 mM) were added after removing the culture medium and rinsing the culture dishes with PBS. After the incubation for 15 min, the staining reagent solution was discarded and the cell were rinsed twice by PBS, which were then visualized by CLSM.

10.4 Intracellular reactive oxygen species (ROS) assay

To visualize the intracellular reactive oxygen species (ROS) generation by CLSM, 1×10^5 of 4T1 cancer cells were seeded into CLSM-exclusive culture dishes in DMEM medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and incubated at 37 ℃ for 24 h to attach. Thereafter, the medium was discarded and the disks were rinsed twice by PBS, following the addition of DMEM high glucose (pH 7.4, 1 mL) containing SMG nanocatalysts (100 μg mL^{-1}). Finally, the co-incubated medium was discarded and replaced by the fluorescence probe after PBS rinsing. In this assay, 2',7'-Dichlorofluorescin diacetate (DCFH-DA, 100 mM) was used for ROS detection, leading to the engendering of fluorescent agent DCF (λ_{Ex} = 480 nm and λ_{Em} = 525 nm), which could be observed by CLSM for intracellular ROS labeling. For the accuracy of fluorescent intensity obtained by CLSM, the above medium was discarded, followed by twice PBS rinsing.

10.5 Cell apoptosis assay

The cell apoptosis was assessed by annexin V-FITC/PI double staining according to the apoptotic assay kit. 4T1 cells cultured in 6-well plates were incubated with SPION@PEG NPs, SPION-MMC@PEG NPs, SPION@PEG-GOD NPs and SMG nanocatalysts at the concentration of 2.54 μg mL^{-1} for 24 h. The cells were digested and washed with PBS buffer for several times. The collected cells were resuspended in 500 μL of binding buffer and stained with 5 μL of annexin V-FITC and 5 μL of PI for 15 min in the dark. The stained cells were analyzed by flow cytometry.

10.6 In vitro and in vivo T_2-weighted MR imaging

A 3.0 T clinical MRI instrument (GE signa) was applied for the in vitro and in vivo MRI assessments of SMG nanocatalysts. Despite with predominant dispersity in water-based solvent, SMG nanocatalysts at various concentration of iron was dispersed in aqueous xanthan gum solution, a representative suspending agent employed in MRI that causes little impact on image results, based on the typical in vitro MRI experimental procedure. For quantitative analysis, the values of $1/T_2$ were linear fitted to corresponding iron concentrations to calculate the r_2 value of SMG nanocatalysts. For in vivo MRI dynamic assessment, 4T1 mammary tumor xenograft was applied for establishing 4T1 mammary tumor xenograft tumor models by subcutaneous injecting 4T1 cells into a female BALB/c nude mouse, and the scanning interval was fixed to 30 min. The SMG nanocatalysts were intravenously injected into tumor-bearing mice at the dose of 30 mg kg^{-1}.

11. Animal experiments

Female Kunming mice and female BALB/c nude mice of 6 weeks old were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All the animal procedures were performed under the protocols accredited by Department of Laboratory Animal Science, Fudan University. All animal experiments were in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Department of Laboratory Animal Science, Fudan University.

11.1 In vivo biosafety evaluation

Six weeks-old female Kunming mice were divided into 6 groups (n = 5) randomly: control, 5 mg kg^{-1}, 10 mg kg^{-1}, 15 mg kg^{-1}, 20 mg kg^{-1} and 30 mg kg^{-1} groups. Saline and corresponding doses of SPION@PEG-GOD NPs were injected intravenously. After the further feeding for 30 days, all Kunming mice were executed and dissected. The collected blood samples were utilized for blood analysis including blood biochemistry and routine while the major organs (heart, liver, spleen, lung, and kidney) of mice were dissected and stained with hematoxylin and eosin (H&E) for pathological analysis.

11.2 In vivo pharmacokinetic evaluation

For in vivo tissue distribution assessment of SMG nanocatalysts, BALB/c nude mice at the age of 6 weeks were stochastically allocated into 3 groups (n = 4 in each group), following with the subcutaneously injection of 4T1 mammary cancer cell (1×10^6 cells per site). Tumors were allowed to proliferate to approximate 60 mm^3, afterwards all mice were intravenously injected with SMG nanocatalysts at the dose of 30 mg kg^{-1}. All mice were dissected at corresponding time post injection (2 h, 24 h and 48 h), and the dissected tumors and major organs including heart, liver, spleen, lung and kidneys were rinsed with PBS, weighted and digested with aqua regia. The biodistributions in various organs and tumors were determined by Fe percentage of injected dosage as determined by ICP-OES test.

To evaluate elimination half-time, female Kunming mice (n = 4) aged 4 weeks were intravenously injected with SMG nanocatalysts. At 2 min, 5 min, 10 min, 30 min, 1 h, 2 h, 4 h, 8 h, and 24 h, 10 μL blood was extracted by gashing the tail vein and immediately dispersed into 990 μL physiological saline containing heparin sodium injection (100 units mL^{-1}). The concentrations of Fe were determined by ICP-OES, and the in vivo terminal half-time of SMG nanocatalysts in blood ($\tau_{1/2}$) was calculated.

The evaluation of the metabolic elimination circumstances of SMG nanocatalysts was conducted on female Kunming mice (n = 3) aged 4 weeks. After the intravenous injection of

SMG nanocatalysts, the mice were promptly settled in metabolic cage, and their urine and feces were collected at 2 h, 6 h, 12 h, 24 h, 36 h, and 48 h. The concentrations of Fe were determined by ICP-OES and the data were recorded for analysis.

11.3 In vivo therapeutic assay

For in vivo therapeutic assessment, BALB/c nude mice were stochastically assigned (n = 4) into 5 groups: control group, SPION-MMC@PEG group, SPION@PEG-GOD group, free MMC group and SMG nanocatalyst group, followed by the subcutaneous injection of 1×10^6 4T1 mammary cancer cells per mouse. To initiate the therapy, tumors were allowed to grow to approximate 60 mm^3. The relative tumor volume V_R is defined as $V_R = V V_0^{-1}$ (V_0: Tumor volume on day 0). Saline and corresponding agents were injected intravenously at the dosage of 2.5 mg MMC per kg, among which the doses of SPION@PEG-GOD were equivalent to SMG nanocatalysts except MMC. The body weights and tumor volumes were obtained in the interval of two or four days to assess the curative effect, meanwhile the digital photos of tumor-bearing mice were taken. The injection of agents was performed only once, and all BALB/c nude mice were executed for anatomy and histopathological assessment after the therapeutic period.

12. Statistical analysis

The significance of the data is analyzed according to a Student's *t* test: *p <0.05, **p < 0.01 and ***p < 0.001. The samples/animals were allocated to experimental groups and processed randomly.

B: Supplementary figures

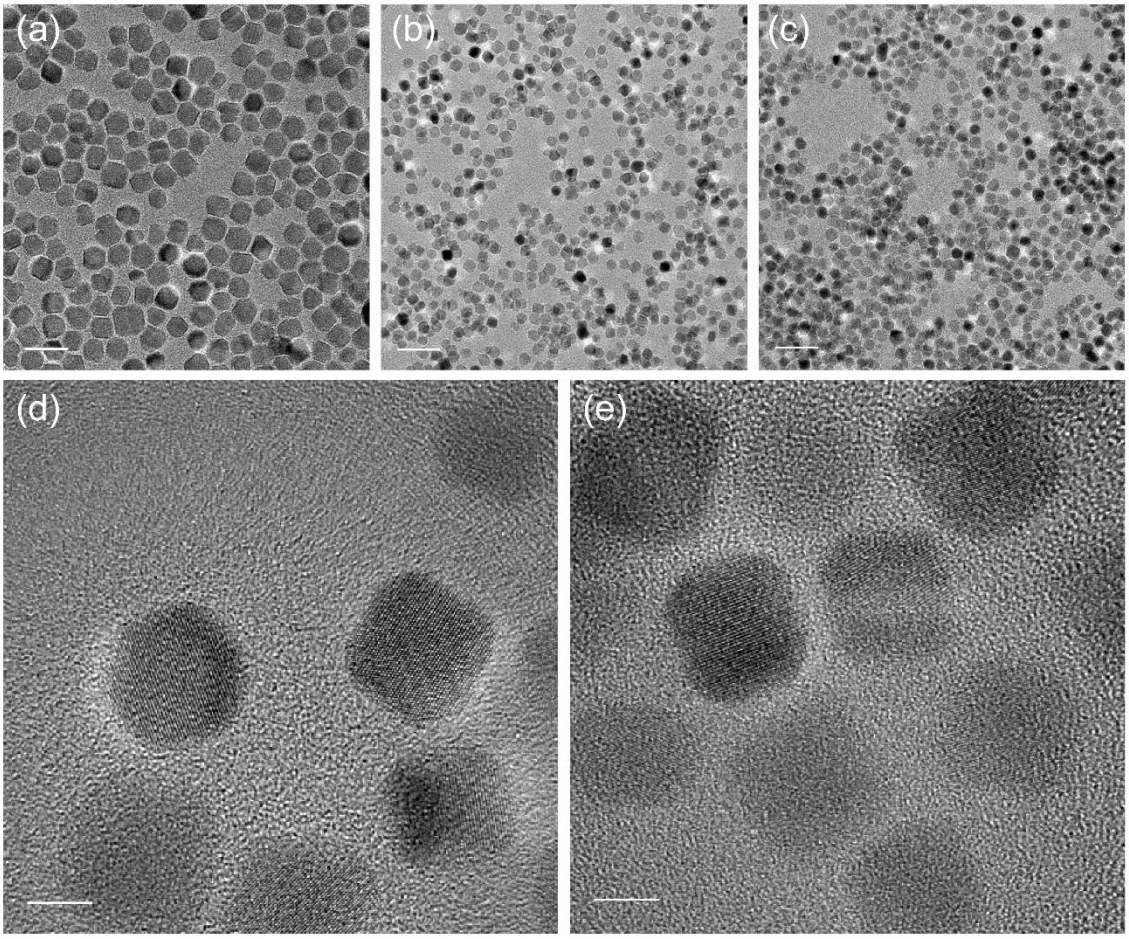

Figure S1. TEM images of (a) SPIONs, (b, d) SPION@PEG NPs and (c, e) SMG nanocatalysts at varied magnifications. Scale bar: a: 20 nm; b, c: 100 nm; d, e: 5 nm.

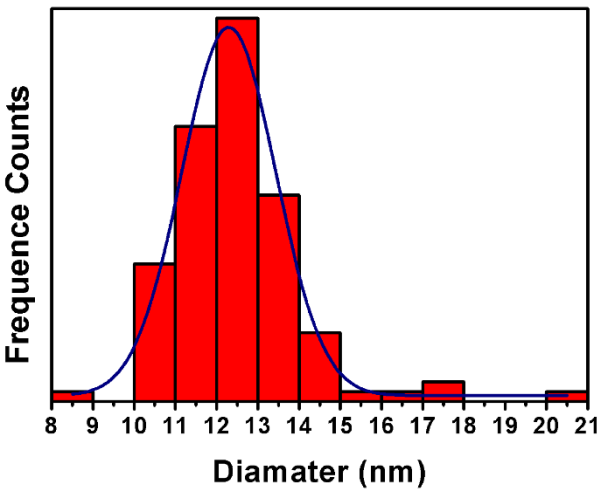

Figure S2. The frequency distribution histogram of 12 nm-sized SPIONs obtained by the statistics from TEM image.

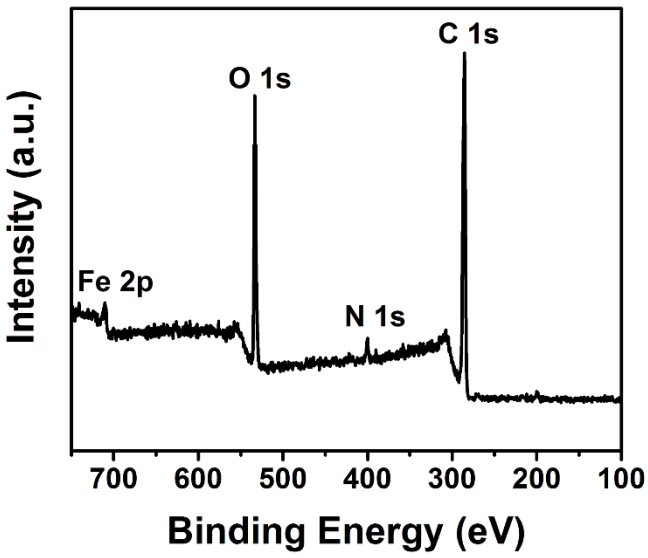

Figure S3. X-ray photoelectron spectroscopy (XPS) spectrum of SMG nanocatalysts.

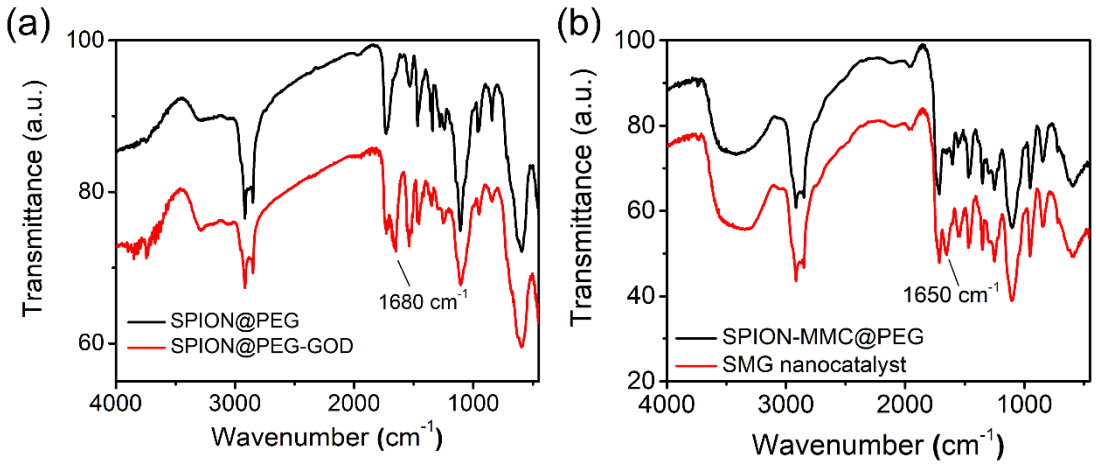

Figure S4. FT-IR spectra of (a) SPION@PEG with or without GOD and (b) SPION-MMC@PEG with SMG nanocatalysts.

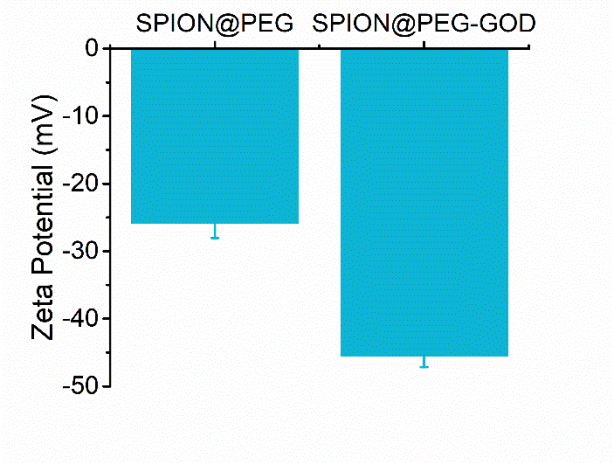

Figure S5. Zeta potentials of SPION@PEG and SPION@PEG-GOD. Data was presented as mean ± s.d (n =3).

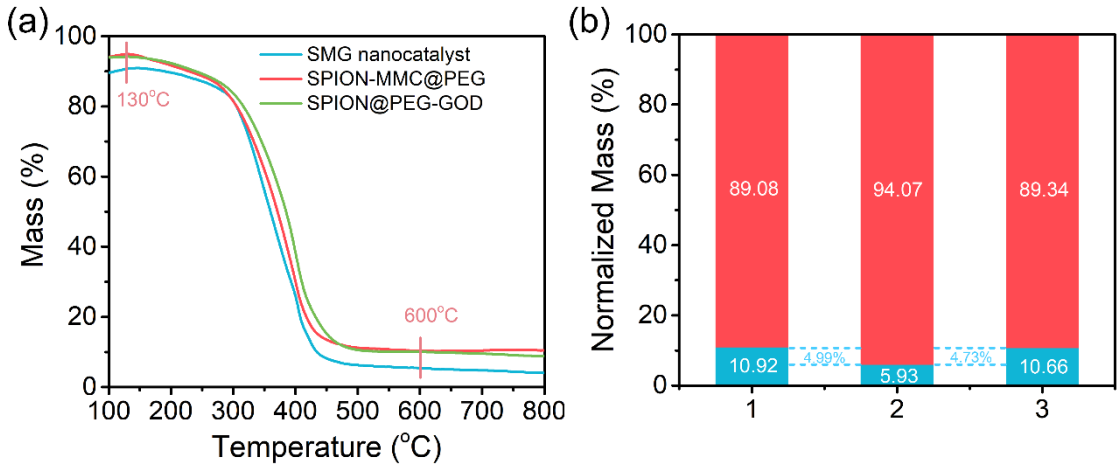

Figure S6. Thermogravimetric analysis (TGA) curves and corresponding normalized mass loss plot of SPION-MMC@PEG NPs, SPION@PEG-GOD NPs and SMG nanocatalysts. 1: SPION-MMC@PEG NPs; 2: SMG nanocatalysts; 3: SPION@PEG-GOD NPs.

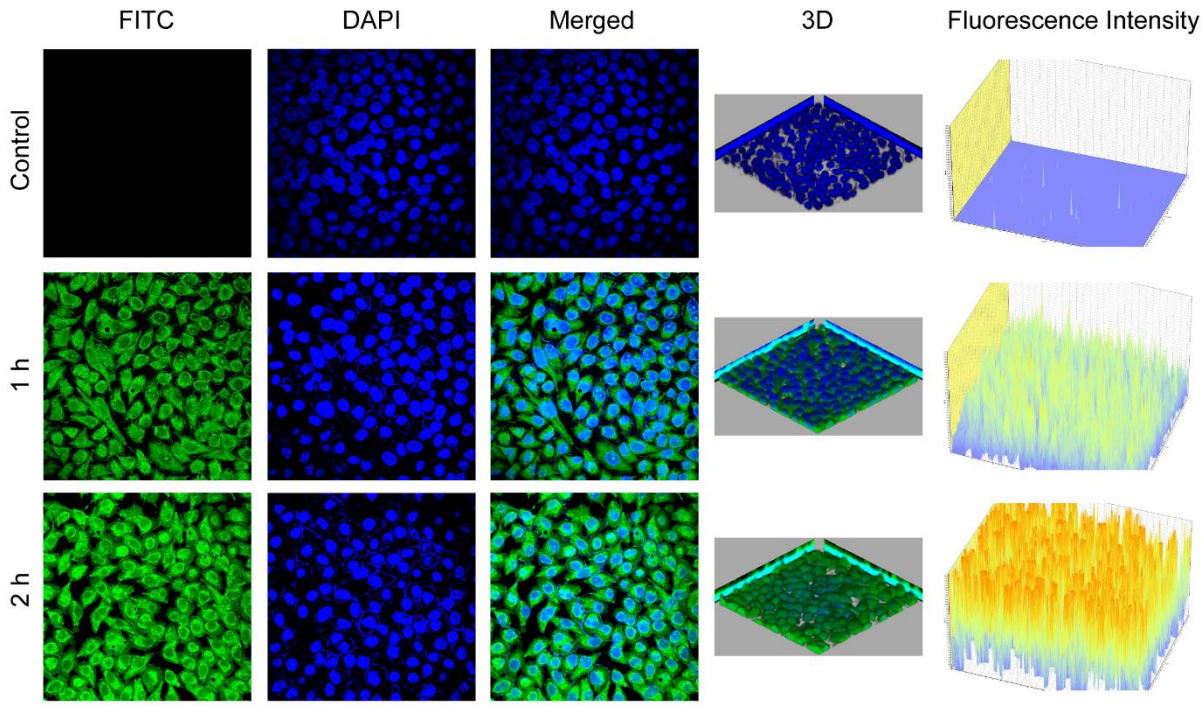

Figure S7. CLSM images of 4T1 cells co-incubated with FITC-labeled SPION@PEG NPs at the concentration of 1 mg/mL at varied time points (1 h, 2 h) for intracellular uptake assessment.

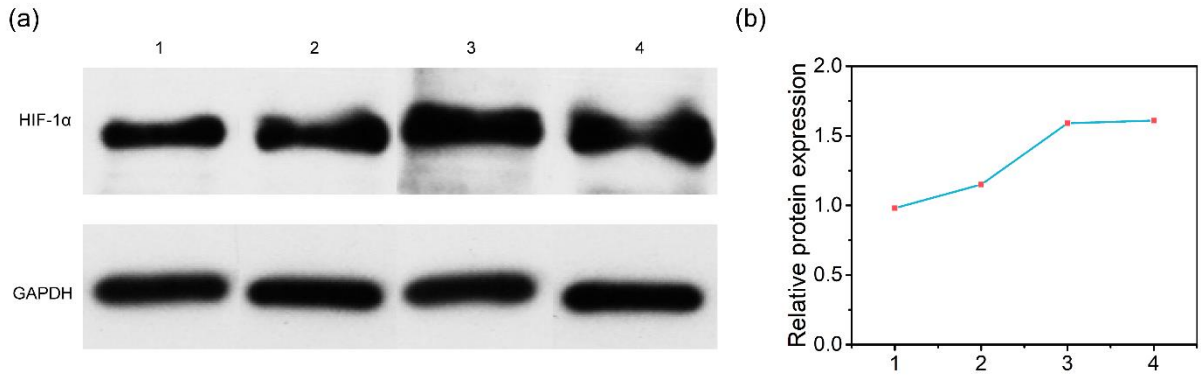

Figure S8. (a) HIF-1α-specific immunoblotting (IB) with GAPDH serving as internal reference and (b) corresponding relative protein expression diagram. 1: SPION@PEG; 2: SPION-MMC@PEG; 3: SPION@PEG-GOD; 4: SMG nanocatalysts.

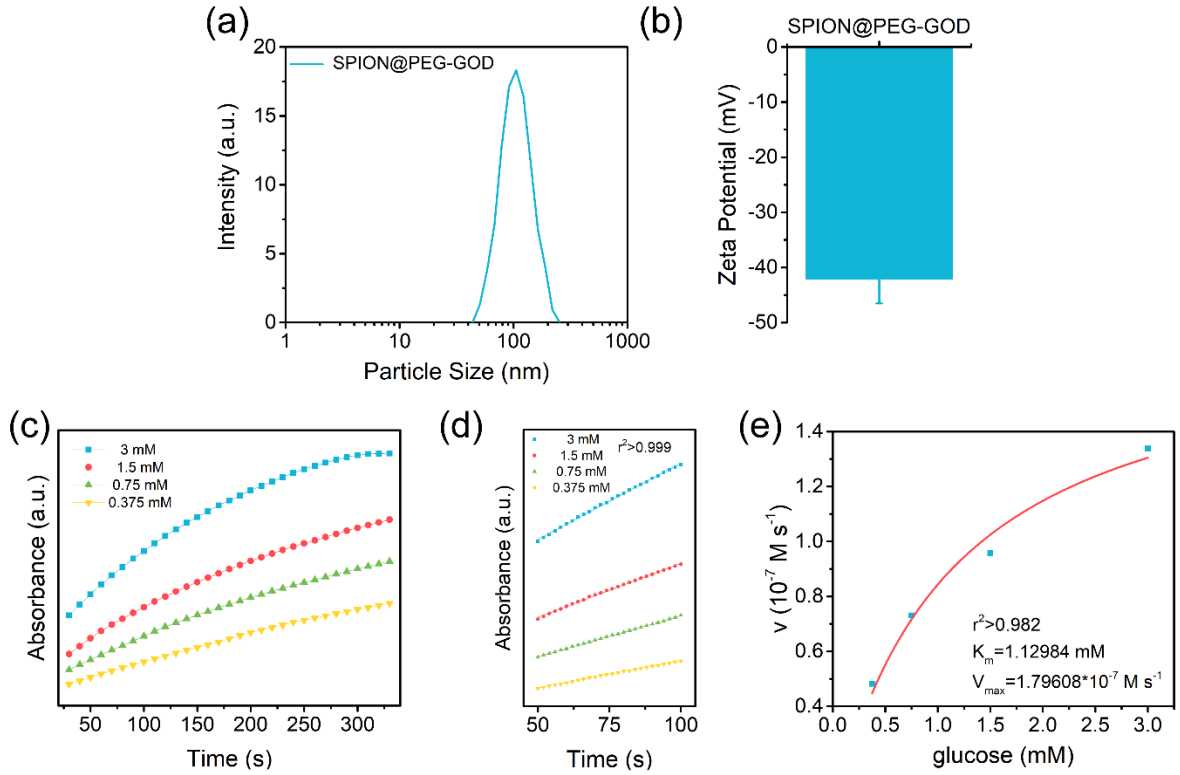

Figure S9. Characterizations of SPION@PEG-GOD NPs stored for several months. (a) Hydrodynamic diameter distribution. (b) Zeta potentials. Data was presented as mean ± s.d (n =3). (d) Time-relied absorbance of TMB in the presence of glucose at elevated concentrations (0.375 mM, 0.75 mM, 1.5 mM, and 3 mM). (e) Linear portions of absorption curves during the time period from 50 s to 100 s. (f) The fitting curve on the basis of Michaelis-Menten kinetic under the catalysis of SPION@PEG-GOD NPs.

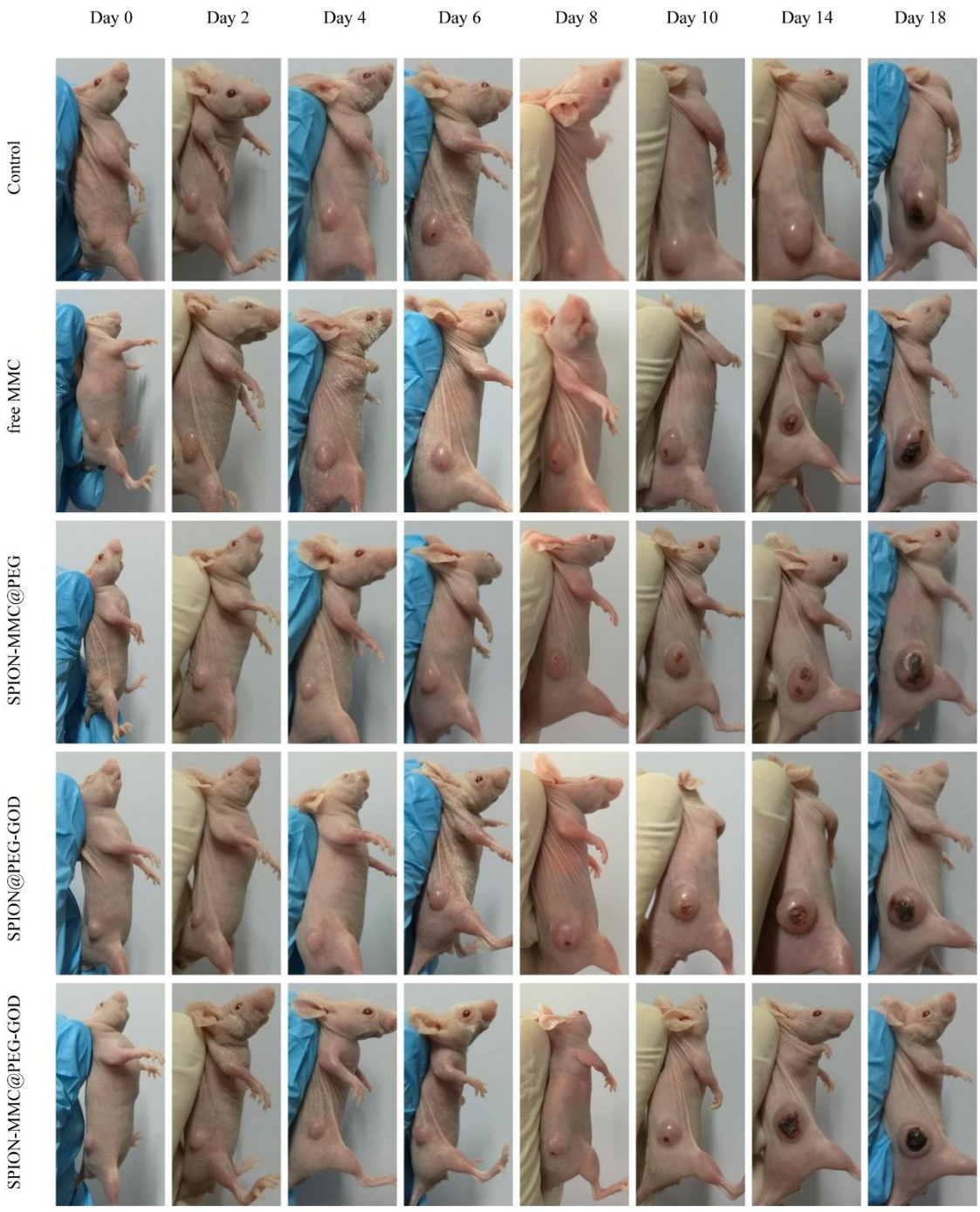

Figure S10. Digital photos of tumor-bearing mice in diverse groups during the therapeutic period.

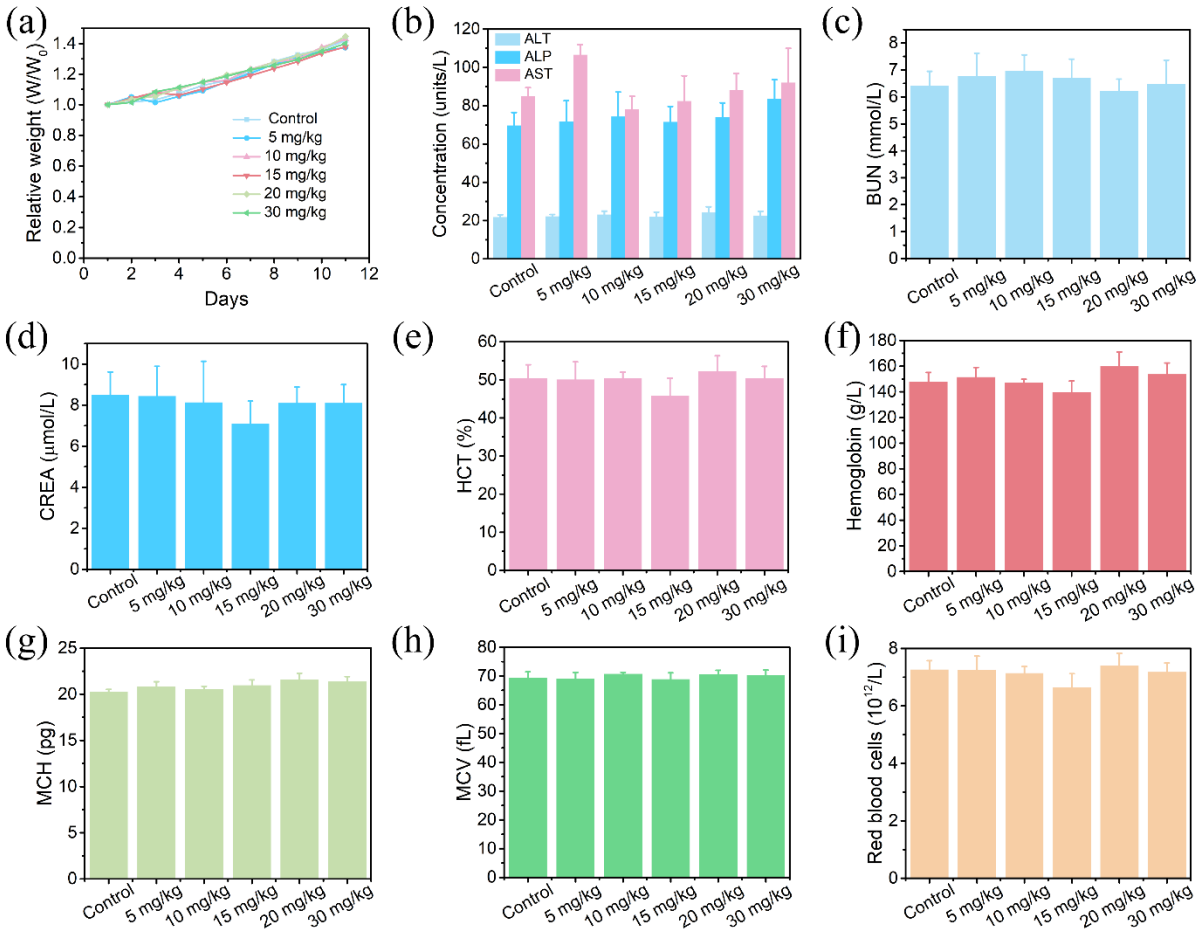

Figure S11. Routine blood test and blood biochemical analysis of SMG nanocatalysts.

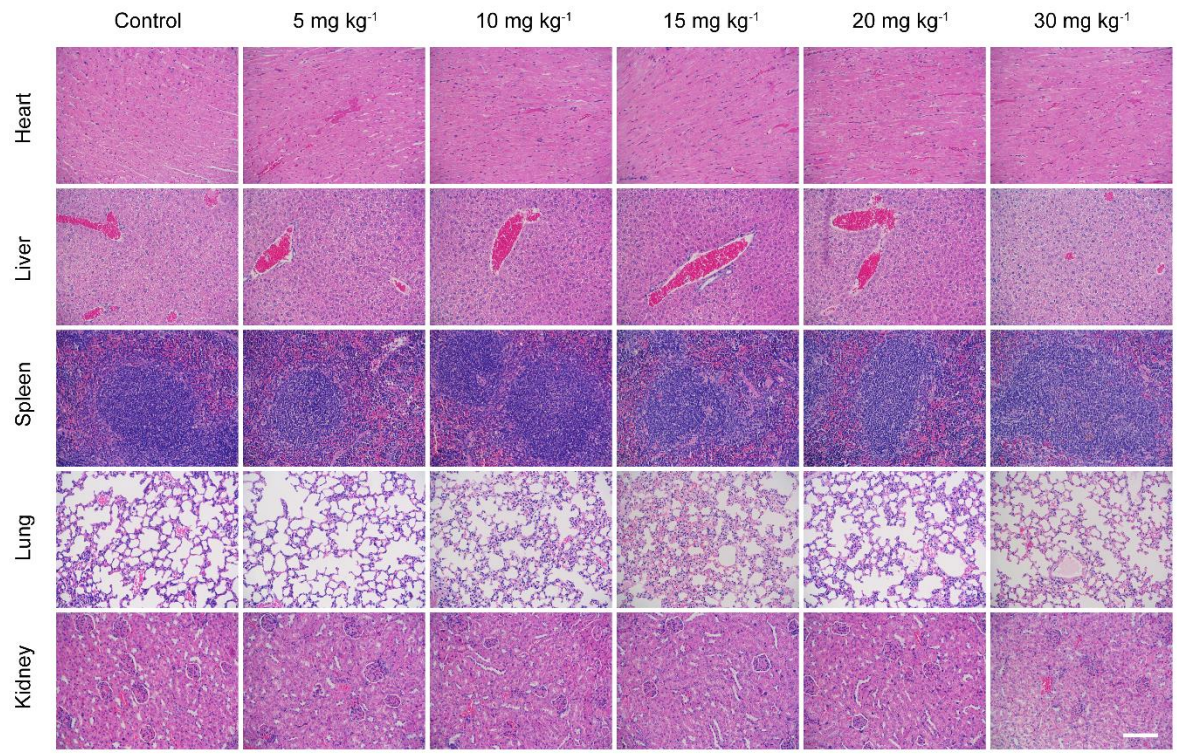

Figure S12. Pathological sections of the major organs stained with H&E for in vivo histocompatibility evaluation of SPION@PEG-GOD NPs. Scale bar: 100 μm.