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Electronic Supplementary Information

Polyaniline-loaded γ-polyglutamic acid nanogels as a platform for photoacoustic imaging-guided tumor photothermal therapy†

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Part of experimental details:

Materials

γ-polyglutamic acid (γ-PGA, Mw = 1000 kDa) was acquired from Nanjing Saitesi Co., Ltd. (Nanjing, China). Aniline hydrochloride (99%) was obtained from Alfa Aesar Chemicals Co., Ltd. (Shanghai, China). Cystamine dihydrochloride (Cys, 99%) was supplied by Energy Chemical Co., Ltd. (Shanghai, China). 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) was purchased from J&K Chemical Ltd. (Shanghai, China). Dioctylsodium sulfosuccinate (AOT), sodium sulfite (Na_2SO_3), and sodium bicarbonate ($NaHCO_3$) were obtained from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). Dichloromethane (DCM) was purchased from Shanghai Lingfeng Chemical Reagent Co., Ltd. (Shanghai, China). Poly(vinyl alcohol) (PVA, 87-89% hydrolyzed, Mw = 85-124 kDa) and ammonium persulfate (APS) were supplied by Aldrich (St. Louis, MO). All chemicals were used without further purification. Cell Counting Kit-8 (CCK-8) was purchased from 7Sea Pharmatech Co., Ltd. (Shanghai, China). 4T1 cells (4T1 murine breast cancer cells) were obtained from Institute of Biochemistry and Cell Biology, the Chinese Academy of Sciences (Shanghai, China). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), penicillin, and streptomycin were obtained from Hangzhou Jinuo Biomedical Technology (Hangzhou, China). Water used in all experiments was purified by a Milli-Q Plus185 water purification system with a resistivity higher than 18.2 MΩ.cm (Millipore, Bedford, MA).

Characterization techniques

UV-vis spectra were collected using a Lambda 25 UV-vis spectrophotometer (PerkinElmer, Boston, MA). Samples were dispersed in water before measurements. Dynamic light scattering (DLS) and zeta potential measurements were carried out using a Malvern Zetasizer Nano ZS model ZEN3600 (Worcestershire, UK) coupled with a standard 633 nm laser. Thermal gravimetric analysis (TGA) was performed using a TG 209 F1 thermo gravimetric analyzer (NETZSCH Instruments Co., Ltd., Selb/Bavaria, Germany) under N_2 atmosphere with a temperature range of 26-900 ^oC and heating rate of 20 ^oC/min. The morphology of the NPs was observed using transmission electron microscopy (TEM, JEOL 2010F analytical electron microscope, Tokyo, Japan) at a voltage of 200

kV or scanning electron microscope (SEM, S-4800 analytical electron microscope, Tokyo, Japan) at a voltage of 15 kV. TEM samples were prepared by transferring a diluting NP suspension (100 μg/mL, 5 μL) onto a carbon-coated copper grid and air dried before measurements, while SEM samples were prepared by dropping an NG suspension (100 μg/mL, 5 μL) onto aluminum foil and air dried before measurements. To calculate the size distribution, TEM and SEM images were randomly selected and at least 300 NPs from different images were measured using an ImageJ software (http://www.rsb.info.nih.gov/ij/download.html) for each sample. Fourier transform infrared (FTIR) spectra were recorded on a Nicolet Nexus 670 FTIR spectrophotometer (Thermo Nicolet Corporation, Madison, WI). Samples were dried and mixed with ground KBr crystals and pressed as pellets before measurements. For PA imaging, each individual hole on a home-made agar plate was filled with 0.3 mL of NGs at different concentrations (0, 0.1, 0.25, 0.5 and 1.0 mg/mL, respectively). The samples were monitored using multispectral optoacoustic tomography system (MSOT, iThera Medical GmbH, Munich, Germany) with a 785 nm laser. To determine the concentration-dependent photothermal conversion property, a series of specimens with different NG concentrations (0, 0.5, 1, 2 and 4 mg/mL, respectively) were put into quartz cuvettes, respectively, and then irradiated by a 785 nm laser for 300 s (facula area: 0.25 cm^2, output power density: 1.5 W/cm^2) using a laser device (Shanghai Xilong Optoelectronics Technology Co. Ltd., Shanghai, China). In addition, a series of specimens with the same NG concentration (1 mg/mL, 0.1 mL) were put into quartz cuvettes, and irradiated by a 785 nm laser for 300 s with different output power densities (0.5, 1.0, 1.5 and 2.0 W/cm^2, respectively) to further determine the laser power density-dependent photothermal conversion property. A DT-8891E thermocouple thermometer (Shenzhen Everbest Machinery Industry Co., Ltd., Shenzhen, China) was used to record the *in situ* temperature change of different samples every 5 s.

Hemolysis assay

Fresh human blood stabilized with heparin was kindly supplied by Shanghai Tenth People's Hospital (Shanghai, China) with the approval of the Shanghai Tenth People's Hospital Ethical Committee. Human red blood cells (HRBCs) were collected according to our previous work.[1]

Hemolysis assay was performed using a standard protocol reported in the literature.[2, 3] For hemolysis assay, 0.1 mL HRBC suspension diluted 10 times with PBS was gently mixed with 0.9 mL water (positive control), 0.9 mL PBS (negative control), and 0.9 mL PBS containing γ-PGA/Cys@PANI NGs with varying concentrations (0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 mg/mL, respectively) for 2 h at ambient temperature. After that, the samples were centrifuged (10000 rpm, 1 min), photographed, and the absorbance of the supernatants (hemoglobin) was recorded by a Perkin Elmer Lambda 25 UV-vis spectrophotometer. The hemolysis percentages (p) of different samples were calculated according to equation (1):

$$p = \frac{OD_{sam} - OD_{neg}}{OD_{pos} - OD_{neg}} \times 100\% \quad (1)$$

where OD_{sam}, OD_{neg} and OD_{pos} represent the absorbance of sample, negative control, and positive control, respectively at 541 nm.

Cytotoxicity assay

CCK-8 cell viability assay was employed to evaluate the *in vitro* cytotoxicity of the γ-PGA/Cys@PANI NGs under standard manufacturer's instructions. In brief, 4T1 cells at a density of 1×10^4 cells per well in 200 μL DMEM were seeded into a 96-well plate. After incubation at 37 ^oC and 5% CO_2 overnight, the adherent cells were incubated with 200 μL fresh medium containing normal saline (NS, control) and γ-PGA/Cys@PANI NGs at different concentrations (0.1, 0.2, 0.5, 1, 2, 3, 4 and 5 mg/mL, respectively). After additional 24 h incubation, 20 μL of CCK-8 was added into each well, and the 4T1 cells were then incubated for another 4 h at 37 ^oC and 5% CO_2. After that, the absorbance of each well at 450 nm was recorded using a Thermo Scientific Multiskan MK3 ELISA reader (Thermo scientific, Hudson, NH). Mean and standard deviation (SD) of 6 parallels were reported for each sample.

Photothermal ablation of cancer cells *in vitro*

4T1 cells at a density of 1×10^4 cells per well in 200 μL medium were seeded into a 96-well plate and cultured at 37 ^oC and 5% CO_2 overnight. After that, the medium was replaced with 200 μL fresh medium containing NS (control) or γ-PGA/Cys@PANI NGs (0.2, 0.5, 1, 2, and 4 mg/mL,

respectively) and the cells were incubated for 4 h under regular cell culture conditions. After being rinsed with NS for 3 times, the cells was irradiated by a 785 nm laser for 5 min with a facula region of 0.25 cm^2 and at an output power density of 1.5 W/cm^2. After incubation of the cells for an additional 2 h, the cell viability was evaluated by CCK-8 assay using protocols reported in the literature.[4]

To qualitatively confirm the photothermal ablation of cancer cells *in vitro*, 4T1 cells with a density of 1×10^5 cells per well in 1 mL medium were seeded into 24-well plate. After overnight incubation at 37 ^oC and 5% CO_2, the medium of cells was replaced with fresh medium containing NS or γ-PGA/Cys@PANI NGs at the concentration of 1, 2, and 4 mg/mL, respectively. After 6 h incubation, the medium was carefully discarded and the cells were rinsed with NS for 3 times, incubated with fresh medium, irradiated with a 785 nm laser at an output power density of 1.5 W/cm^2 for 5 min, and stained with calcein-AM (living cells showing green color) and propidium iodide (PI) (dead cells showing red color) according to the standard procedure.[5] The cells were observed using an Axio Vert. A1 inverted fluorescence microscope (Carl Zeiss, Jena, Germany) with a magnification of 100 × for each sample.

Statistical analysis

One-way ANOVA statistical analysis was performed to evaluate the significance of the experimental data. A value of 0.05 was selected as the significance level, and the data were indicated with (*) for $P < 0.05$, (**) for $P < 0.01$, and (***) for $P < 0.001$, respectively.

Table S1. Zeta potential, hydrodynamic size, and polydispersity index (PDI) of the γ-PGA/Cys NGs with different γ-PGA/Cys mass ratios.

γ-PGA/Cys mass ratio	Hydrodynamic diameter (nm)	Zeta potential (mV)	PDI
1 : 1	282.70 ± 0.89	-4.08 ± 0.17	0.23 ± 0.01
1 : 2	278.70 ± 3.31	-5.98 ± 0.46	0.26 ± 0.02

1 : 3	292.67 ± 0.84	-6.81 ± 0.77	0.17 ± 0.08
1 : 4	1677.67 ± 1096.87	-4.71 ± 0.71	0.87 ± 0.22

Table S2. Zeta potential, hydrodynamic size, and PDI of the γ-PGA/Cys@PANI NGs.

Sample	Hydrodynamic diameter (nm)	Zeta potential (mV)	PDI
γ-PGA/Cys@PANI NGs	689.1 ± 18.5	-4.32 ± 0.16	0.15 ± 0.04

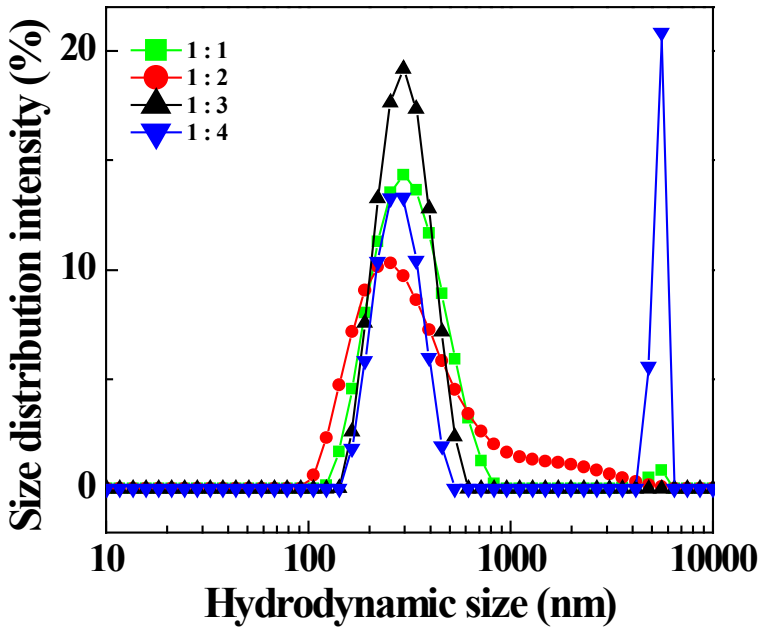

Fig. S1. Hydrodynamic size distribution of the γ-PGA/Cys NGs with different γ-PGA/Cys mass ratios.

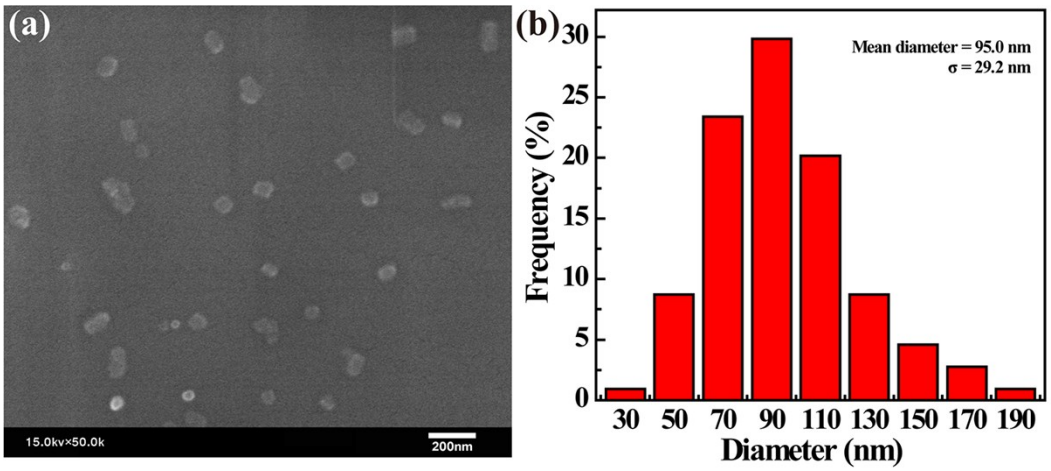

Fig. S2. SEM image and size distribution histogram of the γ-PGA/Cys NGs.

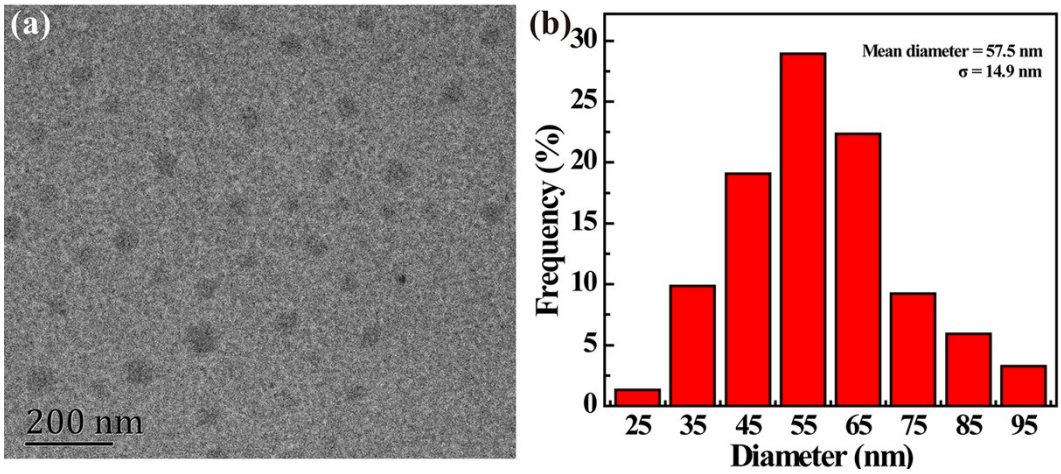

Fig. S3. TEM image and size distribution histogram of the γ-PGA/Cys NGs.

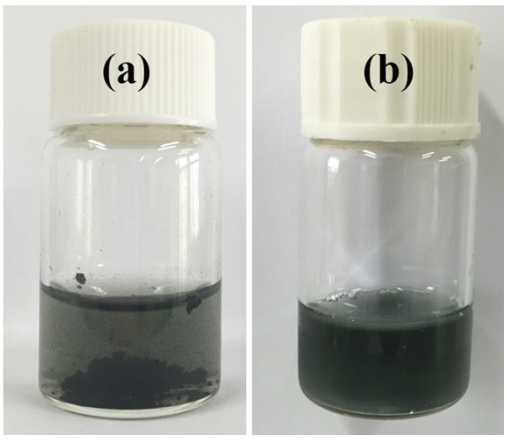

Fig. S4. The photographs of PANI (a) and γ-PGA/Cys@PANI NGs (b).

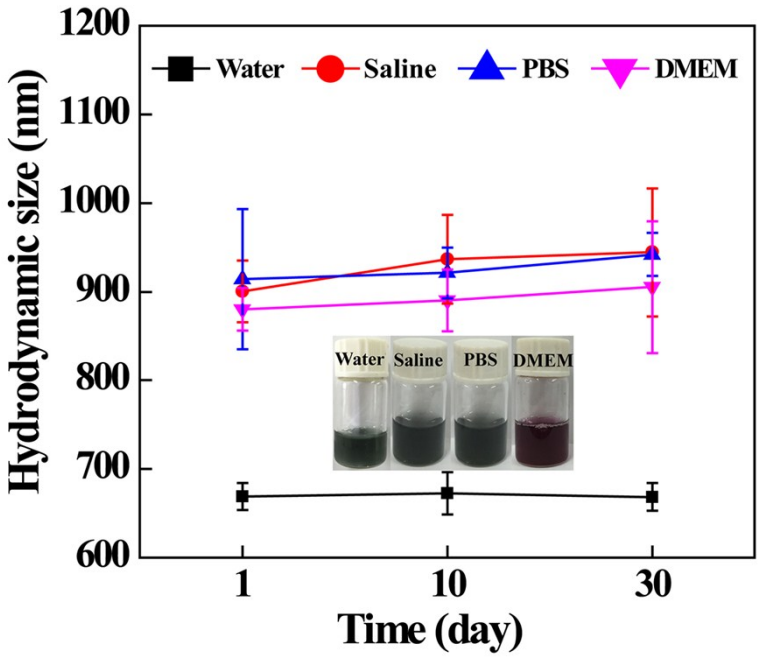

Fig. S5. Hydrodynamic size of the γ-PGA/Cys@PANI NGs dispersed in water, NS, PBS, and DMEM containing 10% FBS at different storage time periods.

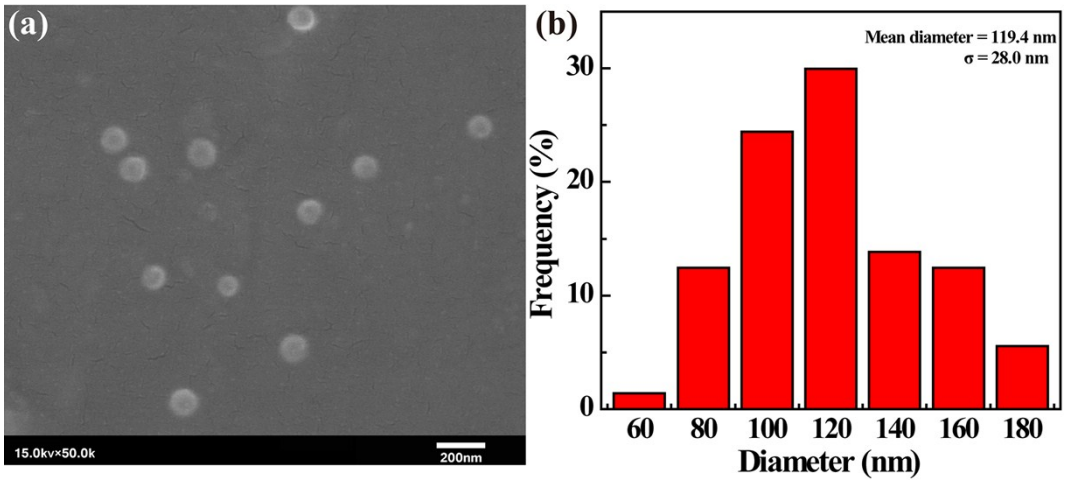

Fig. S6. SEM image and size distribution histogram of the γ-PGA/Cys@PANI NGs.

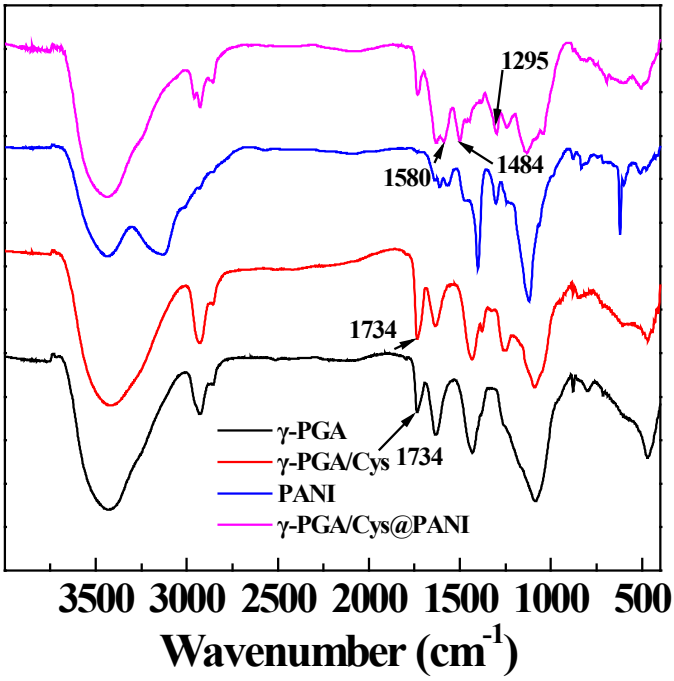

Fig. S7. FTIR spectra of γ-PGA, γ-PGA/Cys NGs, PANI, and γ-PGA/Cys@PANI NGs.

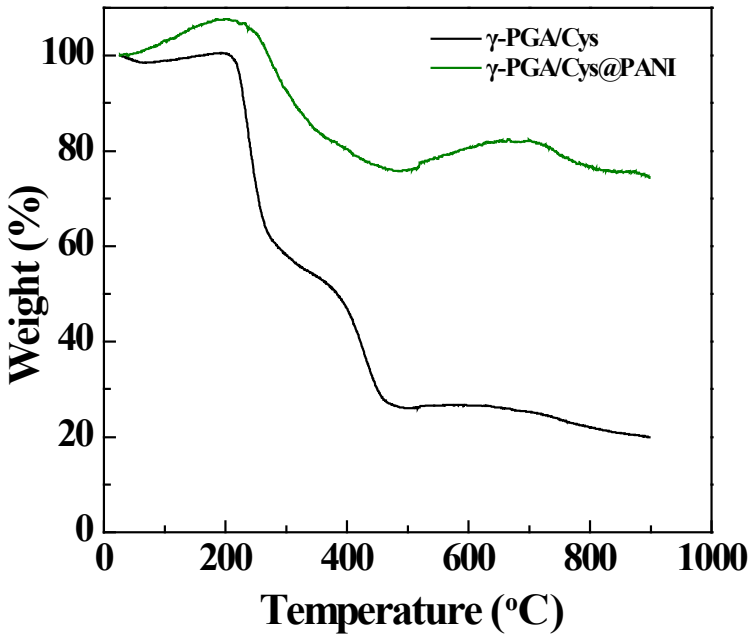

Fig. S8. TGA curves of γ-PGA/Cys and γ-PGA/Cys@PANI NGs.

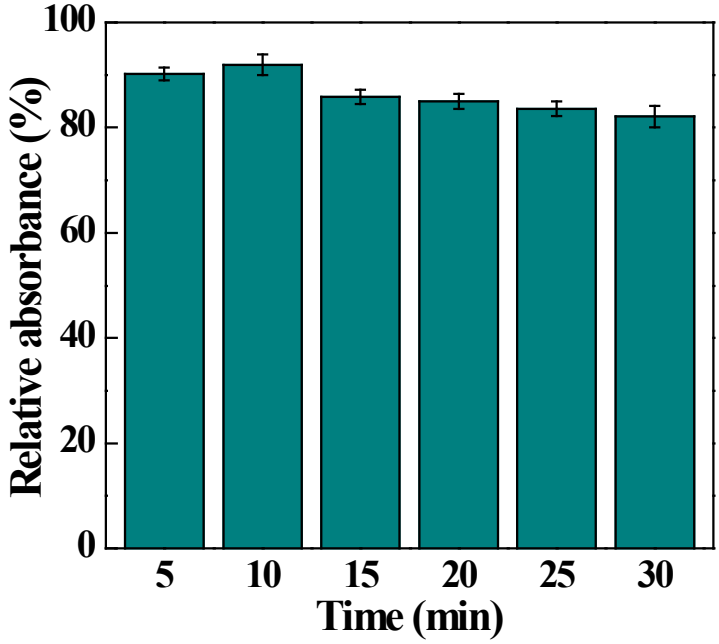

Fig. S9. Relative absorbance of γ-PGA/Cys@PANI NGs (1 mg/mL, in 1 mL water) at 700 nm after the NG suspension was laser irradiated (785 nm laser, power density of 1.5 W/cm^2) for different time periods.

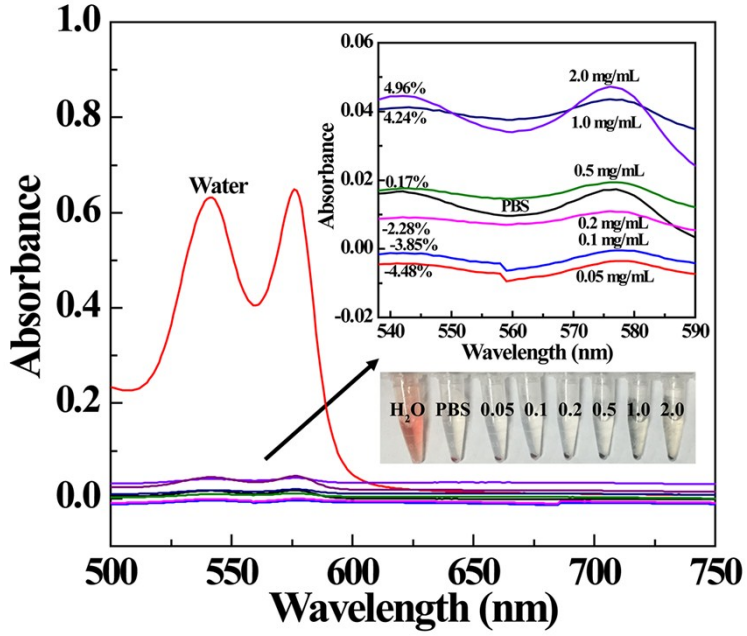

Fig. S10. Hemolytic assay of the γ-PGA/Cys@PANI NGs at different concentrations (0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 mg/mL, respectively).

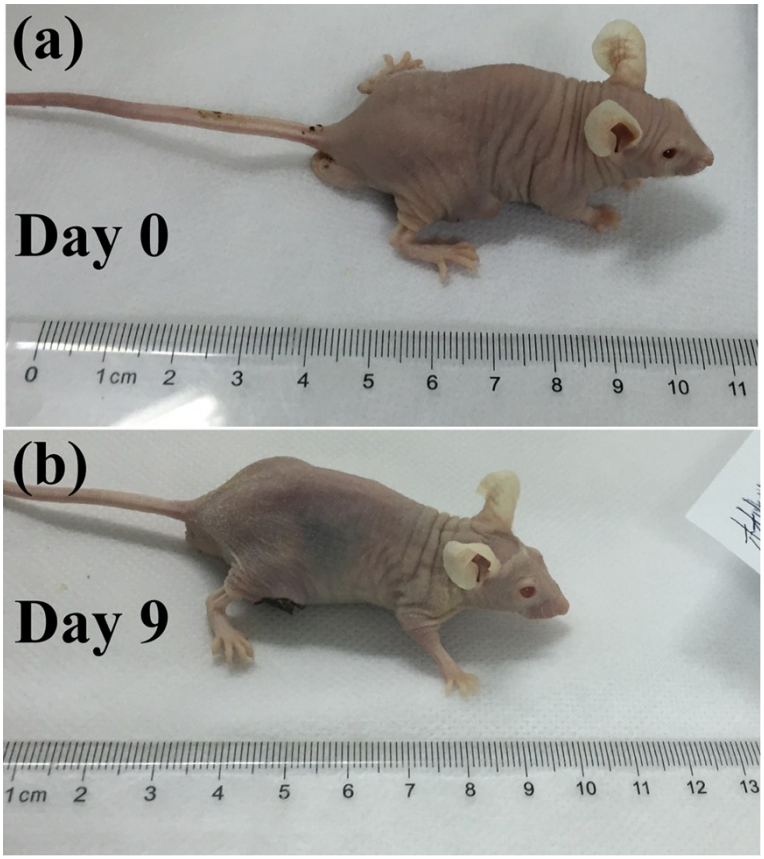

Fig. S11. Photographs of tumor-bearing mice treated with the γ-PGA/Cys@PANI NGs plus laser irradiation at day 0 (a) and day 9 (b).

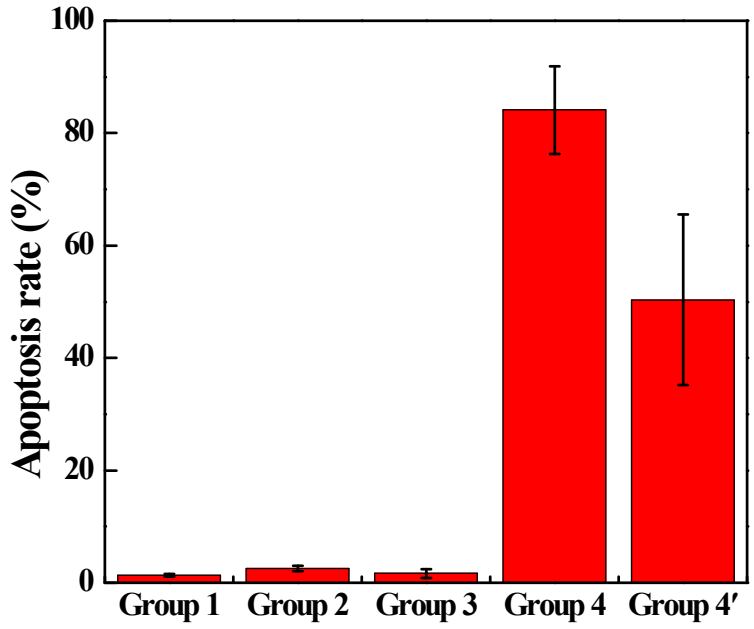

Fig. S12. Quantitative analysis of the apoptotic cells in the tumors for Group 1, Group 2, Group 3, Group 4, and Group 4′, respectively. Definition of the groups can be seen in experimental section.

References

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