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Intracellular fluorescent thermometry and photothermal-triggered drug release developed from gold nanoclusters and doxorubicin dual-loaded liposomes

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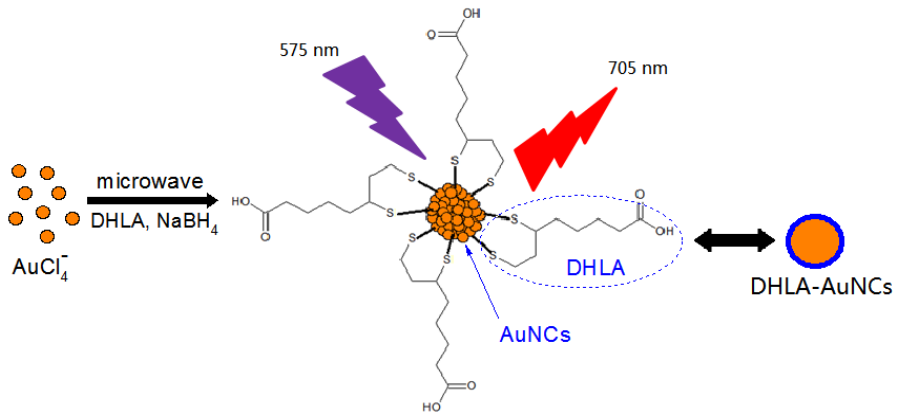

Fig. S1 Schematic illustration of the microwave-assisted synthesis of fluorescent AuNCs

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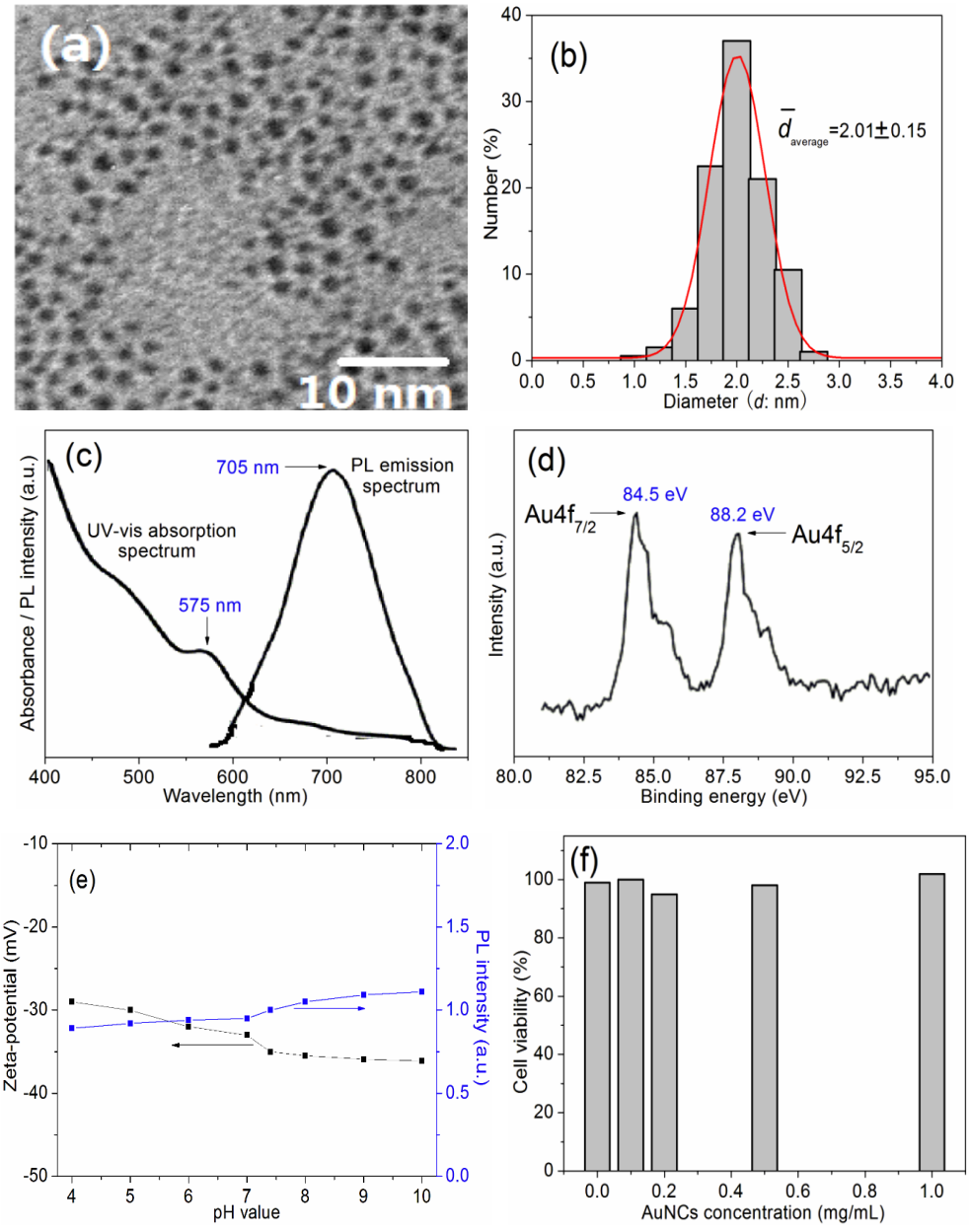
Part S1. Synthesis of DHLA-stabilized AuNCs

In a typical experiment, 10.4 mg (0.05 mol) DHLA was added to 19.5 mL of aqueous solution containing NaOH (0.1 mL, 2 M). The mixture was stirred for 5 min, followed by adding 0.2 mL of aqueous solution of $HAuCl_4$ (wt. 2%). After that, an aqueous solution of sodium borohydride ($NaBH_4$, 0.2 mL, 0.1 M) was dropwise added to the above mixture solution to form a reaction solution under rapid stirring. The reaction solution was irradiated by using a microwave (180 W) for 5 min in a microwave oven. Finally, the reaction solution was allowed to cool to the room temperature (~25 ^{o}C), and further purified by triple centrifugation and filtration using a filter with a molecular mass cut-off of 10 kDa to remove impurities. The brownish AuNCs were cautiously collected from the filter, and re-dispersed in phosphate buffer solution (PBS, 1 mM, pH 7.4) prepared *via* adjusting the concentration ratio of Na_2HPO_4 and NaH_2PO_4, and stored at 4 ^{o}C for further use in following experiments.



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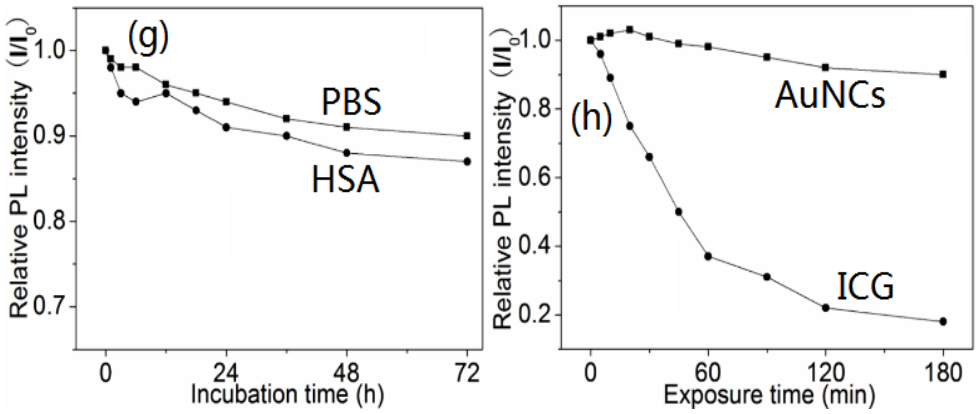

Fig. S2 (a) Wide-filed transmission electron microscope (TEM) images of the as-prepared AuNCs, showing well dispersed spherical particles. (b) Diameter analysis of the observed particles in TEM images, as judged from more than 200 individual particles, showing a narrow size distribution and a small average size of 2.01 nm. (c) Normal UV-vis absorption and PL emission spectra of AuNCs, displaying a distinct absorption band around 575 nm and a sharp emission peak at 705 nm upon the excitation at 575 nm. (d) X-ray photoelectron spectroscopy (XPS) showing the binding energy of $Au4f_{7/2}$ and $Au4f_{5/2}$ of AuNCs at 84.5 eV (metallic gold) and 88.2 eV (gold thiolate), respectively, suggesting the existence of both Au^0 and Au^+ in AuNCs, (e) Zeta potentials and PL intensity of AuNCs, implying a high stability of PL intensity and good colloidal stability at the entire physiologically relevant pH range. (f) Viability of HepG2 cells after 24 h incubation with different concentrations of AuNCs in the cell medium as determined by MTT assay, indicating a low cytotoxicity of AuNCs in the concentration range of 0-1 $mg{\cdot}mL^{-1}$. (g) Colloidal stability of AuNCs in PBS (1 mM, pH 7.0) and human serum albumin (HSA, 10 mM) at 37 ^oC. PL intensities were recorded at different time intervals, denoting a high stability that is suitable for bio-applications. (h) Photostability of AuNCs and indocyanine green (ICG) (a commercial NIR-emitting fluorescent dye) in water. AuNCs were continuously excited with a 405 nm laser (50 mW), and ICG was continuously excited with a 722 nm laser (50 mW), suggesting that the AuNCs possessed a high colloidal stability in aqueous systems, and outstanding photostability or anti-photobleaching capacity. Therein, all experimental results in (e)-(h) were determined repeatedly, and each result was expressed as man of six determinations.

Part S2. Synthesis of AuNCs/Dox dual-loaded liposome

Briefly, yolk lecithin and cholesterol (with a molar ratio of 3:1) were dissolved in chloroform-methanol mixed solvent (with a volume ratio of 3:1) to form a homogeneous solution. Then, the mixed solvent was removed from the homogeneous solution by rotary evaporation at 37 ^oC under N_2 atmosphere. Afterward, a thin lipid film was formed on the bottom side of round flask. The formed thin film was dried in a vacuum tank to completely remove residual solvent by pumping for 2 h, and then kept overnight (about 12 h). The as-obtained dry film, aqueous suspension of AuNCs (0.1 $mg{\cdot}mL^{-1}$,1 mL) and aqueous solution of Dox (1 $mg{\cdot}mL^{-1}$, 1 mL) were transferred into a high-pressure cell and then incubated by introducing CO_2 at 45 ^oC and a pressure of 16 MPa for 30 min. After this incubation, supercritical CO_2 gas in the cell was released carefully and slowly. A limpid AuNCs/Dox-liposome solution was observed in the cell, and collected it for subsequent experiments.

Part S3. Synthesis of blank liposome without AuNCs and Dox

Briefly, yolk lecithin and cholesterol (with a molar ratio of 3:1) were dissolved in chloroform-methanol

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mixed solvent (with a volume ratio of 3:1) to form a homogeneous solution. Then, the mixed solvent was removed from the homogeneous solution by rotary evaporation at 37 ^{o}C under N_2 atmosphere. Afterward, a thin lipid film was formed on the bottom side of round flask. The formed thin film was dried in a vacuum tank to completely remove residual solvent by pumping for 2 h, and then kept overnight (about 12 h). The as-obtained dry film and 2 mL of deionized water were transferred into a high-pressure cell and incubated by introducing CO_2 at 45 ^{o}C and a pressure of 16 MPa for 30 min. After this incubation, supercritical CO_2 gas in the cell was released carefully and slowly. A limpid and transparent liposome solution was observed in the cell, and collected it for following experiments. Due to the absence of AuNCs and Dox, the liposome was named as the blank liposome in this paper.

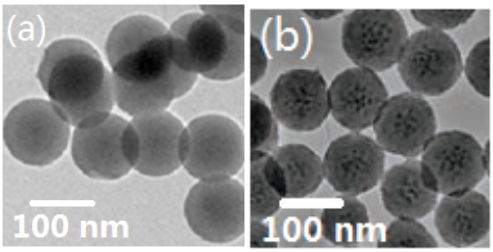

Fig. S3 Wide-filed TEM images of the blank liposome (a) and AuNCs/Dox-loaded liposome (b). In the image (a), the observed particles have a uniform spherical shape with an average diameter of ~105 nm. In the image (b), the observed particles have a uniform and discrete spherical shape, and the average diameter of particles is about 110 nm. Many black dots in the internal cavity or center of individual particle can be watched, which are ascribed to AuNCs (several units of particles) according to the synthesis procedure of AuNCs/Dox-loaded liposome in Part S2. The results indicate that AuNCs were embedded into the internal aqueous cavity of liposome, and AuNCs/Dox-loaded liposome was successfully prepared.

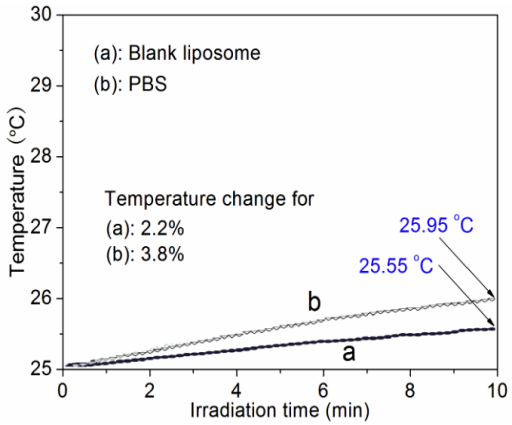

Fig. S4 Temperature elevation in aqueous solutions containing the blank liposome (0.1 mg·mL^{-1}) and PBS (1 mM, pH 7.4) under the laser irradiation (575 nm, 50 mW) measured every 10s by using a digital thermometer, over a period of 10 min. The light irradiation-induced media temperature changes of aqueous samples for the blank liposome and the PBS were calculated according to the following equation as below: Temperature change (T_c, %) = 100 × (T_b – T_a) / T_b, where T_b and T_a stand for the temperatures of aqueous solutions before (T_b) and after (T_a) 10 min irradiation, respectively.

Part S4. Loading content and efficiency of AuNCs and Dox

To study the relationship between the PL intensity of AuNCs/Dox-liposome and the media temperature, a series of Dox-liposome and AuNCs/Dox-liposome samples with different amount of Dox and AuNCs were

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prepared using the same method as referred in Part S2. Dox-liposome samples were prepared by incubating the dry film into 1 mL of aqueous solution of Dox (0.05, 0.1, 0.2, 0.3, and 0.5 mg·mL^{-1}). The as-obtained aqueous suspension of Dox-liposome was centrifuged, and washed with water twice to remove un-loaded and surface-absorbed Dox. The mass of Dox loaded in the liposome was calculated by subtracting the mass of Dox in the supernatant from the total mass of Dox in initial solution. The mass of free Dox ($M_{\text{free-Dox}}$) in solution was determined by UV-vis spectrophotometer at 480 nm using the Lambert-Beer law. The loading efficiency (LE) and loading content (LC) of Dox were calculated by the following equations;

$$\text{Dox-LE (\%)} = 100 \times (M_{\text{total-Dox}} - M_{\text{free-Dox}}) / M_{\text{total-Dox}} \quad (1)$$

$$\text{Dox-LC (\%)} = 100 \times (M_{\text{total-Dox}} - M_{\text{free-Dox}}) / M_{\text{total-liposome}} \quad (2)$$

AuNCs/Dox-liposome samples were prepared by incubating the dry film into 1 mL aqueous suspension of AuNCs (0.1, 0.2, 0.4, 0.6, and 1.0 mg·mL^{-1}) and 1 mL of aqueous solution of Dox (1.0 mg·mL^{-1}). The obtained aqueous suspension of AuNCs/Dox-liposome was centrifuged, and washed with water twice to remove un-loaded and surface-absorbed AuNCs and Dox. The LE and LC of Dox in the liposome could be calculated using the equation (1) and (2) referred above. The mass of free AuNCs ($M_{\text{free-AuNCs}}$) in aqueous solution was determined by UV-vis spectrophotometer at 575 nm using the Lambert-Beer law. The LE and LC of AuNCs in the liposome were calculated by the following equations;

$$\text{AuNCs-LE (\%)} = 100 \times (M_{\text{total-AuNCs}} - M_{\text{free-AuNCs}}) / M_{\text{total-AuNCs}} \quad (3)$$

$$\text{AuNCs-LC (\%)} = 100 \times (M_{\text{total-AuNCs}} - M_{\text{free-AuNCs}}) / M_{\text{total-liposome}} \quad (4)$$

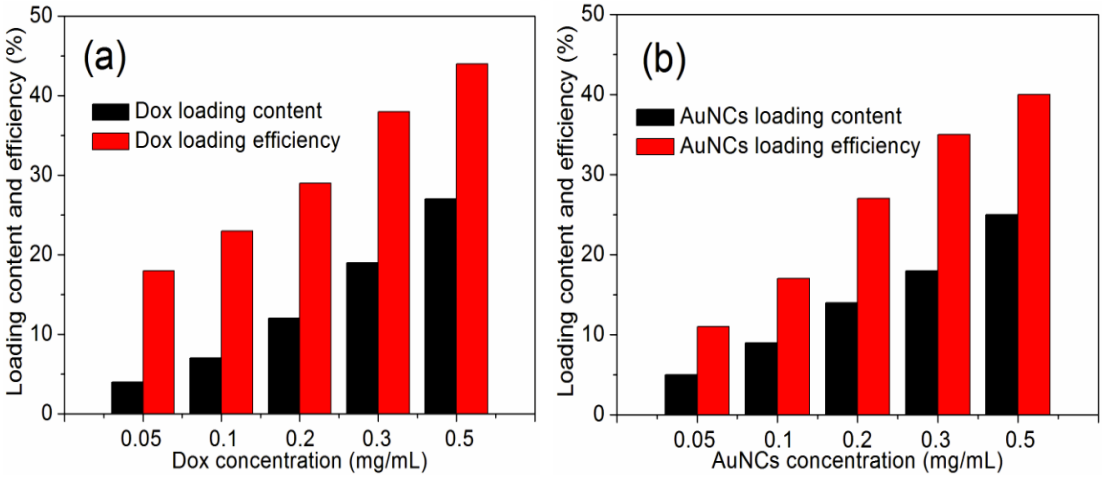

Fig. S5 (a) Effects of the incubation concentration of Dox on Dox-loading content (LC) and Dox-loading efficiency (LE) of the Dox-liposome. Both the LC and LE increased with Dox concentration increasing. When Dox concentration increased to 0.5 mg·mL^{-1}, both the LC and LE increased to the maximum values, i.e., 27% and 44%, respectively. (b) Effects of the incubation concentration of AuNCs on AuNCs-LC and AuNCs-LE of the AuNCs/Dox-liposome. The incubation concentration of Dox was 0.5 mg·mL^{-1}, and the LC and LE of Dox in the liposome were calculated to be 22% and 41%, respectively. Upon the increase of AuNCs concentration (0.05, 0.1, 0.2, 0.3, and 0.5 mg·mL^{-1}), both the LC (5, 9, 14, 18, and 25%) and LE (11, 17, 27, 35, and 40%) of AuNCs increased gradually.

Part S5. Relationship between the relative PL intensity of AuNCs/Dox liposome and the media temperature

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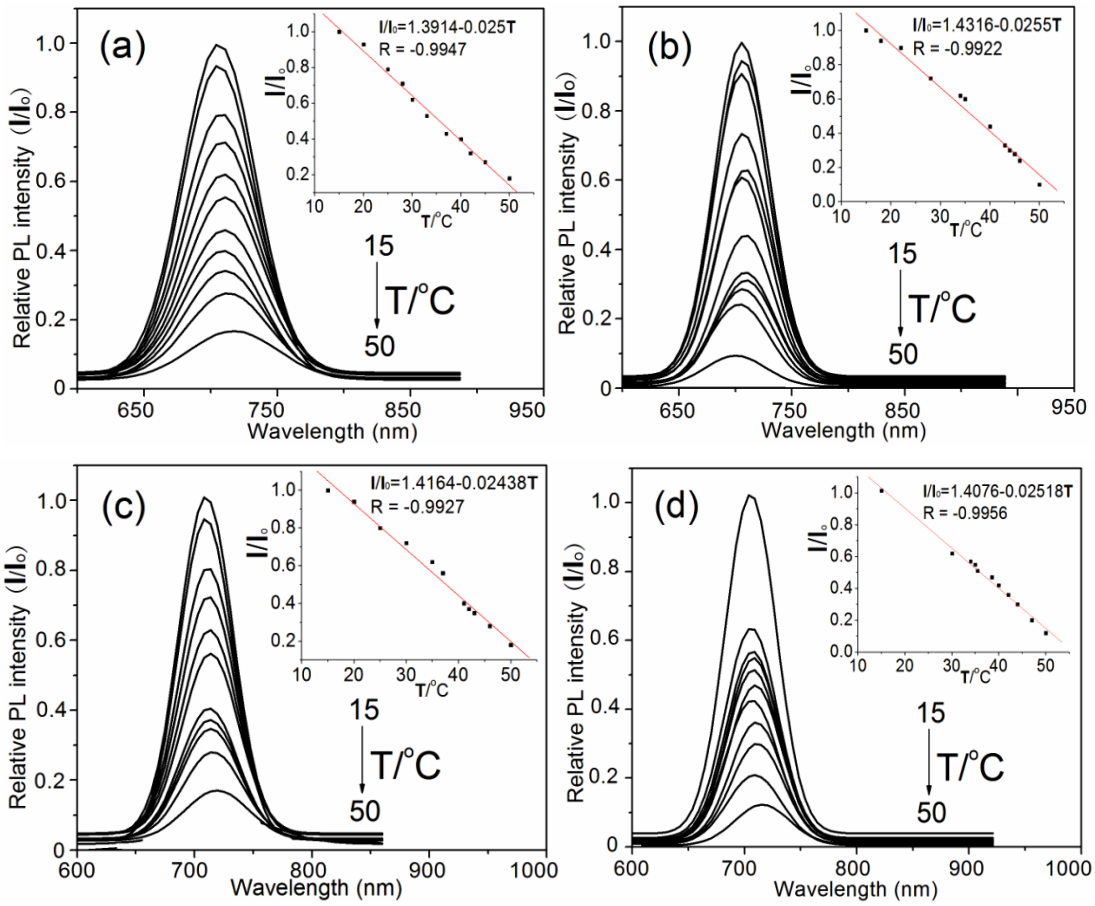

Fig. S6 Temperature dependence of PL emission from AuNCs/Dox-liposome dissolved in 1 mM of PBS (pH 7.4), and the relative PL intensity of AuNCs/Dox-liposome at 705 nm is plotted *versus* temperature, as inserted in each figure. (a) AuNCs/Dox-liposome sample was prepared by incubating into 1 mL aqueous suspension of AuNCs (0.2 mg·mL^{-1}) and 1 mL of aqueous solution of Dox (1.0 mg·mL^{-1}). (b) The same conditions as the (a) except for AuNCs (0.4 mg·mL^{-1}). (c) The same conditions as the (a) except for AuNCs (0.6 mg·mL^{-1}). (d) The same conditions as the (a) except for AuNCs (1.0 mg·mL^{-1}). The LC and LE of Dox and AuNCs in the liposome could be obtained in Fig. S3. All samples were excited at 575 nm.

In addition, the AuNCs/Dox-liposome sample was prepared by incubating into 1 mL aqueous suspension of AuNCs (0.1 mg·mL^{-1}) and 1 mL of aqueous solution of Dox (1.0 mg·mL^{-1}), and the relationship between the relative PL intensity of the AuNCs/Dox-liposome (with 5% of AuNCs-LC, 11% of AuNCs-LE, 22% of Dox-LC, and 41% of Dox-LE) and the media temperature had been provided in the manuscript (Fig. 2b). Apparently, the four samples of AuNCs/Dox-liposome in Fig S4 (a-d) showed the temperature dependence of PL emission, and good linear relationships between the relative PL intensity and media temperature. The corresponding linear coefficients (R) of regression equations were calculated to -0.9947, -0.9922, -0.9927, and -0.9956, respectively. However, the sample of AuNCs/Dox-liposome in the manuscript (Fig. 2b) had a better linear coefficient (-0.9985). Therefore, this sample was selected as the typical AuNCs/Dox-liposome for subsequent experiments.

Part S6. Stabilities of the AuNCs and Dox in AuNCs/Dox-liposome under light irradiation

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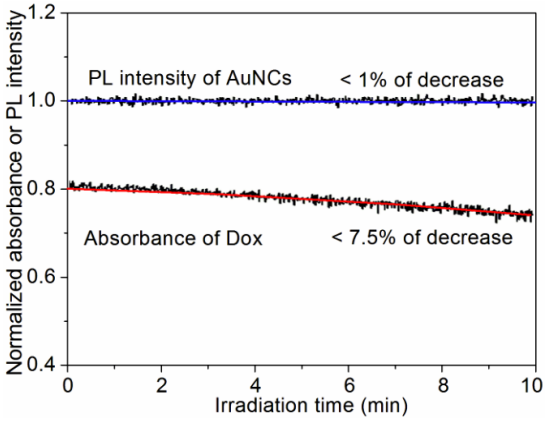

Fig. S7 Normalized absorbance of Dox (at 480 nm) and normalized PL intensity of AuNCs (at 705 nm, excited at 575 nm) in the AuNCs/Dox-liposome under a laser irradiation (575 nm, 50 mW) for 10 min. For the AuNCs loaded into the liposome, PL intensity of AuNCs/Dox-liposome was highly stable and almost no decrease (<1 %) was observed. For the Dox loaded into the liposome, no more than 7.5% of decrease in the absorbance of Dox was watched after 10 min irradiation. These results testified that AuNCs loaded into the liposome was highly stable, and the Dox loaded into the liposome still maintained relatively high stability (< 7.5%) after 10 min of continuous laser irradiation.

Part S7. Sample preparation and Dox concentration calculation

The as-prepared AuNCs/Dox-liposome (in Part S2) was ultrasonically dispersed in 10 mL of PBS (1 mM, pH 7.4), and the concentration of AuNCs/Dox-liposome was regulated to 0.1 mg·mL^{-1}. Under continuous stirring, the liposome-PBS mixture solution was prepared at room temperature (25 ^{o}C). Then, the mixture solution was continuously irradiated by using a laser (575 nm, 50 mW) for different times (0~8 min). After irradiation for a period of time (*e.g.* 4 min), the suspension of mixture solution was centrifuged, and washed with water twice to remove free Dox that was released from AuNCs/Dox-liposome. Both the initial (before irradiation) mass and the residual (after irradiation) mass of Dox loaded in the liposome were calculated by UV-vis spectrophotometer (absorbance) at 480 nm using Lambert-Beer law. The concentration of released Dox (%) was calculated by the following equation as below:

$$\text{Released Dox (\%)} = 100 \times (M_{\text{initial-Dox}} - M_{\text{residual-Dox}}) / (M_{\text{initial-Dox}}) \quad (5)$$

Part S8. Tumor cellular uptake of AuNCs/Dox-liposome

We have selected HeLa, L929 and HepG2 cells (bough from the cell bank of Shanghai Science Academe) as the typical samples of tumor cells to evaluate the analytical performance of AuNCs/Dox-liposome-based PL sensor of temperature. In detail, 6 × 10^4 cells/well (*e.g.* HepG2 cells) was seeded on a 6-well plate at 37 ^{o}C for 24 h. Then, 0.1 mg·mL^{-1} of AuNCs/Dox-liposome aqueous suspension was added into the cell dishes. After 24 h incubation, AuNCs/Dox-liposome-loaded cells were triple washed with PBS (1 mM, pH 7.4) to remove free AuNCs/Dox-liposome absorbed or attached on the outer surface of cell membranes. Then, the targeting cells were respectively irradiated for 3 and 6 min, using a laser (575 nm, 50 mW). After the laser irradiation for some time (*e.g.* 3 min), PL spectrum of the prepared cell solution was detected by using fluorescence spectrophotometer (excited at 575 nm). The environmental temperature of cell solution was calculated according to the PL intensity (at 705 nm) of cell solution, using the linear relationship inserted in Fig. 2b. In the cell solution, the concentration of Dox released from AuNCs/Dox-liposome due to the light irradiation-triggered photothermal effect was measured by the method referred in Part S7.

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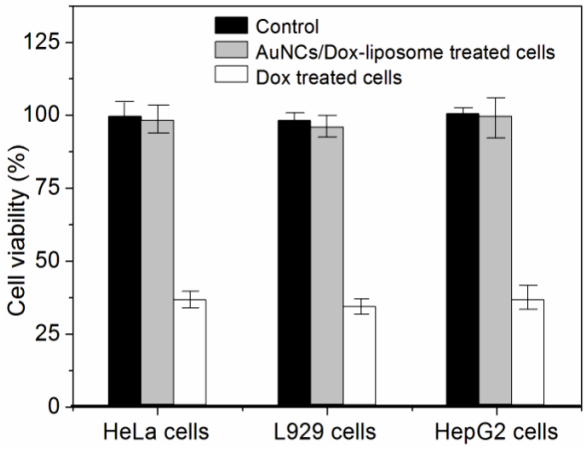

Fig. S8 *In vitro* cytotoxicity of the Dox and AuNCs/Dox-liposome against HeLa, L929, and HepG2 cells after 24 h incubation at 37 ^{o}C. The controls were prepared in the absence of Dox and AuNCs/Dox-liposome. The concentrations of Dox and AuNCs/Dox-liposome in each group of tumor cells were fixed to be 1.0 and 0.1 mg·mL^{-1}, respectively. As a result, after 24 h incubation, the AuNCs/Dox-liposome (as a PL sensor for temperature detection of tumor cells in this manuscript) produced neglectful effect (< 5%) on cell viability, indicating excellent biocompatibility of AuNCs/Dox-liposome and potential application as an intracellular fluorescent thermometry to analyze tumor cells.

Part S9. Photothermal cytotoxicity of AuNCs/Dox-liposome

To test the photothermal cytotoxicity of AuNCs/Dox-liposome *in vitro*, HepG2 cells as the typical tumor cells were selected and corresponding experiments of cell viability were regularly conducted. Cytotoxicity studies of the free Dox and the AuNCs/Dox-liposome were assessed by 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay. Typically, HepG2 cells (1×10^4 cells/well) were incubated in the Dulbecco's modified Eagle medium (DMEM) containing calf serum (wt. 10 %) and 100 units mL^{-1} penicillin in a fully humidified incubator with CO_2 (vol. 5%) at 37 ^{o}C. When the cells reached 80% of confluence with a normal morphology, the free Dox and AuNCs/Dox-liposome were added to cell dishes, respectively. The concentrations of free Dox and AuNCs/Dox-liposome in cell solution were respectively adjusted to be 1 and 0.1 mg·mL^{-1}. Then, the cell dishes containing additives were put into incubators for 24 h at 37 ^{o}C. Afterward, the treated cell dishes were irradiated for 0, 180 and 250s. In the absence of free Dox and AuNCs/Dox-liposome, the control groups were prepared by the above method. After 24 h incubation, the culture medium was replaced by 20 μL of MTT reagent (diluted in the culture medium, 0.5 mg·mL^{-1}), followed by an additional 2 h incubation. After that, the MTT medium was carefully removed and 150 μL of dimethyl sulfoxide (DMSO) was added into each well for dissolving crystals, and the absorbance (A) of colored solutions (the individual wells) was recorded at 570 nm with a Multiskan MK3 enzyme-labeled Instrument. All experiments were performed in triplicate, and each result was averaged. The cell viability (or survival) rate was determined by using the following equation as below:

Cell viability rate (%) = $100 \times (A_{\text{test cells}} / A_{\text{control cells}})$

In addition, after 180s and 250s light irradiation (575 nm, 50 mW), the environmental temperatures in cell solution were detected by using the PL sensor of temperature developed in this manuscript (Fig. 2b).

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Table S1 Dox concentrations and temperatures in tumor cells incubated with AuNCs/Dox-liposome (0.1 $mg \cdot mL^{-1}$) after light irradiation (575 nm, 50 mW) were measured by the HPLC, digital thermometer and liposome-based PL sensor of temperature.

	Irradiation time / 3 min				Irradiation time / 6 min			
Cells	HPLC, Dox/%	Thermometer, T/^{o}C	PL sensor, T/^{o}C	RSD, [a] /%	HPLC, Dox/%	Thermometer, T/^{o}C	PL sensor, T/^{o}C	RSD, [a] /%
HeLa	7.4	37.1	37.2	1.5	71.9	47.9	48.0	3.3
L929	6.8	36.9	36.9	2.8	72.0	48.3	48.4	1.0
HepG2	7.1	37.3	37.4	2.3	71.7	48.0	48.2	2.5

[a] The relative standard deviation (RSD) from the PL sensor measured results of temperatures was defined as (standard deviation/mean)×100%. Dox concentrations and temperatures were all determined repeatedly, and each result was expressed as mean of six determinations.