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Supporting Information

Salt-Induced Aggregation of Gold Nanoparticles for Photoacoustic Imaging and Photothermal Therapy of Cancer

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Materials

All chemical reagents were purchased from Sigma Aldrich and used as received, unless otherwise specified. C26 murine colon carcinoma cell line was purchased from cell bank of Chinese Academy of Medical Sciences. Female BALB/c nude mice were purchased from Vital River Laboratories (Beijing, China) and accommodated in animal research facility of Tsinghua University. The Laboratory Animal Facility at the Tsinghua University is accredited by AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International), and all animal use protocols used in this study are approved by the Institutional Animal Care and Use Committee (IACUC).

Synthesis of Gold Nanoparticles and Gold Nanorods

Gold nanoparticles (GNPs) with an average diameter of 15 nm were synthesized as previously reported.¹ Briefly, 45 mg $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ in 250 mL ultrapure water was brought to boil, to which 1 mL of 15% w/v trisodium citrate dihydrate was added to react for another 5 min under vigorous stirring. The obtained solution was cooled down to room temperature. The GNPs were purified by centrifugation and re-dispersed in pure water or 5% glucose for further use.

The purified GNPs were coated with thiolated polyethylene glycol 5000 kD (HS-PEG) to form PEG-GNPs as a control. Briefly, an aqueous solution of HS-PEG was added drop-wise to the purified GNP solution with a final HS-PEG concentration of 1 μM , and the resulting solution was stirred vigorously at room temperature for 12 h. The PEG-GNPs were separated by centrifugation (20,000 rpm for 1 h) to remove excess polymers and re-dispersed in pure water or 5% glucose for further use.

The gold nanorods (GNRs) possessing a maximal absorbance at 790 nm were synthesized by the seed-mediated method, as described by El-Sayed et. Al.² A solution of GNRs was centrifuged at 14,000 rpm for 15 min to remove the CTAB, followed by reacting with HS-PEG with a final concentration of 1 μM for 12 h. The PEG-GNRs were separated by centrifugation (14,000 rpm for 15 min) to remove excess polymers and re-dispersed in pure

water or 5% glucose for further use.

Characterization of GNPs and PEG-GNPs

Hydrodynamic sizes of GNPs and PEG-GNPs were determined by dynamic light scattering (DLS), which were carried out by a Nano ZetaSizer (Malvern Instruments, Worcestershire, UK) with a 90° scattering angle at 25 °C. The data were analyzed with Zetasizer software 6.32.

UV-vis spectra of the GNPs and PEG-GNPs samples were recorded on a SpectraMax® M3 Microplate Reader (Molecular Devices) in concert with *Softmax Pro*. The absorption values were read from 200 to 1000 nm wavelength, with a step size of 5 nm. All results were obtained by using 2 mL of solutions in cuvettes, and the solvents were used as the blank in all cases. The presence of an absorbance peak at 520 nm was used as an indicator of gold nanoparticles formation.

Transmission electron microscopy (TEM) was carried out using a Hitachi-H-7650B operating at 120 kV. Samples for TEM were prepared by casting 20 µL of GNP or PEG-GNP aqueous solution on 300 mesh copper grids covered with carbon film.

Photothermal Heating Experiments

All photothermal materials were dispersed in PBS (0.1 mM, pH 7.4) to the gold concentration of 186 µg/mL, PBS was used as the control. The heating characteristics were measured in disposable cuvette containing samples (1.0 mL). Then the cuvettes were irradiated for 5 min with an 808 nm laser at a power of 1.5 W/cm² (0.5 cm² laser area). The temperature change was recorded every 10 s by a digital thermometer with an accuracy of ± 0.1 °C.

Photothermal Conversion

1 mL of GNPs, PEG-GNPs or PEG-GNRs at the concentration of 186 µg Au /mL in PBS was exposed to an 808 nm NIR laser (1.5 W/cm²) to the maximum temperature, and then the

laser was shut off. The temperature change was recorded once every 10 s during the heating and cooling periods. The photothermal conversion efficiency (η) was determined by Equation 1 and 2³.

$$\eta = \frac{hS(T_{Max} - T_{Surr}) - Q_{Dis}}{I(1 - 10^{-A_{808}})} \quad (1)$$

$$\tau_s = \frac{\sum_{i=1}^n m_i C_{p,i}}{hS} \quad (2)$$

in which h is heat transfer coefficient, S is the surface area of the container, T_{Surr} is ambient temperature of the surroundings, and T_{max} is the equilibrium temperature. I is 1.5 W/cm², A_{808} is the absorbance at 808 nm. Q_{Dis} expresses heat dissipated from light absorbed by the quartz sample cell itself. The variable τ_s is the sample-system time constant, where m and C_p are the mass (1 g) and heat capacity (4.2 J/g) of the PBS used as the solvent³.

Cytotoxicity and *In vitro* PTT

The C26 murine colon carcinoma cells were cultured in RPMI 1640 medium supplemented with 2.2 mg/mL sodium carbonate, 50 µg/mL penicillin, 50 µg/mL streptomycin and 10% fetal bovine serum (FBS) at 37 °C and 5% CO₂, in accordance with standard cell media by American type culture collection (ATCC).

The cytotoxic effect of GNPs plus laser irradiation was determined by standard MTT assay in 96 well plates. Approximately 4,000 cells were placed in each well and cultured for 48 h at 37 °C and 5% CO₂, followed by addition of 40 µL GNPs samples to different final gold concentrations (0, 30, 60, 120, 180 and 300 µg/mL). After incubation for another 3 h, the medium was removed, and cells in 100 µL of medium were exposed to laser irradiation (808 nm, 3.5 W/cm²) for 5 min, followed by recovery for 1 h at 37 °C and 5% CO₂. The MTT reagents of 10 µL per well were added, followed by reaction for 4 h at 37 °C. Then the mixture absorbance was measured at 490 nm on a SpectraMax® M3 Microplate Reader. Meanwhile, the cytotoxicity of GNPs was determined by incubating cells with GNPs only.

For *in vitro* PTT, 1×10^5 C26 cells were placed in a 35-mm diameter dish and cultured for 24 h at 37 °C and 5% CO₂. After rinsed with PBS (pH 7.4), the cells were incubated with GNPs (186 µg Au/mL), PEG-GNPs (186 µg Au/mL) or PEG-GNRs (186 µg Au/mL) for 3 h. Then cells were rinsed again with PBS and immersed in 500 µL of medium before exposed to an 808 nm laser with a power density of 3.5 W/cm² for 5 min. After the irradiation, cells were cultured for another 30 min at 37 °C. For identifying the cell viability, the cells were co-stained by Calcein AM and propidium iodide (PI) to determine the live and dead cells with a laser scanning confocal microscope.

Photothermal and Photoacoustic Imaging *In vivo*

Animal experiments were carried out in accordance with a protocol approved by the Institutional Animal Care and Use Committee. Female Balb/c nude mice of 6 weeks were subcutaneously injected with a suspension of C26 tumor cells (2×10^6) in RPMI 1640 medium into the back. When the tumor size reached 80-100 mm³, 150 µL of GNPs (186 µg Au/mL), PEG-GNPs (186 µg Au/mL) and PEG-GNRs (186 µg Au/mL) in 5% glucose was intratumorally injected into these anaesthetic mice, and the glucose solution was used as a control.

Photothermal imaging was recorded by a GF300 infrared camera (FLIR) when the tumor exposed to an 808 nm laser (BWT Beijing LTD) at a power density of 1.0 W/cm² for 5 min. These mice were anesthetized by intraperitoneal injection of 0.4 % pentobarbital sodium (200 µL/20 g).

Photoacoustic imaging was performed by a *MSOT inVision 512-echo* system (iTheraMedical, German) equipped with a 5 MHz, 128-element linear array transducer on tumors. These mice were anesthetized by continuous isoflurane anesthesia.

Photothermal Therapy

The C26 tumor-bearing mice were divided to 4 groups (n = 3-7 per group) when the tumor volume reached 80 -100 mm³. For the treatment group, 150 µL of GNPs (186 µg Au/mL) was

intratumorally injected with an incubation time of 1.5 h, followed by laser irradiation (808 nm) for 5 min at a power density of 1.0 W/cm². Other control groups were mice with glucose solution (150 µL) and laser irradiation (1.0 W/cm²) for 5 min, mice with PEG-GNPs (150 µL, 186 µg Au/mL) administration and irradiation (1.0 W/cm²) for 5 min, mice with PEG-GNRs (150 µL, 186 µg Au/mL) administration and irradiation (1.0 W/cm²) for 5 min and mice with GNPs (150 µL, 186 µg Au/mL) administration only. The tumor sizes and body weights were measured every three days after treatment. Tumor volume (V) was determined by the following equation: $V = AB^2/2$, A and B are the length and width of tumors, respectively. The mice with tumor sizes over 1000 mm³ or more than 15% weight loss were executed.

H&E staining and TEM assay of tumors

The mice with C26 tumors (80-100 mm³) on back were intratumorally injected with GNPs, PEG-GNPs, PEG-GNRs (150 µL, 186 µg Au /mL) and glucose solution, to incubate for 1.5 h. For H&E staining, after laser irradiation for 5 min, the obtained tumors were fixed in a formaldehyde solution for 24 h, then dehydrated, sliced into 5.0 µm sections and subjected to H&E staining assay. All stained tumor sections were imaged with an invert fluorescence microscope (Eclipse 90i, Nikon, Japan). For TEM assay, the obtained tumors were fixed in 0.05 M PBS with 2.5 % glutaraldehyde for 12 h, and then cut up. Pellets were fixed again, dehydrated, embedded in Epon, and sliced to a thickness of 70 nm. Images and EDS analyses of the slices were taken with a JEM 2010 transmission electron microscope.

Figure S1-9

Table S1. Hydrodynamic sizes of GNPs and PEG-GNPs in different solutions.

	Deionized Water	5% Glucose	PBS	RPMI 1640 with 10% FBS	Tumor Interstitial Fluid
Radius of GNPs (nm)	9.878	11.64	411.7	87.90	148.8
Radius of PEG- GNPs (nm)	25.58	28.05	27.62	29.30	30.39

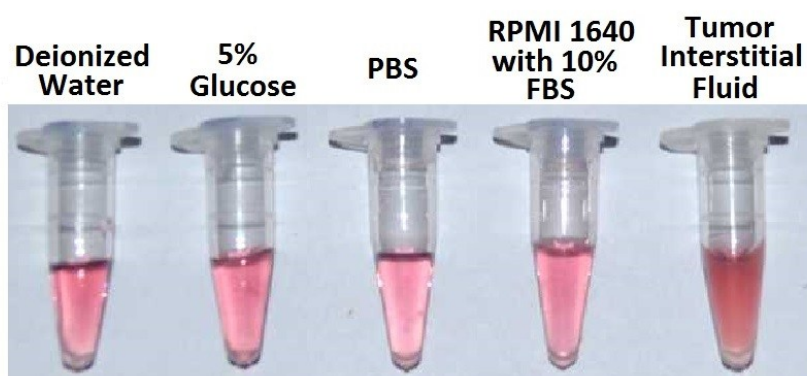


Figure S1. Photographs of the same dose of PEG-GNPs dispersed in different solutions.

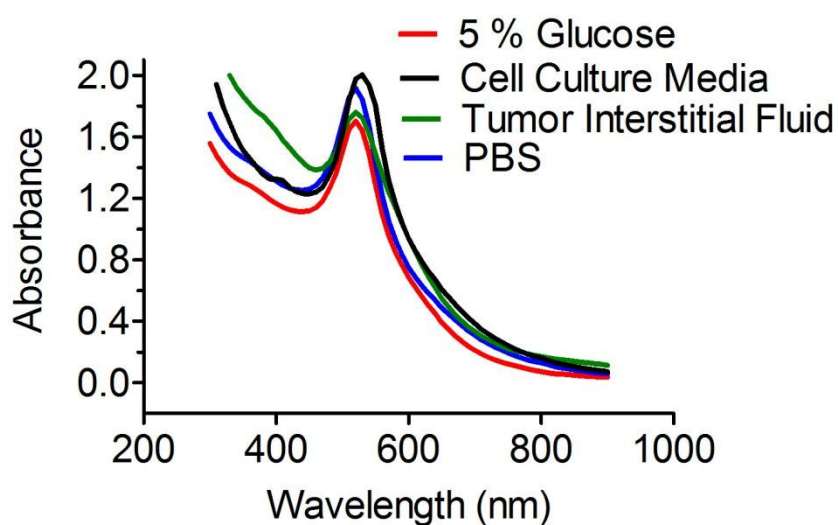


Figure S2. UV-vis-NIR spectra of PEG-GNPs dispersed in different biological media.

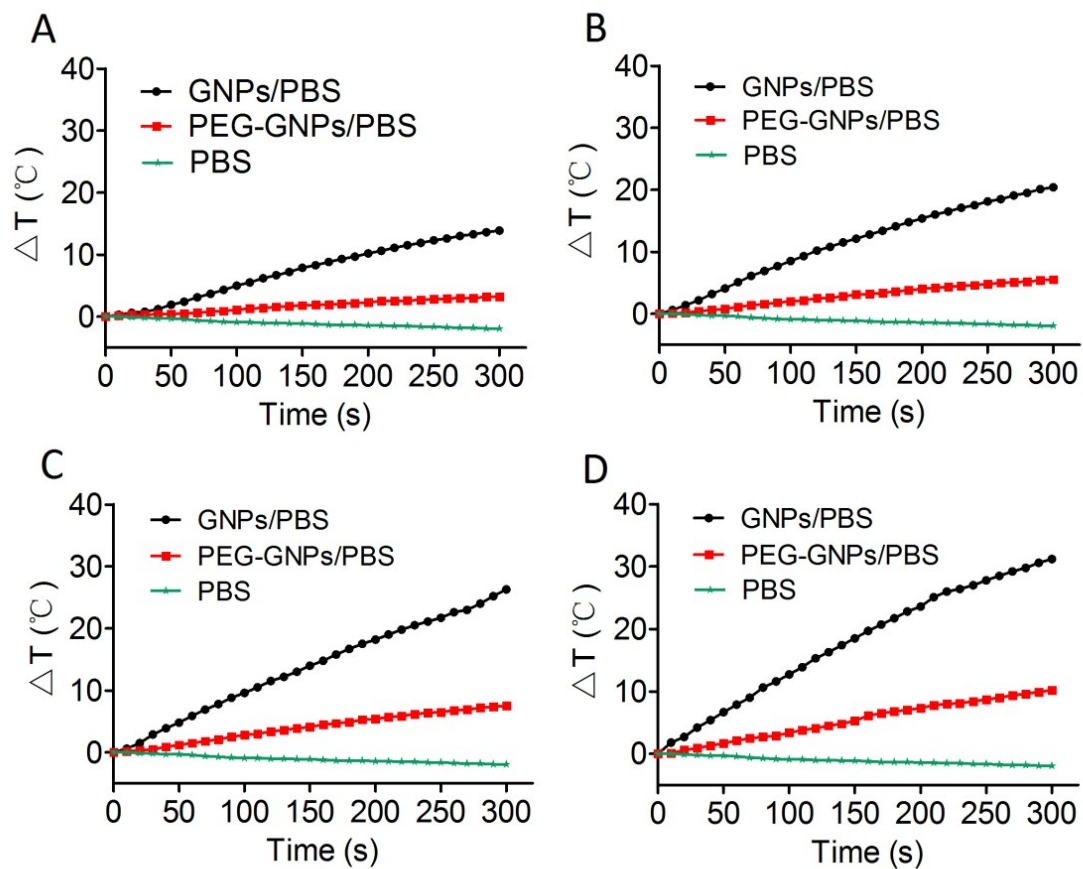


Figure S3. Heating curves of GNPs and PEG-GNPs in PBS at different doses of 50 (A), 100 (B), 150 (C) and 250 (D) $\mu\text{g Au/mL}$ under 808 nm laser irradiation (1.5 W/cm^2 , 5 min).

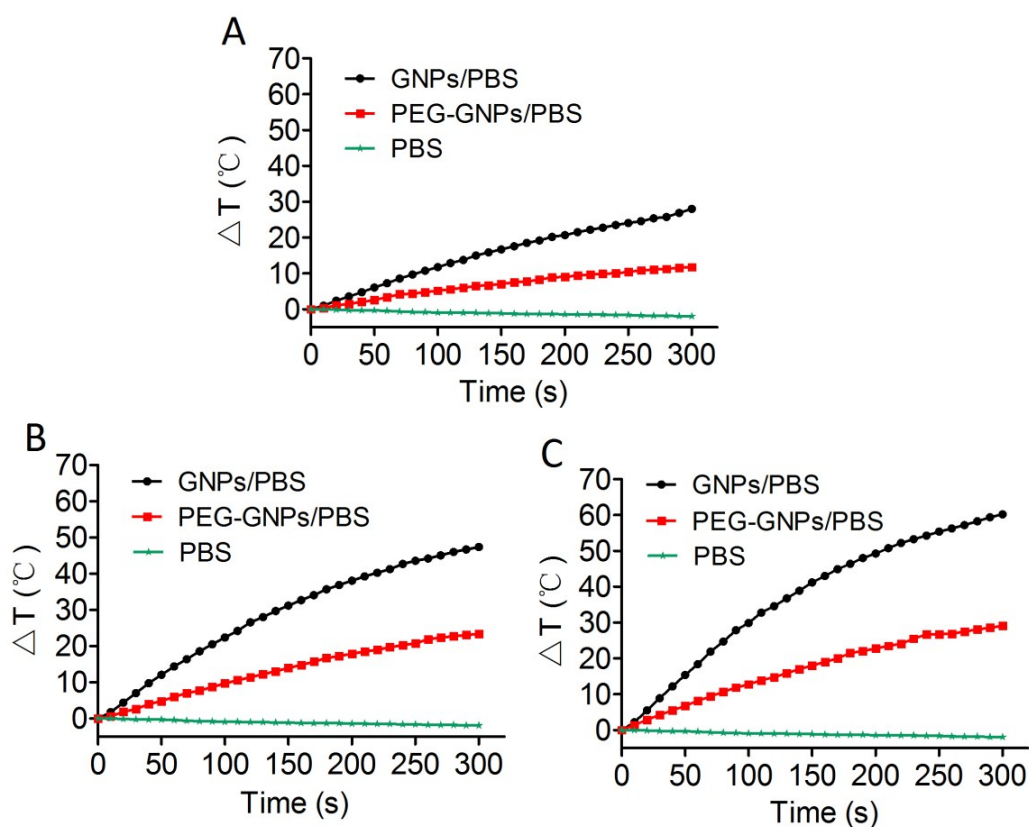


Figure S4. Heating curves of GNPs (186 $\mu\text{g Au/mL}$, 1 mL) and PEG-GNPs (186 $\mu\text{g Au/mL}$, 1 mL) in PBS at different power densities (A-C: 1, 2, 2.5 W/cm^2) under 808 nm laser irradiation.

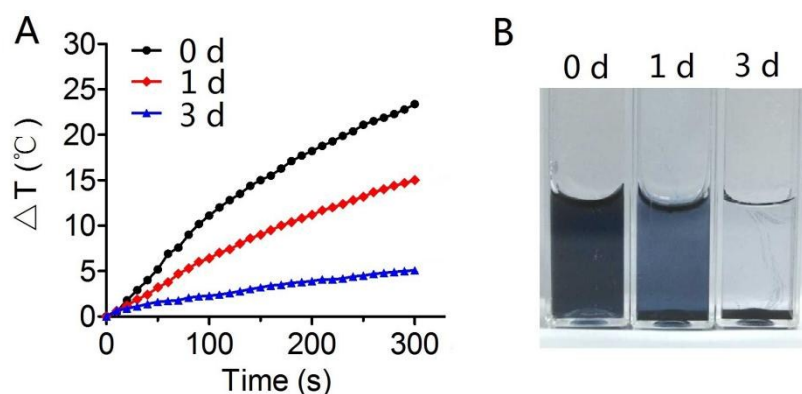


Figure S5. Heating curves (A) and digital pictures (B) of GNPs in PBS (150 $\mu\text{g Au/mL}$, 1 mL) with incubation time of 0 day (0 d), 1 day (1 d) and 3 days (3 d), followed by 808 nm laser irradiation (1.5 W/cm^2 , 5 min).

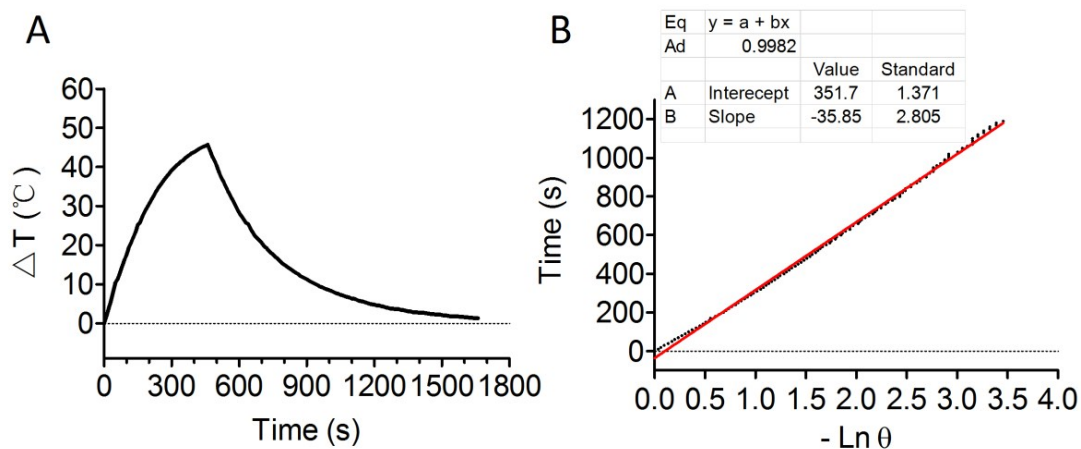


Figure S6. (A) Photothermal effect of GNPs in PBS (186 $\mu\text{g/mL}$, 1 mL) with the NIR laser (808 nm, 1.5 W/cm^2), in which the irradiation lasted for 460 s, and then the laser was turned off. (B) Time constant for heat transfer from the system is determined to be $\tau_s = 351.7$ s by applying the linear time data from the cooling period (after 460 s) versus negative natural logarithm of driving force temperature, which is obtained from the cooling stage of panel A.

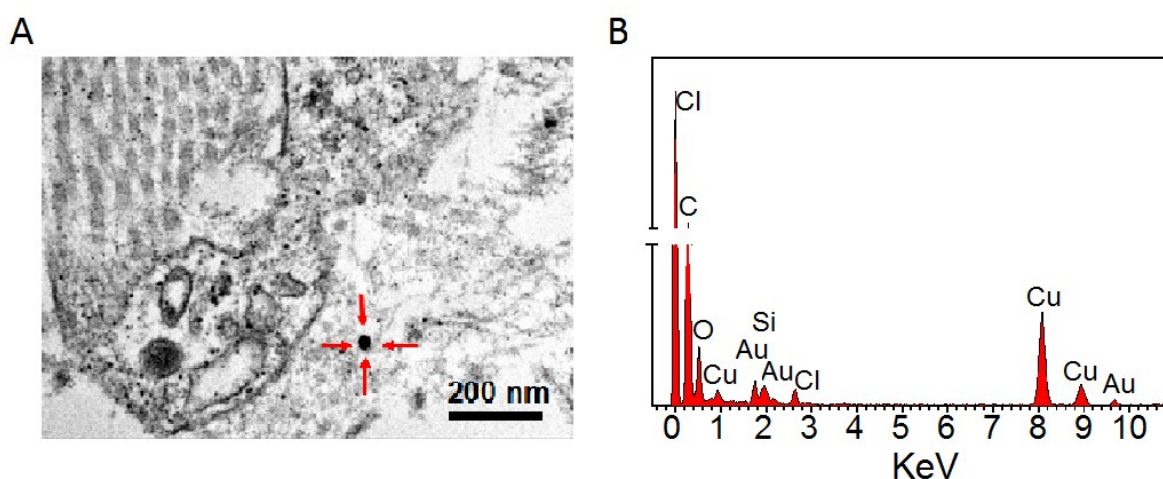


Figure S7. TEM (A) and EDS (B) analyses of tumor tissue containing PEG-GNPs. Arrows in panel A indicate the monodisperse PEG-GNPs whose EDS data are shown in panel B.

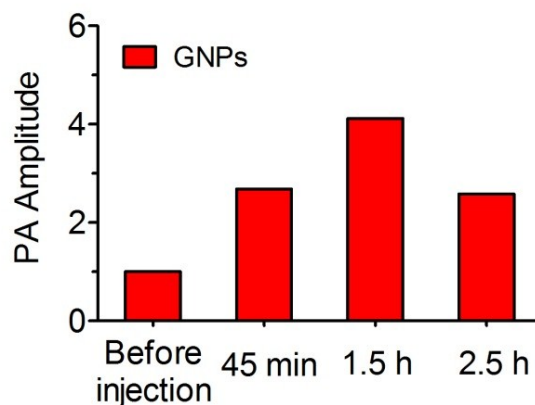


Figure S8. PA intensities of tumor tissues following the intratumoral administration of GNPs (186 $\mu\text{g Au/mL}$, 150 μL) at different incubation time.

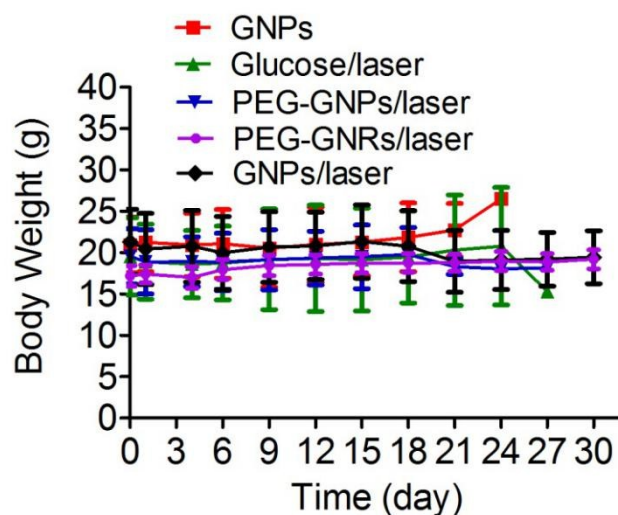


Figure S9. The change of body weight of mice in different groups after the treatments.

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