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# Supplementary Information

## In Vivo Self-assembly Induced Retention of Gold Nanoparticles for Enhanced Photothermal Tumor Treatment

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Keyword: In Vivo, Gold Nanoparticles, Self-Assembly, Retention, Photothermal Therapy

**Figure S15.** Biodistribution of **AuNPs@Pep1/Pep2**, Ctrl 1 and Ctrl 2 in lung injected after 1 h, 4 h, 12 h and 48 h;

**Figure S16.** Biodistribution of **AuNPs@Pep1/Pep2**, Ctrl 1 and Ctrl 2 in kidney injected after 1 h, 4 h, 12 h and 48 h;

**Figure S17.** Temperature rise profiles of Tris buffer, the Tris buffer solutions of Ctrl 1, Ctrl 2 and assemblies of **AuNPs@Pep1/Pep2** as a function of heating time under irradiation by a 660 nm laser (5 W/cm$^2$);

**Figure S18.** Infrared thermal images of PBS injected U87 tumor-bearing mice at different time points under laser irradiation at 660 nm with 5W/cm$^2$;

**Figure S19.** Time-dependent mice weight changes of U87 xenografted mice by treatment of PBS (200 μL), PBS (200 μL) and Laser, Ctrl 1 (1 mg/mL, 200 μL) and Laser, Ctrl 2 (1 mg/mL, 200 μL) and Laser, **AuNPs@Pep1/Pep2** (1 mg/mL, 200 μL), and **AuNPs@Pep1/Pep2** (1 mg/mL, 200 μL) and Laser. The laser irradiation was carried out at 12 h after *i.v.* administration (Laser: 660 nm, 5 W/cm$^2$, 10 min).

**Figure S20.** *In vivo* toxicity investigated the organs (heart, liver, spleen, lung and kidney) of treated mice under different administration. The organs toxicity was studied on pathological features (tissues section) of different organs in representative mice treated with PBS, PBS and Laser, Ctrl 1 and Laser, Ctrl 2 and Laser and **AuNPs@Pep1/Pep2** and Laser. The tissues section was stained with H&E.

## 1. Experiment:

### Chemicals and reagents

Gelatinase (contain MMP-2), tifluoroacetic acid (TFA), 2, 5-dihydroxybenzonic acid (DHB), triisopropylsilane (TIPS), 1.2-Ethanedithiol and ascorbic acid (VC), Trisodium citrate (99.9%), piperidine and 4-methylmorpholine (NMM) were purchased from Sigma-Aldrich Chemical Co., LCC. All Fmoc-amino acids, Wang resins and O-(benzotriazol-1-yl)-N, N, N', N'-tetramethyluronium hexafluorophosphate (HBTU) were obtained from GL Biochem. (Shanghai) Ltd. Hydrogen tetrachloroaurate (Ⅲ) trihydrate ($HAuCl_4 \cdot 4H_2O$, 99%) was purchased from Sinopharm Chemical Reagent Co., Ltd. Phosphate-Buffered Saline (PBS), Fetal

Bovine Serum (FBS), DMEM (Dulbecco's Modified Eagle Medium) medium，McCoy's 5A Medium and Trypsin were obtained from HyClone/Thermo fisher (Beijing, China). U87, MCF-7, HT-29, 293T and LO2 cell lines was purchased from cell culture center of Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China). Cell counting kit assay (CCK-8) was obtained from Beyotime Institute of Biotechnology (Shanghai, China). 96-well culture plates were purchased from Coning Company. 6-8 week-old female BALB/c nude mice were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Inertsil C18 HPLC column (ODS-3, 3 μm, 4.6×250 mm) for peptide analysis was purchased from Shimadzu Corporation. The HPLC grade solvents methanol and acetonitrile were from Aldrich, Fisher Scientific. Ultrapure water (18.2MΩ·cm) was used in all experiments. All the other solvents were purchased from Beijing (China) Chemical Company.

**Methods**

**UV-vis spectra** were recorded using Shimazdu-2600. The spectra were recorded in quartz glass cuvettes and according to Lambert-Beer's law the extinction coefficients were calculated.

**The morphology** of the **AuNPs@Pep1/Pep2** and aggregate was observed by Tecnai G2 20 S-TWIN transmission electron microscopy operating at an acceleration voltage of 200 kV.

**HPLC** was carried out by Shimadzu LC-20A.

**Mass spectra** were obtained by Microflex LRF MALDI-TOF with a matrix of DHB (20 mg/mL) in TA30 ($H_2O$ 70%, acetonitrile 30%, 0.1% TFA, v/v solution) by the drid droplet method on the AnchorChip of a MSP 96 polished plate.

**FTIR spectra** were detected by the Spectrum One, Perkin Elmer Instruments Co. Ltd.

**ICP-MS Sample Preparation and Detection:** Mice were euthanized 1 h, 4 h, 12 h, 48 h after intravenous injection of **AuNPs@Pep1/Pep2**, Ctrl 1 and Ctrl 2 (1mg/mL, 200 μL), and then the tumor tissues and organs were collected. All the samples were completely digested in acid (3:1 mixture of $HNO_3$ and $H_2O_2$) on a hot plate prior to

ICP analysis. The detection of Au contents in samples treated with **AuNPs@Pep1/Pep2** and two controls was performed by mass spectrometry, NexIon 300X, PerkinElmer.

**The morphology of** the aggregate of **AuNPs@Pep1/Pep2** in tumor tissues was observed by Ht-7700, Hitachi, Ltd.

**Synthesis and characterization**

**Synthesis of gold nanosphere:** In a typical method, uniform gold nanoparticles in diameter 13 nm were prepared by injecting a sodium citrate solution(15 mL, 1% w/v) into a boiling aqueous solution of $HAuCl_4$ (100 mL, 1 mM) under vigorous stirring.[1] After boiling for 30 min, heat was removed to allow the reaction solution to cool to room temperature. The solution is kept at 4 °C for long-term storage.

**Synthesis of peptide 1, peptide 2 and peptide 3:** Peptide 1 (CPLGVRGDDRGD), peptide 2 (CKKKLVFF) and peptide 3 (CPIGIRGDDRGD) were synthesized according to the standard solid-phase peptide synthesis techniques using Fmoc-coupling chemistry. Fmoc-Asp (otBu) resin (loading=0.349 mmol/g) for peptide 1 and peptide 3, and Fmoc-Phe resin (loading=0.322 mmol/g) for peptide 2 were used as the solid phase support respectively. Deprotection of the N-terminal Fmoc group was carried out using piperdine (20%, v/v) in anhydrous DMF for 15 min. Fmoc deprotection was confirmed by a qualitative ninhydrin test (ninhydrin:phenol: VC=1:1:1, v/v). Amino acid activation was achieved by NMM (0.4 M) and HBTU (equal molar ratio with the amino acid) in anhydrous DMF. Amino acid coupling was reacted at room temperature for 1 h. Cleavage from the resin and deprotection of the amino acid side chains was carried out with a mixture of TFA (92.5%, v/v), $H_2O$ (2.5%, v/v), TIPS (2.5%, v/v) and 1.2-Ethanedithiol (2.5%, v/v) for 30 min in ice bath and then at room temperature for another 3 h. After separated from the resin, the mixture liquid was vacuum rotary evaporated to remove the TFA. The peptides were then precipitated in cold anhydrous diethyl ether, wash and collected by centrifuge, dried under vacuum. All peptides were confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

**Spherical AuNPs modification with peptide 1 and peptide 2:** Co-coating method was performed under stirring at room temperature for 24 h by mixing a certain volume of spherical AuNPs with peptide 1 and peptide 2. 400 μL AuNPs (200 μg/mL) were diluted by adding ultrapure water to 2 mL. The sols then adjusted to pH 9 by NaOH (90 μL, 0.1 M). Under vigorous stirring, typically, peptide 1 (40 μL, 2 mg/mL in water) and peptide 2 (5 μL, 1 mg/mL in water) were added to the gold sols simultaneously**.** The mixture was stirred for 24 h and then was centrifuged three times for 15 min (15000 rpm, Eppendorf centrifuge) to obtain **AuNPs@Pep1/Pep2**.

Then, the free -SH was evaluated according to the literature.[2] The obtained **AuNPs@Pep1/Pep2** was washed three times by centrifugation and re-dispersed in equal amount of water. A 1 mL of liquid supernatant (after centrifugation with **AuNPs@Pep1/Pep2**) and 20 μL of Ellman's reagent (2 mg/mL) were then added to 2 mL of phosphate buffer (pH = 7.8) to react for 3 min at room temperature. Then the absorbance of the supernatant was measured at a wavelength of 412 nm by a UV-vis spectrophotometer.

**Responsive cleavage of peptide 1 by MMP-2:** The enzyme mixture was prepared: (2 mg/mL) peptide 1, (15 ng/mL) MMP-2 in buffer (20mM Tris·HCl, 50 mM NaCl, 100 mM $CaCl_2$, 0.05% Brij 35, pH 7.4) with a total volume of 0.5 mL. The reaction was followed at 37 °C for 1 h. The original peptide and after exposition for 1 h to MMP-2 were analyzed by HPLC (acetonitrile (0.1% TFA) /water (0.1% TFA) linear gradient from 5%/95% to 60%/40% in 30 min flux 1 mL/min, 220 nm detection). The residual peptides were confirmed by MALDI-TOF-MS.

**Responsive cleavage of AuNPs@Pep1/Pep2 by MMP-2**: The enzyme mixture was prepared: (200 μg/mL) **AuNPs@Pep1/Pep2**, (50 ng/mL) MMP-2 in buffer (20 mM Tris·HCl, 50 mM NaCl, 100 mM $CaCl_2$, 0.05% Brij 35, pH 7.4) with a total volume of 2 mL. The reaction was followed at 37 °C for 4 h. And the residual peptide was confirmed by MALDI-TOF-MS.

**The interaction between extracellular MMP-2 and AuNPs@Pep1/Pep2:** 100 μL (200 μg/mL) **AuNPs@Pep1/Pep2**, Ctrl 1 and Ctrl 2 were incubated with four types original cell culture media (30 μL) in 96-well plates overnight, respectively. The

assembly actions were characterized by colorimetric change and the UV-vis absorbance spectra.

**Cell viability of AuNPs@Pep1/Pep2:** Human kidney cells (293T) and human hepatocytes (LO2) were cultured in DMEM supplemented with 10% fetal bovine serum and 1% antibiotic solution (penicillin and streptomycin) at 37 °C in the environment containing 5% carbon dioxide. Third generation of 293T and LO2 cells (7,000 cells per well) were incubated with different concentrations of **AuNPs@Pep1/Pep2** (62.5, 125, 250, 500, 1000 and 2000 μg/mL) in DMEM medium (200 μL) for 24 h in 96-well plates. After discarding the supernatant, CCK-8/DMEM solution (200 μL, 1/10, v/v) was added and the samples were incubated at 37 °C for 4 h. The absorbance at 450 nm with a reference wavelength of 650 nm was determined using a multifunctional microplate reader.

**In vivo PTT:** Mice bearing U87 cells at 12 h post *i.v.* injection with **AuNPs@Pep1/Pep2** (1 mg/mL, 200 μL), Ctrl 1 (1 mg/mL, 200 μL), Ctrl 2 (1 mg/mL, 200 μL) and PBS (200 μL) were exposed to NIR 660 nm laser (5 W/cm$^2$) for 10 min, respectively. The mice were randomly divided into five groups: only PBS (n = 4), PBS and laser (n = 4), Ctrl 1 and laser (n = 4), Ctrl 2 and laser, and **AuNPs@Pep1/Pep2** and laser (n = 4). The temperature varies of tumor were recorded by Thermal Imager of FLIR E64501. The tumor size were measured by the caliper and calculated by the equation: 1/2 × (length of tumor) × (width of tumor) $^2$. Relative tumor volumes were denoted as $V/V_0$, $V_0$ is the initial volume before treatment.

## 2. Supporting Figures

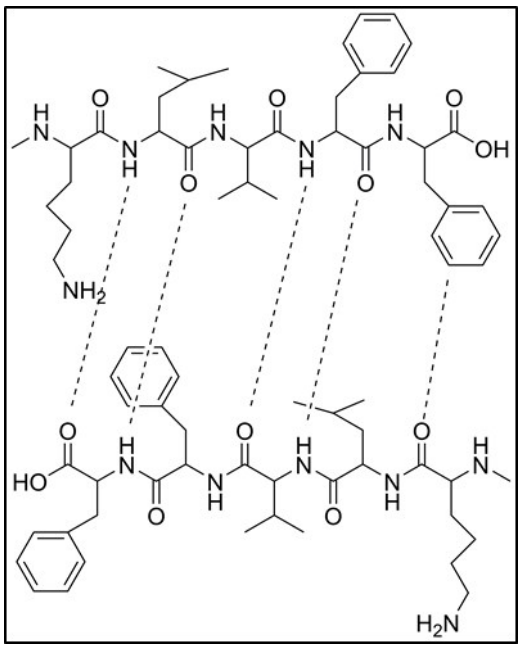

**Figure S1.** The formation of H-bonds between Peptide 2 (KLVFF segment) on another particle.

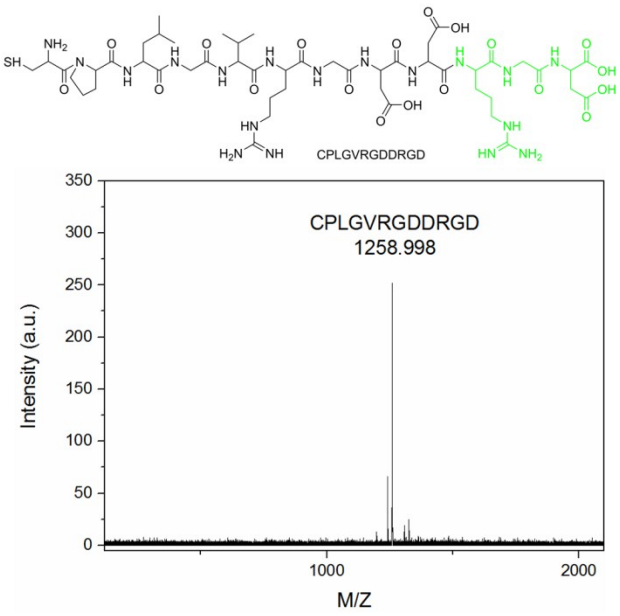


**Figure S2.** The structure formula and MALDI-TOF-MS spectrum of Peptide 1 (CPLGVRGDDRGD).

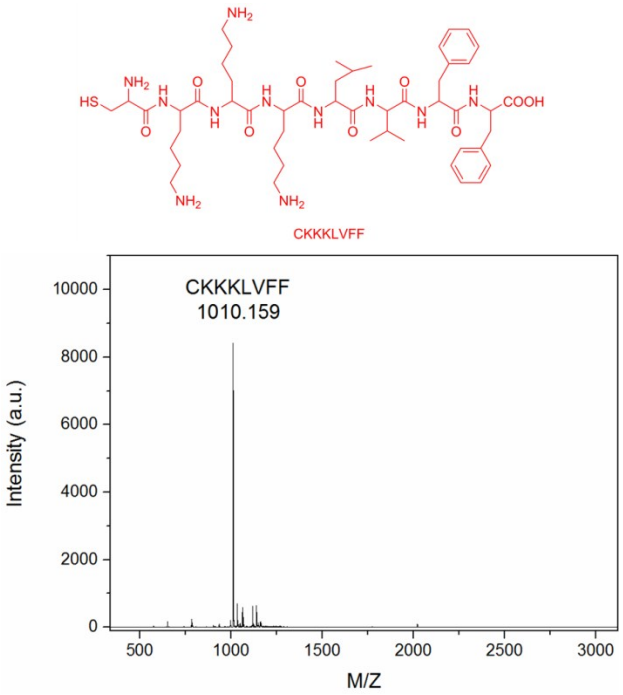


**Figure S3.** The structure formula and MALDI-TOF-MS spectrum of the Peptide 2 (CKKKLVFF).

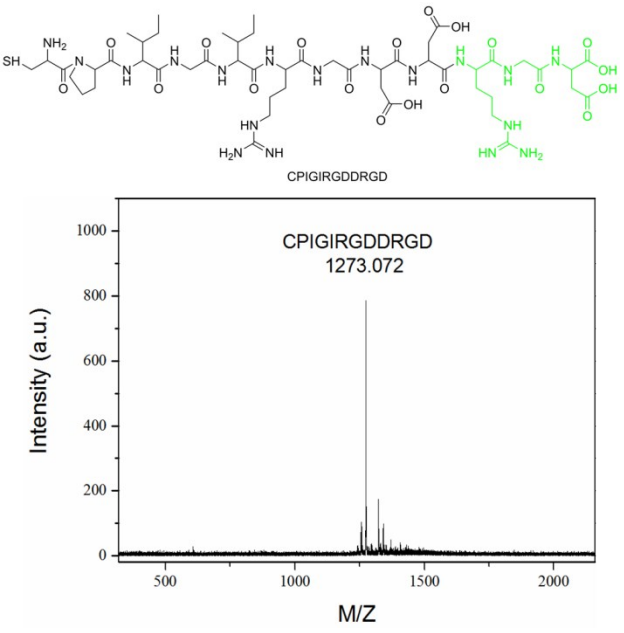


**Figure S4.** The structure formula and MALDI-TOF-MS spectrum of Peptide 3 (CPIGIRGDDRGD).

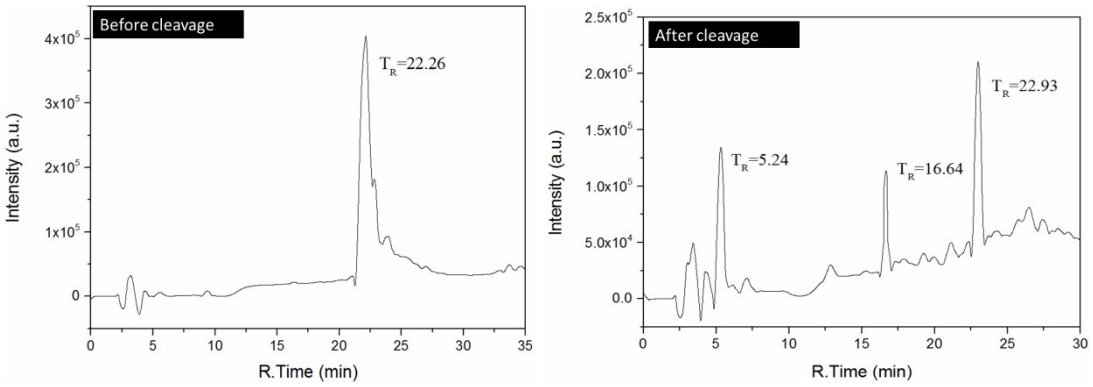


**Figure S5.** HPLC characterization of Peptide 1 before (a) and after (b) incubation with MMP-2.

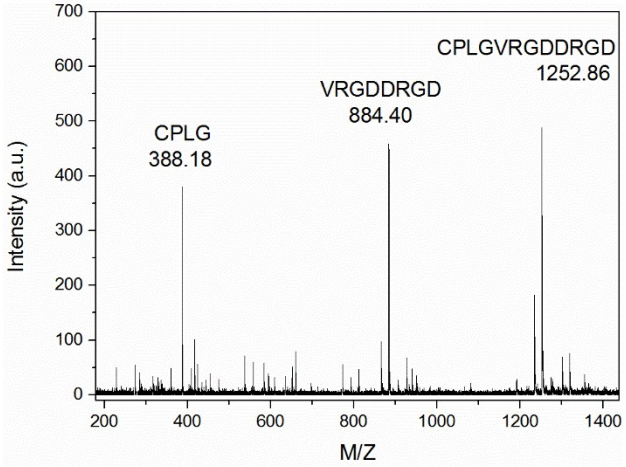


**Figure S6.** MALDI-TOF-MS characterization of Peptide 1 after incubation with MMP-2.

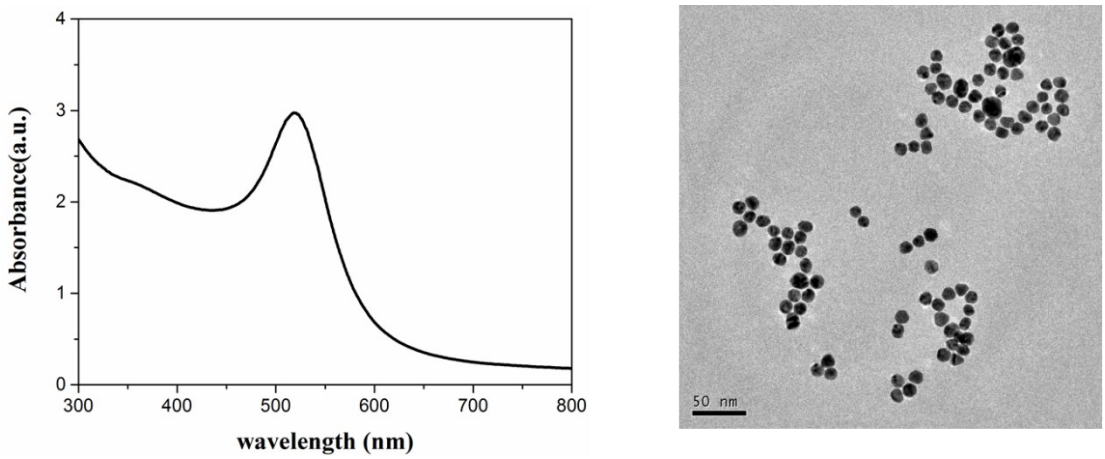


**Figure S7.** UV-vis absorption spectrum and TEM image of spherical gold nanoparticles.

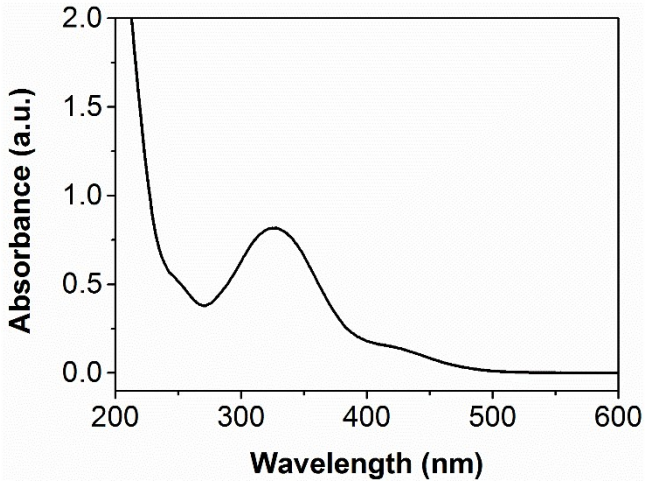


**Figure S8.** UV-vis spectrum of the liquid supernatant (after centrifugation with **AuNPs@Pep1/Pep2**) after reaction with Ellman's reagent. No absorption at 412 nm was detected and the absorption at 325 nm intensified for Peptide 1 or Peptide 2.

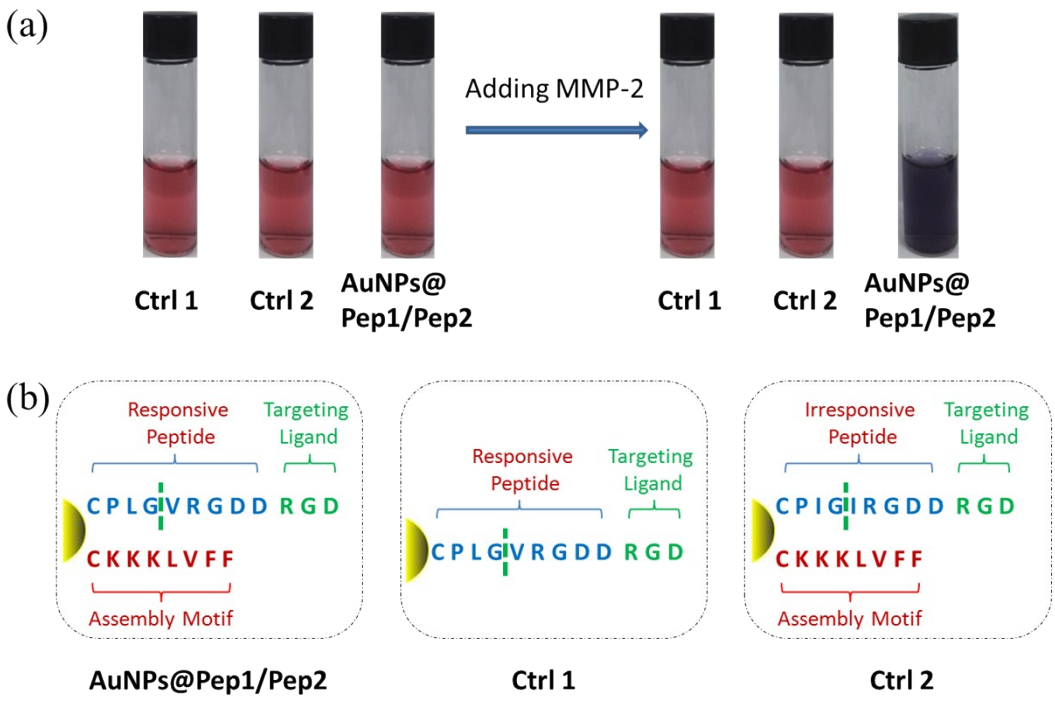


**Figure S9.** (a) The colorimetric change of **AuNPs@Pep1/Pep2**, Ctrl 1 and Ctrl 2 before and after incubation with MMP-2. (b) Structures of **AuNPs@Pep1/Pep2**, Ctrl 1 and Ctrl 2.

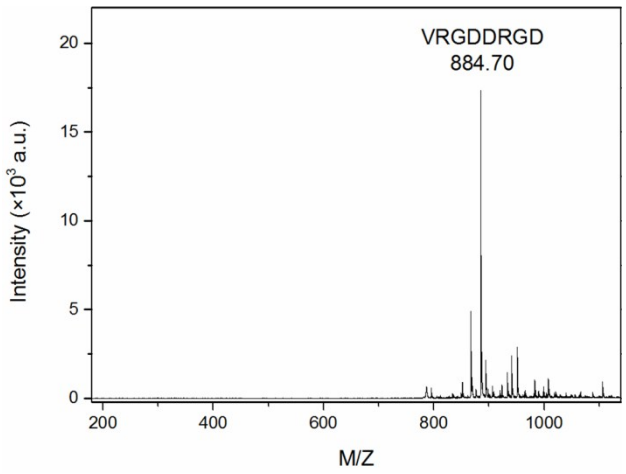


**Figure S10.** MALDI-TOF-MS spectrum of cleavage residual in liquid supernatant from **AuNPs@Pep1/Pep2** enzyme mixture.

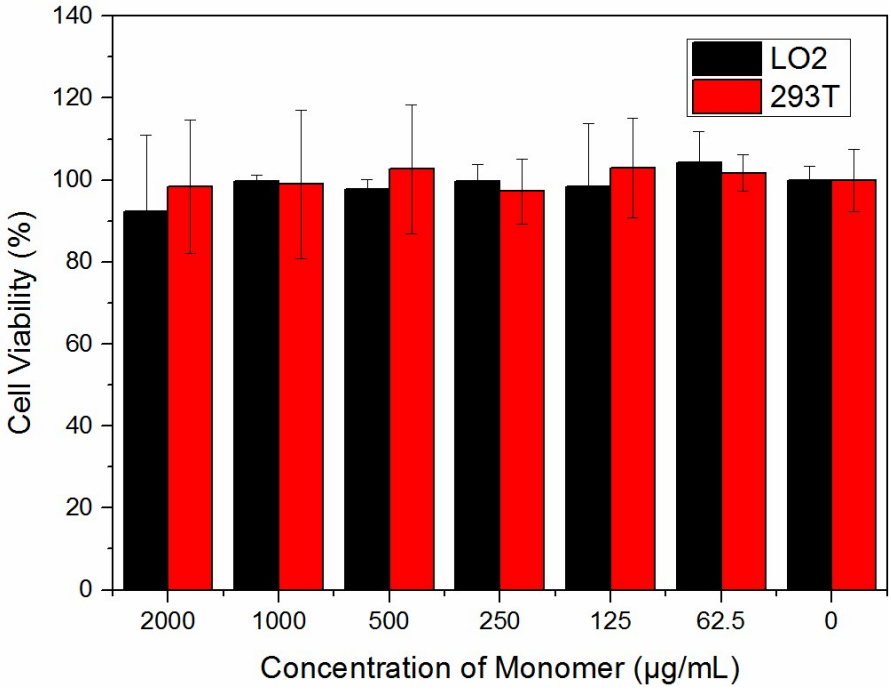


**Figure S11.** Viability of Human kidney cells (293T) and human hepatocytes (LO2) cells upon treating with **AuNPs@Pep1/Pep2** at different concentrations (0-2000 μg/mL).

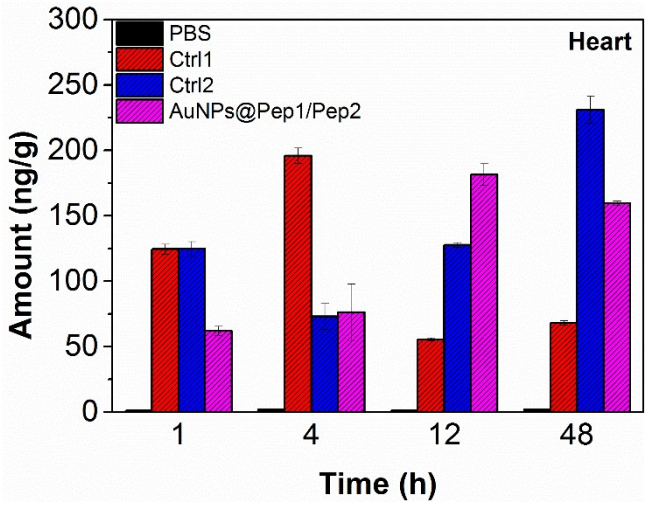


**Figure S12.** Biodistribution of **AuNPs@Pep1/Pep2**, Ctrl 1 and Ctrl 2 in heart injected after 1 h, 4 h, 12 h and 48 h.

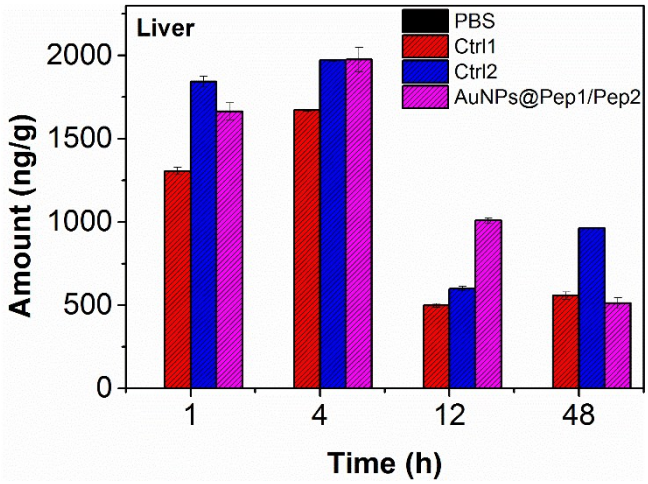


**Figure S13.** Biodistribution of **AuNPs@Pep1/Pep2**, Ctrl 1 and Ctrl 2 in liver injected after 1 h, 4 h, 12 h and 48 h.

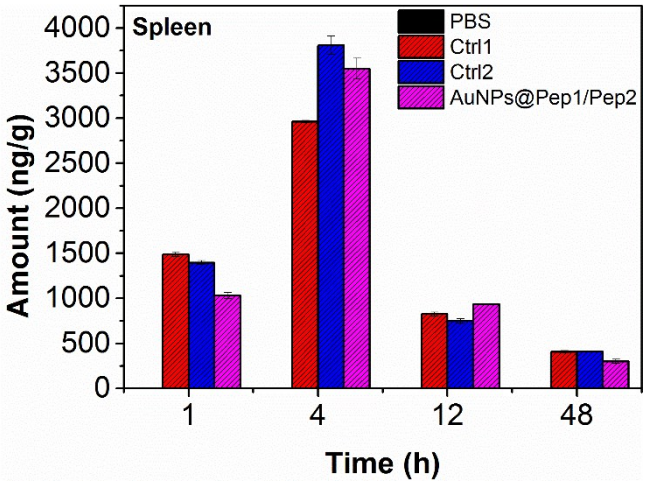


**Figure S14.** Biodistribution of **AuNPs@Pep1/Pep2**, Ctrl 1 and Ctrl 2 in spleen injected after 1 h, 4 h, 12 h and 48 h.

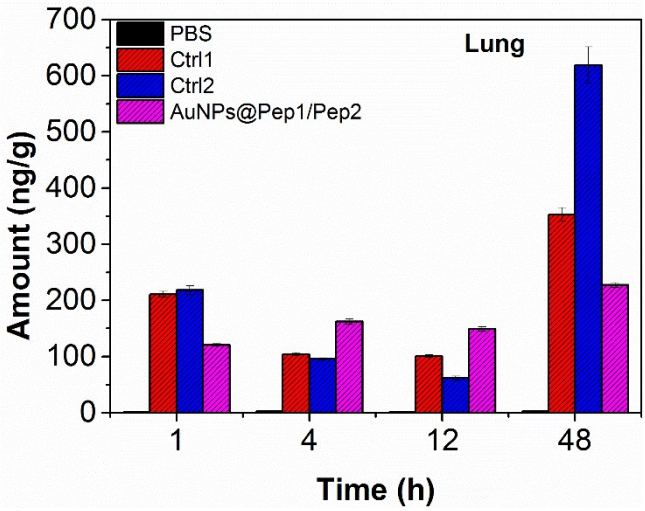


**Figure S15.** Biodistribution of **AuNPs@Pep1/Pep2**, Ctrl 1 and Ctrl 2 in lung injected after 1 h, 4 h, 12 h and 48 h.

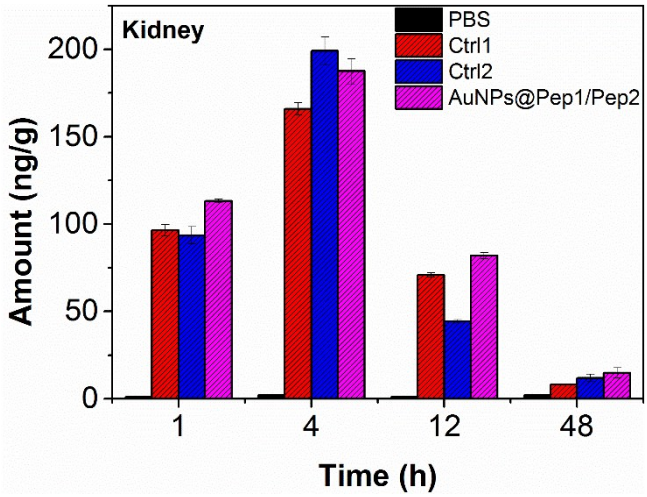


**Figure S16.** Biodistribution of **AuNPs@Pep1/Pep2**, Ctrl 1 and Ctrl 2 in kidney injected after 1 h, 4 h, 12 h and 48 h.

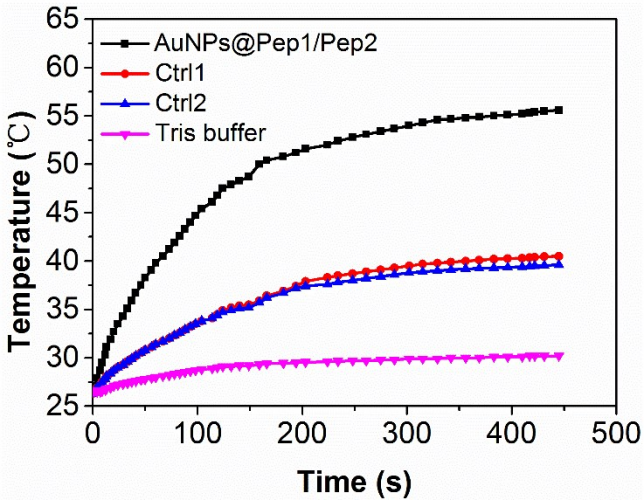


**Figure S17.** Temperature rise profiles of Tris buffer, the Tris buffer solutions of Ctrl 1, Ctrl 2 and assemblies of **AuNPs@Pep1/Pep2** as a function of heating time under irradiation by a 660 nm laser (5 W/cm$^2$).

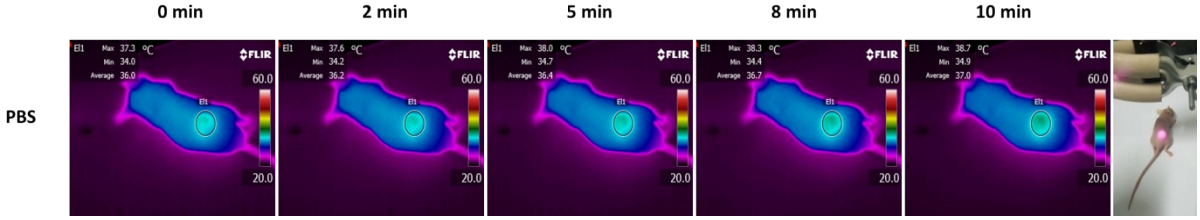


**Figure S18.** Infrared thermal images of PBS injected U87 tumor-bearing mice at different time points under laser irradiation at 660 nm with 5W/cm$^2$.

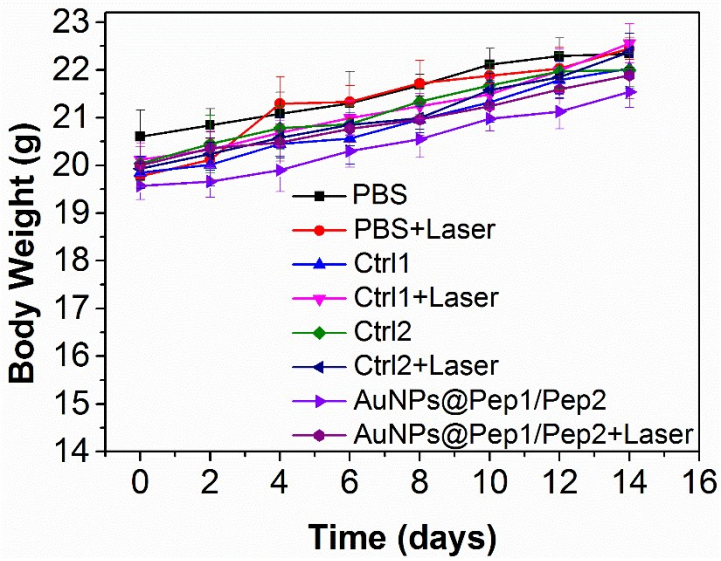


**Figure S19.** Time-dependent body weight changes of U87 xenografted mice by treatment of PBS (200 μL), PBS (200 μL) and Laser, Ctrl 1，Ctrl 1 (1 mg/mL, 200 μL) and Laser, Ctrl 2，Ctrl 2 (1 mg/mL, 200 μL) and Laser, **AuNPs@Pep1/Pep2**，**AuNPs@Pep1/Pep2** (1 mg/mL, 200 μL), and **AuNPs@Pep1/Pep2** (1 mg/mL, 200 μL) and Laser. The laser irradiation was carried out at 12 h after *i.v.* administration (Laser: 660 nm, 5 W/cm$^2$, 10 min).

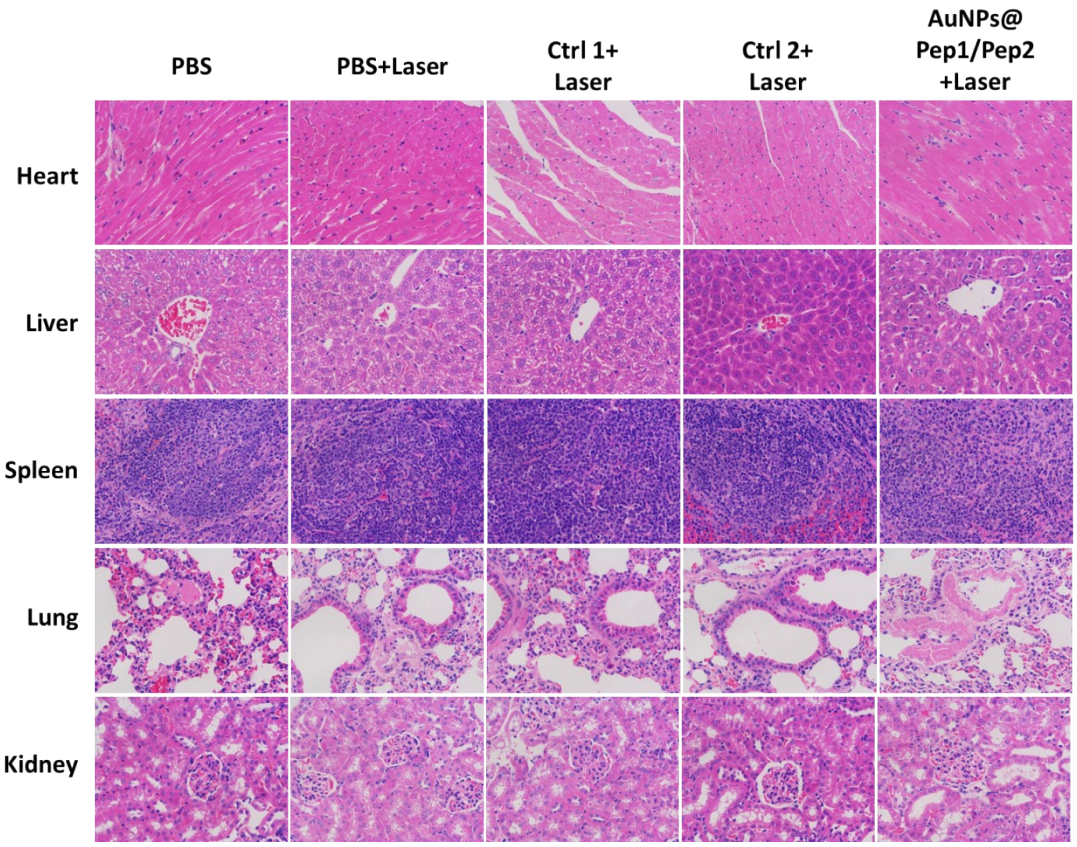


**Figure S20.** *In vivo* toxicity investigated the organs (heart, liver, spleen, lung and kidney) of treated mice under different administration. The organs toxicity was studied on pathological features (tissues section) of different organs in representative mice treated with PBS, PBS and Laser, Ctrl 1 and Laser, Ctrl 2 and Laser and **AuNPs@Pep1/Pep2** and Laser. The tissues section was stained with H&E.

1 G. Frens, *Nature*, 1973, **241**, 20-22.
2 Y. Gao, J. Gu, L. Li, W. Zhao and Y. Li, *Langmuir*, 2016, **32**, 2251-2258.