Electronic Supplementary Information
Remotely Controlled NIR-II Photothermal-Sensitive Transgene System for Hepatocellular Carcinoma Synergetic Therapy

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Experimental Section

Materials

(2,3-Dioleoyloxy-propyl)-trimethylammonium (DOTAP) was purchased from Shanghai Advanced Vehicle Technology Pharmaceutical Ltd. The nucleic acid dye Hoechst 33342 was purchased from Sigma Aldrich Co., Ltd. Gibco DMEM was purchased from Thermo Fisher Scientific Co., Ltd. Fetal bovine serum (FBS) was purchased from PAN Biotech. Phosphate buffer saline (PBS) was purchased from HyClone company (USA). Dichloromethane was purchased from Sinopharm Chemical Reagent Co. Ltd. LIVE/DEAD® Viability/Cytotoxicity Kit and Annexin V-Fluoroisothio cyanate (FITC)/propidium iodide (PI) apoptosis detection kit was purchased from Dojindo Molecular Technologies. The CCK-8 kit was purchased from TransGen Biotech. Ultrapure water was prepared by a Milli-Q Gradient System (18.2 M Ω resistivity, Millipore Corporation, Bedford, MA, USA).

Preparation of SPN and SPNHT

The SPN was synthesized by microemulsion method. Firstly, 1 mg BBTDTS, 10 mg PCL-PEG and 10 mg DOTAP were dissolved in 2 mL CH_2Cl_2. The obtained organic solution was added to 10 mL PVA solution (2 $mg \cdot mL^{-1}$), and then ultrasonic

emulsification with microprobe probe (JY92-Iin, Scientz Biotechnology, Ningbo, China) was performed for 2 minutes (work for 9.5 s and rest for 0.5 s). Subsequently, CH_2Cl_2 was removed by stirring at room temperature for more than 12 hours. The SPN solution was ultrafiltration at 4500 rpm for 30 minutes and then washed three times with ddH_2O to remove excess PCL-PEG.

Moreover, the positively charged SPN could bind to the pDNA through electrostatic adsorption reaction. SPN/pDNA of different mass ratios were oscillated on the vortex oscillator for about 30 s and then incubated at room temperature for 30 min to obtain the final SPN/pDNA mixtures.

Characterization

Zeta potential/particle sizer Malvern 3600 (U.K.) was selected to measure zeta potential and dynamic light scattering (DLS) particle size distribution. The UV-Vis-NIR absorption spectra were measured on the Fourier transform infrared spectrometer (Perkin-Elmer, Spectrum-2000, USA).

Photothermal effect evaluation

The photothermal effect of the SPNHT aqueous solution was studied by irradiating various concentrations (0, 5, 10, 20, 40 and 80 $\mu g \cdot mL^{-1}$) with a 1064 nm laser at a power density of 1 $W \cdot cm^{-2}$ for 10 min. The temperature was monitored and periodically measured by a digital thermometer with a thermocouple probe (ϕ = 0.5 mm) (STPC-510P, China) and IR thermal camera (Ti25 Fluke Co, USA). To explore the photothermal stability of SPNHT nanoparticles, five photothermal cycles (laser on/off) were performed. In each cycle, the aqueous solution of SPNHT (40 $\mu g \cdot mL^{-1}$) was irradiated under the 1064 nm laser for 10 min, then naturally cooling down to room temperature without laser irradiation.

In order to analyze the photothermal conversion efficiency, an aqueous solution of 1 mL SPN (20 $\mu g \cdot mL^{-1}$) was irradiated under the 1064nm-laser (1 $W \cdot cm^{-2}$, 25 min) until the temperature reached the platform, and then it naturally drops to room temperature. Next, we calculated the photothermal conversion efficiency (η) using the following formula:

$$\eta = (hA\Delta T_{max} - Q_s)/I(1 - 10^{-A\lambda}) \quad (1)$$

$$\tau_s = m_D C_D/hA \quad (2)$$

$$\theta = \Delta T/\Delta Tmax \quad (3)$$

where h was the heat transfer coefficient, A was the surface area of a cuvette, T_{max} was the highest temperature when equilibrium is reached, T_{sur} was the ambient temperature, $\Delta T_{max} = T_{max} - T_{sur}$, I was the incident laser power (1 $W \cdot cm^{-2}$), and A_λ was the absorbance of 20 $\mu g \cdot mL^{-1}$ aqueous solution of SPN at 808 nm. Q_s was the heat associated with the light absorbance of the solvent, which measured in ddH_2O at 25.2 mW. The value of hA was determined by formula 2, in which the time constant of the sample system stood for τ_s, 1g of ddH_2O stood for m_D (m_D = 1g,), and the heat capacity of ddH_2O stood for C_D (C_D = 4.2 $j \cdot g^{-1}$). The ratio of ΔT to ΔT_{max} is defined as θ. τ_s determined by applying linear time data on the cooling time versus -lnθ. Combining formula 2 and formula 3, the photothermal conversion efficiency (η) could be obtained.

Gel retardation assay

We further used agarose gel electrophoresis to estimate the binding capacity of the synthesized SPNs nanocarriers with pDNA (HSP70-TRAIL-GFP). SPN mixed with pDNA at different mass ratios (0:1, 2:1, 4:1, 8:1, 16:1, 20:1, 24:1, 28:1). For electrophoresis, 1% agarose gel was mixed in 1×TAE buffer solution at 100 V for 20 min. The gel was finally imaged with the ChemiDocTM MP imaging system.

Cell culture

The Mouse hepatocellular carcinoma line Hepa1-6 cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) solution at 37 ℃ in a humidified incubator containing 5% CO_2.

***In vitro* gene transfection**

Hepa1-6 cells were seeded onto 24-well plates in DMEM medium with 10 % FBS and 1% penicillin /streptomycin solution for 24 hours. Once the cells reached 60 % - 70 % density, they were ready for transfection.

Firstly, Hepa1-6 cells incubated with SPNs/pDNA at different mass ratios (10:1, 20:1, 40:1, 80:1, 120:1), and the cells were placed in a 42 ℃ incubator to thermal-

stimulate (at room temperature as control) after incubating 8 h. Then the GFP fluorescence was observed to search for a suitable complexation ratio of SPN/pDNA by the fluorescence microscope.

Secondly, pDNA was combined to the SPNs in FBS-free DMEM at a mass ratio of 1:80 for 30 min at room temperature (Lip3000 transfection as control), and the product was then incubated Hep1-6 cells for another 8 h. After 1064nm-laser irradiation ($1W \cdot cm^{-2}$) for 0 min (37 ℃) and 5 min, the cells were cultured with fresh FBS-DMEM for 24 h. We monitored the gene expression efficiency by the fluorescence microscope and Western-blot.

Western-blot

We also detected protein expression by Western-blot. The concentration of the acquired protein extracts was measured by the Easy Ⅱ Protein Quantitative Kit (BCA, TransGen Biotech, DQ111-01). The protein extracts were separated by 10% SDS-PAGE gels and transferred to nitrocellulose blotting membranes. Then, the membrane was incubated overnight 4 ℃ with primary antibody (TRAIL mouse antibody, 1:1000 dilution, β-actin mouse antibody, 1:5000 dilution) and anti-mouse secondary antibody (1:1000 dilution) for another 2 h. Finally, the signal was observed by the ChemiDoc™ MP imaging system.

Cellular uptake

In order to investigate cellular uptake, we seeded Hepa1-6 cells into Confocal dish overnight. Once Hepa1-6 cells reached approximate 80% density, the cells were incubated with SPNHT (SPN combined with pDNA at a ratio mass of 80 : 1) dispersed in DMEM for 0, 2, 4, 6 and 8 h. Subsequently, the cells were fixed 4% Paraformaldehyde and washed three times with PBS. Thirty minutes before imaging, YoYo-3 and DAPI were respectively added to dye pDNA and cell nucleus for 15 min. Finally, Hepa1-6 cells were washed with PBS three times and imaged by CLSM (Zeiss LSM780).

***In vitro* synergetic anti-tumor efficiency**

We seeded Hepa1-6 cells onto 96-well plates and cultured overnight to a density of

80% for following experiments. To evaluate cytotoxicity, the cells incubated with SPNHT (0, 0.3, 0.6, 1.2, 2.4, 3.6 and 4.8 μg·mL^{-1}) for 24 h. Cell viabilities were estimated by CCK8 assay and the absorption value was measured by a Spectra Max M5 microplate reader (Molecular Devices, USA) at 450 nm. (Cell survival rate = ($A_{sample}/A_{control}$) × 100%, where A_{sample} and $A_{control}$ represent the absorbencies of the sample and control wells, respectively).

To evaluate Synergetic therapy Efficiency, the cultured Hepa1-6 cells divided into 9 group: (1) Free medium (control), (2) SPV/HSP70-TRAIL-GFP (SPVHT without BBTDTS), (3) SPN/HSP70-GFP (SPNHG), (4) SPN/HSP70-TRAIL-GFP (SPNHT), (5) Free medium + Laser, (6) SPVHT + Laser, (7) SPVHT + 42 ℃ (GT), (8) SPNHG + Laser (PTT), (9) SPNHT + Laser (PTT/GT). When co-incubation with these experimental materials for 8 h, Hepa1-6 cells washed with PBS and cultured in fresh DMEM media of 10% FBS. After 1064 nm-laser irradiation for 5 min (0.8 W·cm^{-1}), all groups of the cells were incubated for another 24 h. Finally, the cells were stained according to the instructions provided by the manufacturer of the Live-Dead Cell Staining Kit and imaged through the fluorescence microscope (Zeiss Axio Vert.A1, Germany). For the apoptosis assay, the Hepa1-6 cells were treated by the above 9 groups, then stained with Annexin V-FITC/PI and quantitatively analyzed by flow cytometry (BD FAC-SAria TM III, USA).

Animal tumor model

All animal experiments were performed strictly under the guidelines of the National animal management regulations of China and the animal study guidelines of Fujian Medical University, as well as approved by the Animal Ethics Committee of Mengchao Hepatobiliary Hospital of Fujian Medical University. To establish a Hepa1-6 tumor model, 2×10^6 Hepa1-6 cells were subcutaneously injected into the right hip of healthy BALB/c nude mice weighing approximate 20 g. When the tumors reached an about size of 70-100 mm^3 in volume, the mice could be used for the following experiments.

Biodistribution of SPNHT

In order to evaluate the biodistribution of SPNHT in vivo, The SPNHT was labeled

with ICG, and ICG-labeled SPNHT (100 μL, 1 mg·mL^{-1} of BBTDTS concentration) was injected into the caudal vein of the Hepa1-6 tumor-bearing mice. Then the PSL Viewer system was used to observe the ICG fluorescence signal after 0, 4, 8, 12, 24, and 48 h injection. Subsequently, the mice were sacrificed at 48 h post-injection and the main organs (heart, liver, spleen, lung and kidney) were took out and imaged by the PSL Viewer system.

***In Vivo* synergetic anti-tumor efficiency**

In order to evaluate the effect of synergetic therapy, Hepa1-6 tumor-bearing mice were randomly divided into 8 groups (n = 5): (1) PBS (as control), (2) SPV/HSP70-TRAIL-GFP (SPVHT without BBTDTS), (3) SPN/HSP70-GFP (SPNHG), (4) SPN/HSP70-TRAIL-GFP (SPNHT), (5) PBS + Laser, (6) SPNHG + Laser (PTT), (7) SPVHT + Laser, (8) SPNHT + Laser (PTT/GT), and the materials was injected into the tail vein (100 μL, 1 mg·mL^{-1} of BBTDTS concentration, SPN : pDNA = 80:1). The tumor-bearing mice were irradiated by 1064 nm laser (0.8 W·cm^{-2}, 10 min) at 24 h post-injection. The body weight and tumor volume of mice in the treatment group were measured every 2 days to assess the efficacy of synergetic therapy. Tumor volume is calculated according to the formula: tumor volume = length × width^2/2. The length and width are measured with the vernier calipers.

One tumor-bearing mouse in each group was sacrificed after 48 h treatment for examining the histological changes of the tumor. The acquired tumor was fixed with 4 % formalin, embedded in paraffin, and sectioned for immunohistochemical analysis (H&E, Ki67, TUNEL).

In addition, to further analyze the function of gene therapy, the tumor was taken out after 48 h treatment and ultrasonically ground. The mixture was centrifuged at 10000 g for 15 min at 4 ℃ and the protein supernatant was collected. Through the Easy Ⅱ protein quantitative kits, protein immunoblot experiment was used to verify the thermal controlled gene expression.

***In Vivo* safety evaluation**

In order to evaluate the long-term biosafety of SPNHT, mice of all groups were

sacrificed after 18 days of treatment, and the main organs (heart, liver, spleen, lung, kidney) were removed, fixed with 4 % formalin, and H&E was performed to evaluate the toxicity of the nanoparticles.

Moreover, to better estimate the biosafety, the normal BABL/c nude mice were respectively injected the same dose (100 μL, 1 $mg \cdot mL^{-1}$ of BBTDTS concentration) of PBS or SPNHT, and collected blood samples at different time points (0, 3, 7 and 14 days) for blood routine and biochemical analysis.

Statistical analysis

The experiments results are reported as the mean ± standard deviation (SD). A one-way analysis of variance (ANOVA) method was used for statistical significance analysis by the Prism 6 software (GraphPad). The P-value < 0.05 was considered statistically significant.

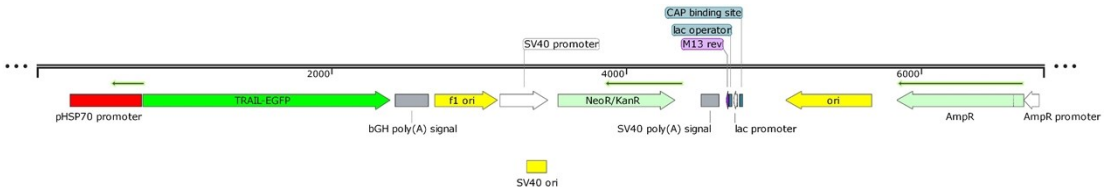

Fig. S1 Map of the HSP70-TRAIL-GFP plasmid. The HSP70 promoter is marked in red and the region in green is TRAIL-GFP gene fragment.

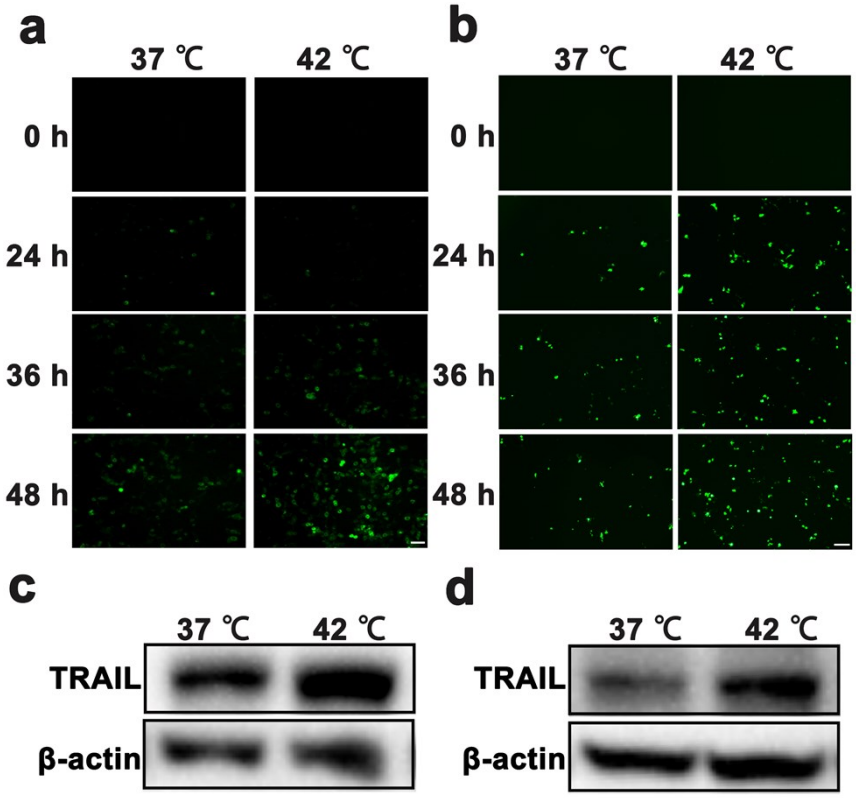

Fig. S2 (a) The GFP expression under HSP70 promoter was triggered by pDNA (HSP70-TRAIL-GFP) transfection in HSK293-FT cells cultured at 42 ℃ incubator (scale bars = 100 μm). (b) The GFP expression under HSP70 promoter was triggered by pDNA (HSP70-GFP) transfection in Hepa1-6 cells cultured at 42 ℃ incubator (scale bars = 100 μm). (c) Western blotting in (a). (d) Western blotting in (b).

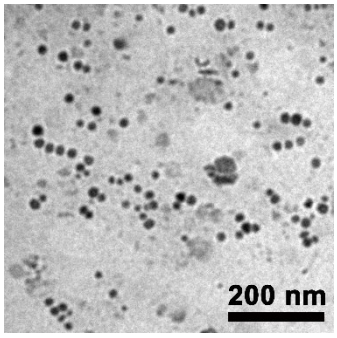

Fig. S3 TEM image of the SPNHT.

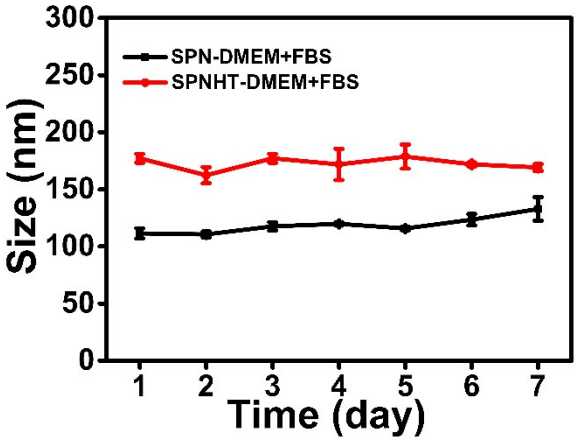

Fig. S4 Size distributions of SPN and SPNHT in 10% FBS measured by DLS.

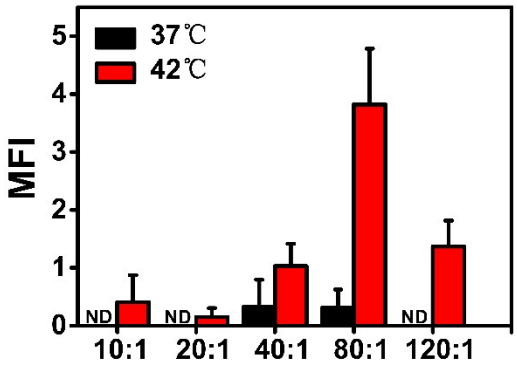

Fig. S5 The relative fluorescence intensity of f GFP expression in Hepa1-6 cells treated with SPN/pDNA (HSP70-GFP) at different mass ratios.

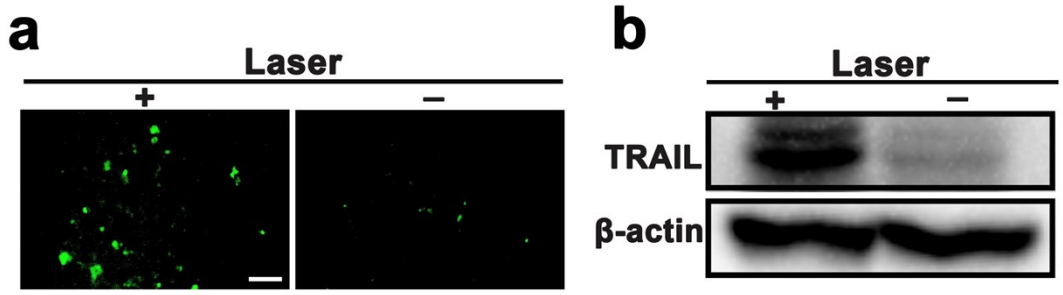

Fig. S6 (a) Fluorescence images and (b) Western blot were used to detect the TRAIL expression after SPNHT incubated Hepa1-6 cells with or without 1064 nm laser irradiation (scale bars = 100 μm).

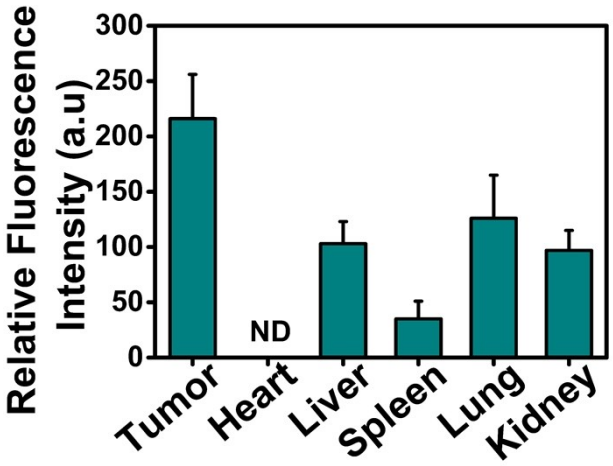

Fig. S7 Mean fluorescence quantitative analysis of tumor and main organs in vitro 48 h-post injection.

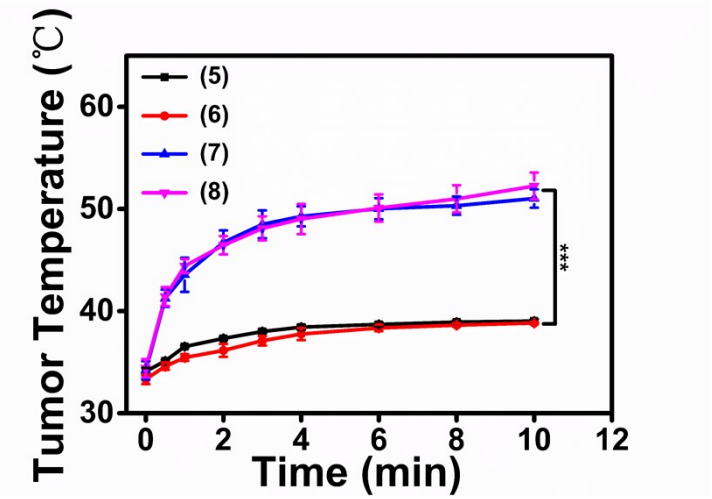

Fig. S8 The temperature changes of tumor sites after 1064 nm laser irradiation with different treatments for 8 h post-injection. ((5) PBS + Laser, (6) SPVHT + Laser, (7) SPNHG + Laser (PTT), (8) SPNHT + Laser (PTT/GT)).

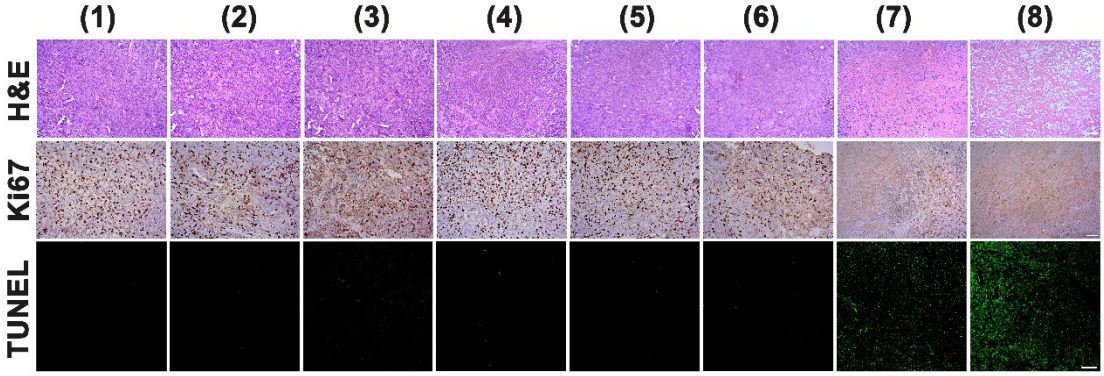

Fig. S9 Uncropped images of Fig 4h. Scale bar:50 μm. Related to Fig 4. ((1) PBS, (2) SPVHT, (3) SPNHG, (4) SPNHT, (5) PBS + Laser, (6) SPVHT + Laser, (7) SPNHG + Laser (PTT), (8)

SPNHT + Laser (PTT/GT)).

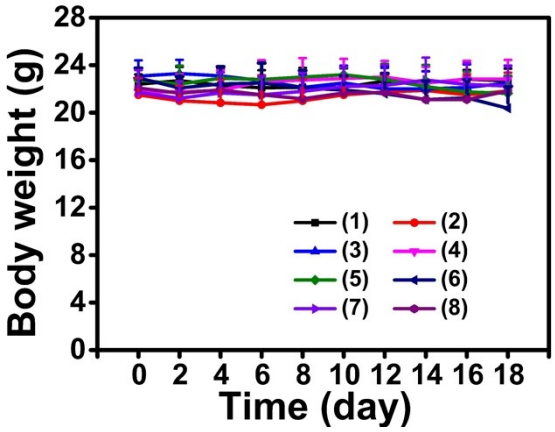

Fig. S10 Body weight changes of BALB/c nude mice after various treatments. ((1) PBS, (2) SPVHT, (3) SPNHG, (4) SPNHT, (5) PBS + Laser, (6) SPVHT + Laser, (7) SPNHG + Laser (PTT), (8) SPNHT + Laser (PTT/GT)).

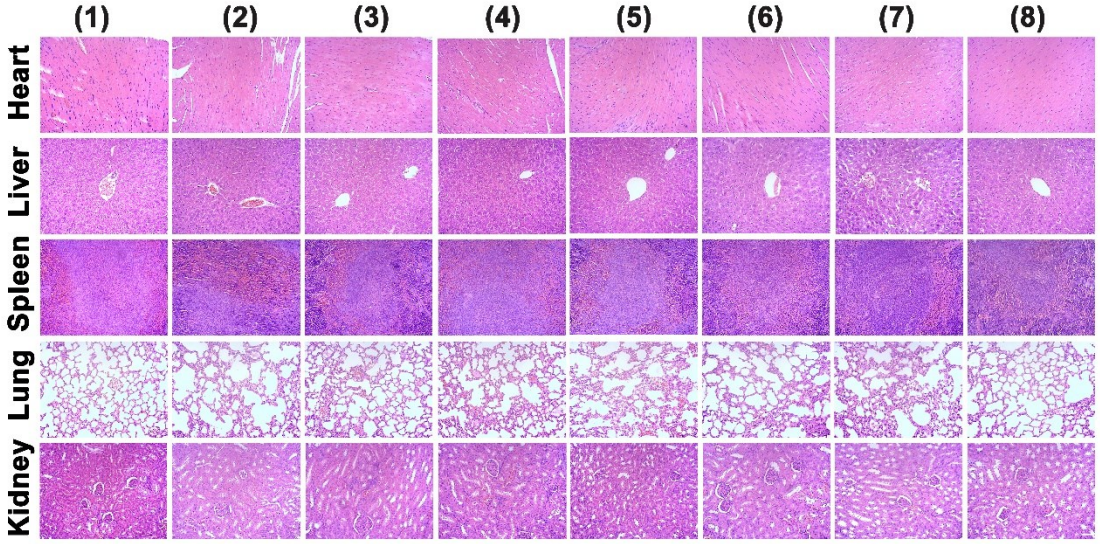

Fig. S11 H&E staining of major organs of BALB/c nude mice after various treatments. (bars = 50 μm). ((1) PBS group, (2) SPVHT, (3) SPNHG, (4) SPNHT, (5) PBS + Laser, (6) SPVHT + Laser, (7) SPNHG + Laser (PTT), (8) SPNHT + Laser (PTT/GT)).