Electronic Supplementary Information (ESI)

Facile synthesis of hollow Au nanoflowers for synergistic chemo-photothermal cancer therapy

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Experimental Section

Chemicals: Hydroge tetrachloroaurate trihydrate ($HAuCl_4 \cdot 3H_2O$), Poly(acrylic acid) (PAA) and L-Ascorbic acid (LAA) were purchased from Sigma-Aldrich (USA). Anhydrous ethanol and aqueous ammonia solution were purchased from Beijing Chemical Works. Isopropyl alcohol (IPA) was purchased from Tianjin Fuyu Fine Chemical Co., Ltd. Anhydrous ethanol, aqueous ammonia solution and IPA were analytically pure. All glassware was first cleaned with freshly prepared aqua-regia and extensively rinsed with water before it was used. Hyperpure water was used in all experiments.

Characterization: Transmission electron microscope (TEM) measurements were carried out on a JEOL-100CX transmission electron microscope at 80 kV (Hitachi, Japan). Scanning electron microscopy (SEM) images were taken with an XL30 ESEM-

FEG field-emission scanning electron microscope (FEI Co.). High-resolution TEM (HR-TEM) characterizations were recorded by a TECNAI G2 F20 transmission electron microscope under 200 kV accelerating voltage. UV-vis absorption spectra were monitored by a U-3010 spectrophotometer (Hitachi, Japan). Confocal laser scanning microscopy (CLSM) was observed by means of an Olympus Fluoview FV1000. ICP-AES was determined by a Leeman ICP-AES Prodigy instrument. FTIR spectra were performed with a Magna 560 FTIR spectrometer (Nicolet, USA).

Synthesis of PAA Nanospheres: Well-dispersed PAA nanospheres were prepared at room temperature. Firstly, PAA solution (100 μL, 0.2 g mL^{-1}) and $NH_3 \cdot H_2O$ (100 μL, 2 mol L^{-1}) were added to 10 mL deionized water with ultrasonic sound for 10 min in a round-bottom flask, and then, 40 mL IPA was dropwise added into the flask by continuously stirring at a rate of 20 $cm^3\ h^{-1}$, which produced an color change from colorless PAA solution to a milk-white color. The obtained PAA nanospheres were stored at room temperature for further experiments.

Synthesis of hollow Au nanoflowers (H-AuNFs): In a 50 mL round-bottom flask, 15 mL IPA was added to 3mL of as-prepared PAA nanosphere solution. After that, 100 μL of 30 mM $HAuCl_4$ was added dropwise to the resulting solution with moderate agitation. After stirring at room temperature for 2 h, 90 μL of 0.1 M LAA was added to the resulting mixture and kept reacting for 1 h under ambient conditions. Finally, H-AuNFs obtained from the above process were collected by centrifugation at 5000 rpm for 8 min and washed with distillated water twice.

DOX loading and pH/NIR dual-responsive controlled release in vitro: For DOX loading, free DOX (10 mg mL^{-1}, 30 μL) was mixed with H-AuNFs solution (1 mg mL^{-1}, 1 mL). Then the mixed solution was stirred at room temperature for 24 h. DOX-loaded H-AuNFs were obtained by centrifugation and washed once with deionized water to remove the DOX adsorbed on the surface. The amount of original DOX and all the supernatants were determined by measuring the absorbance at 490 nm in a UV-Vis spectrophotometer. The DOX loading efficiency (LE) can be calculated as eq 1:

$$\mathrm{LE}(\%) = \frac{m(\text{original DOX}) - m(\text{DOX in supernatant})}{m(\text{original DOX})} \times 100\% \qquad (1)$$

Release profile was assessed at 37 ^{o}C in phosphate buffered saline (PBS, pH = 5.3 and 7.4). 1 mL of H-AuNFs in PBS was placed in centrifuge tubes (in triplicate). Two of them (pH = 5.3 and 7.4, respectively) were kept releasing in a 37 ^{o}C bath without other conditions. To prove that the laser irradiation can promote the drug release, another (pH = 5.3) was exposed to NIR irradiation at selected time interval with the 808 nm NIR light at a power density of 2 W cm^{-2}. All the supernatant was carefully extracted by centrifuging and was resuspended in 1 mL of fresh buffer. DOX concentration in the supernatant was determined by UV-Vis spectrophotometer at the absorbance of 490 nm.

The photothermal effect of H-AuNFs: First, the photothermal effect of H-AuNFs was measured in aqueous solution. Briefly, nanoparticle suspensions (1 mL) with various concentrations were subjected to NIR laser with wavelength of 808 nm and power density of 2 W cm^{-2} for 5 min. The temperature was recorded every 30 s. Deionized water was also exposed to the NIR laser for comparison. Subsequently, the photothermal effect in the cell level was analyzed by using calcein AM staining method. Calcein AM can only penetrate in live cells and emit green fluorescence. The cells were seeded in 96-well plates (2.5 × 10^4 cells per well) and incubated for 24 h to attach to the wells at the environment of 37 ^{o}C and 5% CO_2 using DMEM medium with 10% fetal bovine serum (FBS). Then, the cells treated by DOX-free H-AuNFs (25 μg mL^{-1}) were exposed to NIR laser (2 W cm^{-2}) for 5 min and finally stained with calcein AM. In addition, cells treated with only H-AuNFs or laser irradiation, and the group treated with no H-AuNFs and no laser were investigated as comparison, respectively.

Calculation of the photothermal conversion efficiency (η): To evaluate the photothermal conversion efficiency (η), the time-dependent temperature increment of the aqueous dispersion (0.2 mg mL^{-1}) was recorded under continuous 808 nm NIR laser irradiation with a power density of 2 W cm^{-2}. Subsequently, the irradiation source was shut off, and the temperature decrease of the aqueous dispersion was monitored to determine the rate of heat transfer from the dispersion system to the environment. The photothermal conversion efficiency (η) of H-AuNFs was calculated

using eq 2:

$$\eta = \frac{hS(T_{max} - T_{surr}) - Q_{dis}}{I(1 - 10^{-A_{808}})} \quad (2)$$

where h is heat transfer coefficient, S is the surface area of the container, T_{max} is the equilibrium temperature, T_{surr} is the ambient temperature, Q_{dis} represents the heat dissipation from the light absorbed by container, I is incident laser power, and A_{808} is the absorption intensity of H-AuNFs at 808 nm. The value of hS is derived according to eq 3:

$$\tau_s = \frac{m_D C_D}{hS} \quad (3)$$

where τ_s is the sample system time constant, m_D and C_D are the mass and heat capacity of deionized water used as the solvent, respectively. The Q_{dis} was measured independently using sample cell containing pure water without H-AuNFs.

Cellular uptake: Cellular uptake by HepG-2 cells was examined by using confocal laser scanning microscopy (CLSM). For CLSM, the HepG-2 cells were seeded in 24 well plates with a clean cover-slip per well and incubated with 20 μg mL^{-1} of DOX-loaded H-AuNFs for 1 h, 3 h, 15 h and 24 h in the environment of 5 % CO_2 at 37 ^oC. After incubation, cell supernatant was discarded and the cell monolayer was washed with PBS (pH = 7.4) for three times. Thereafter, cells were treated with formaldehyde for 10 min for fixing and then rinsed three times with PBS again. Subsequently, HepG-2 cells were stained with Hoechst 33342 for 15 min and rinsed with PBS three times. The cover-slips were mounted onto a glass microscope slide and the cells were observed by CLSM.

Cell cytotoxicity in vitro: MTT assays were carried out with HepG-2 cells to quantify the cytotoxicity of DOX-free H-AuNFs, free DOX, DOX-loaded H-AuNFs and DOX-loaded H-AuNFs with NIR laser. The cells were seeded in 96-well plates (2.5×10^4 cells per well) and incubated for 24 h to attach to the wells at the environment of 37 ^oC and 5% CO_2 using DMEM medium with 10% fetal bovine serum (FBS). One row of the 96-well plate was used as a blank control with culture medium only. Then, the suspensions of H-AuNFs with different concentrations were added. After

incubation for another 24 h, the cell viability was determined by MTT assay.

Animal experiment: All the animal experiments were done on Kunming mice and were consistent with the guidelines of institutional animal use committee. H-22 tumor cells were inoculated into the left axillary fossa of Kunming mice in the animal experiment. The tumor-bearing mice were randomly assigned to four groups (n = 5) and each group was numbered one through four. The mice from the first to the third groups were separately treated by tail vein injection of free DOX (4 mg kg^{-1}), DOX-loaded H-AuNFs (containing 4 mg kg^{-1} DOX) and physiological saline (300 μL) served as a control. The mice in the fourth group were intravenously injected with equivalent dose of DOX-loaded H-AuNFs and the tumors were exposed to NIR laser (2 W cm^{-2}, 5 min) after injection for 24 h. The body weights, tumor volumes were monitored every other day after treatment. The tumor dimensions were measured by a vernier caliper and the tumor volume was computed as eq 4:

$$\text{tumor volume} = \frac{(\text{tumor length}) \times (\text{tumor width})^2}{2} \quad (4)$$

After treatment for 11 days, mice of each group, chosen at random, were sacrificed to retrieve tumors and organs (heart, spleen, liver, lung, and kidney). The excised tumors and organs were washed with deionized water and fixed with 4% paraformaldehyde solution. Tumor growth inhibition rate was calculated with eq 5:

$$\text{inhibition}\,(\%) = \frac{W_C - W_T}{W_T} \times 100\,\% \quad (5)$$

Here, W_C is the average tumor weight of the control group and W_T is the average tumor weight of each treated group. The tissues (control and NPs groups) were processed using conventional method and the tissue sections were stained with hematoxylin and eosin.

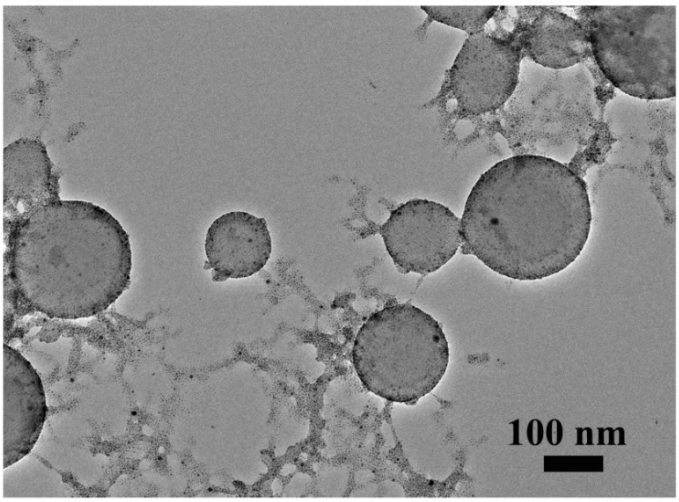

Fig. S1 TEM image of the morphology of the obtained NPs after adding chlorauric acid to PAA nanosphere solution for 2 h.

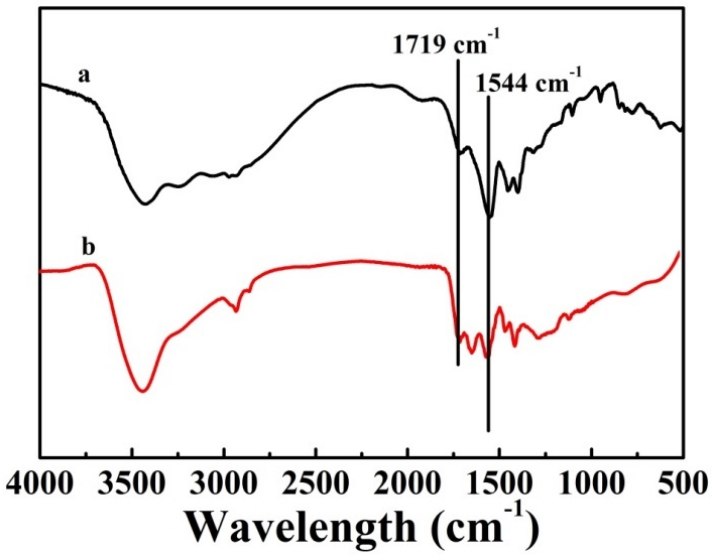

Fig. S2 FTIR spectra of (a) PAA nanosheres and (b) H-AuNFs.

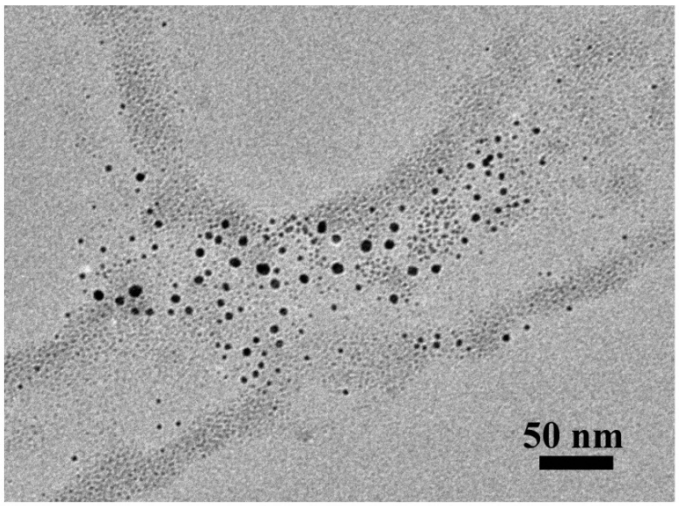

Fig. S3 TEM image of the obtained spherical Au NPs in the absence of PAA nanospheres.

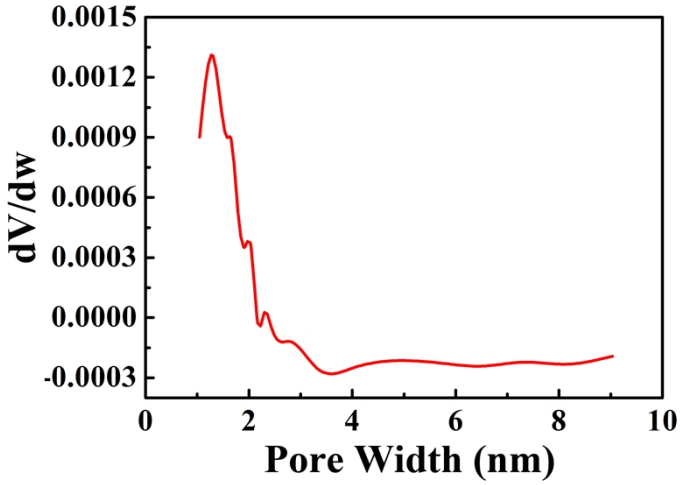

Fig. S4 Pore size distribution of H- AuNFs.

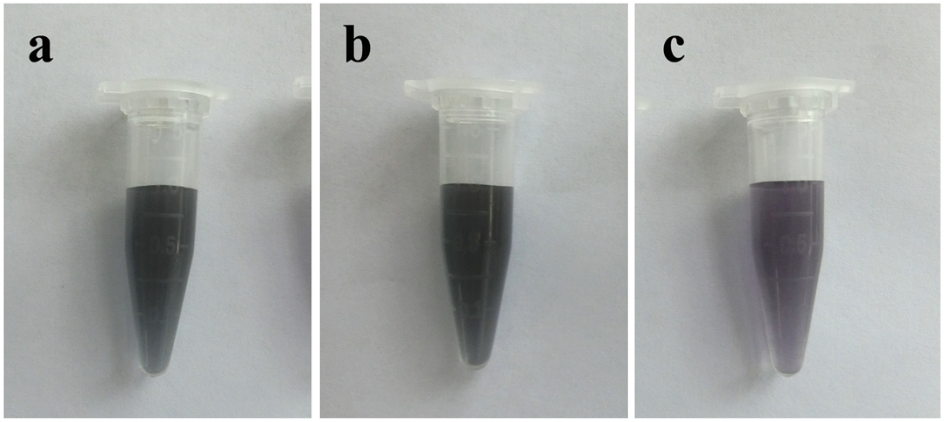

Fig. S5 Photographs of H-AuNFs in (a) water, (b) PBS buffer and (c) culture medium with serum and they were stored for 6 h, respectively.

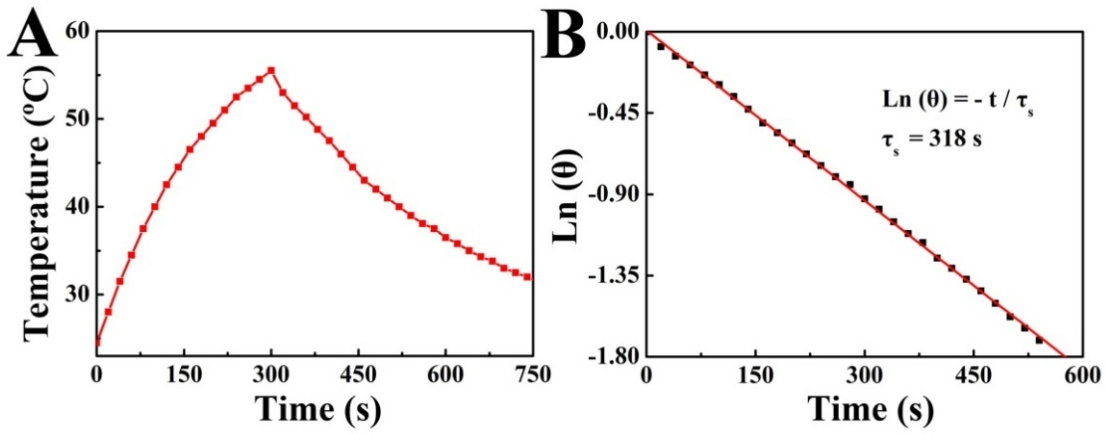

Fig. S6 (a) Photothermal effect of the irradiation of the aqueous dispersion of H-AuNFs with the NIR laser (808 nm, 2 W cm^{-2}), in which the irradiation lasted for 5 min, and then the laser was turned off. (b) Time constant for heat transfer from the system is determined to be τ_s = 318 s by applying the linear time data from the cooling period (after 300 s) versus negative natural logarithm of driving force temperature, which is obtained from the cooling stage of panel a.

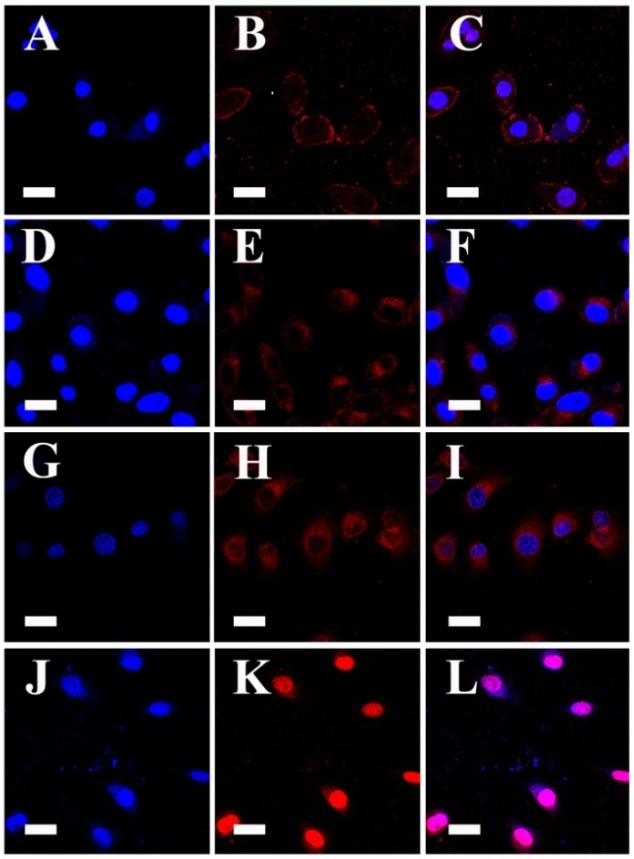

Fig. S7 CLSM images of HepG-2 cells incubated with DOX-loaded H-AuNFs for 1 h (A-C), 3 h (D-F), 15 h (G-I) and 24 h (J-L) at 37 °C, respectively. Each series can be classified to cell nucleus (being dyed in blue by Hoechst 33342), DOX-loaded H-AuNFs and the merged images of both above, respectively. All scale bars are 20 μm.

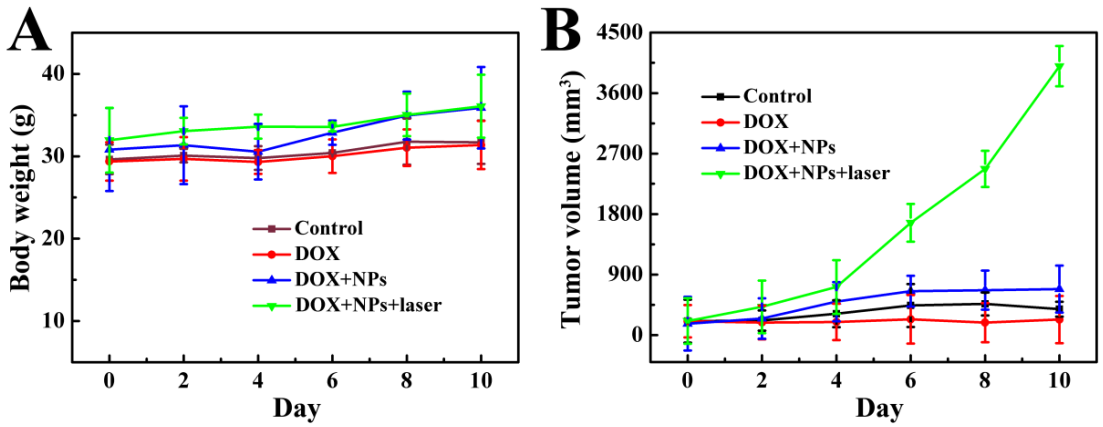

Fig. S8 (A) Body weight and (B) relative tumor volume recorded for mice after treatment with physiological saline as a control, free DOX, DOX-loaded H-AuNFs and DOX-loaded H-AuNFs upon NIR irradiation.

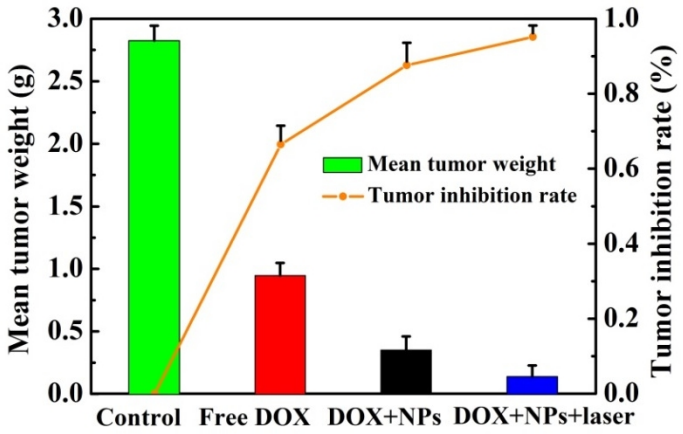

Fig. S9 The mean tumor weights for each group on the last day of experiment and the tumor inhibition rate of each group.