Electronic Supplementary Material (ESI) for Chemical Communications.

Supplementary Information

An injectable nanocomposite alginate-Ca^{2+} hydrogel for melittin-assisted Ca^{2+}-overload and photothermal cancer therapy

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Experimental section

Materials

Calcium chloride ($CaCl_2$, AR), ammonium persulfate (APS, 98%) and glutathione (GSH) were purchased from Sigma-Aldrich. Sodium alginate (ALG, AR), 3,3',5,5'-teramethylbenzidine (TMB, 98%), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and rhodamine B (RB) were purchased from Aladdin (Shanghai, China). Aniline (AN) and sodium bicarbonate ($NaHCO_3$, AR) were purchased from Sinopharm Chemical Regent (Shanghai, China). Tris-HCl (pH 8.5) was purchased from Leagene (Hefei, China). Melittin (95%) was purchased from GL Biochem (Shanghai, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 1,1-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Dil), calcein-AM, propidium iodide (PI), Annexin V-FITC, mitochondrial membrane potential assay kit (JC-10), fluo-4 AM and phosphate buffer solution (PBS) were bought from Keygen Biotechnology (Nanjing, China). Ca^{2+} detection kit, ATP assay kit, 4',6-diamidino-2-phenylindole (DAPI) and Hoechst 33342 were purchased from Beyotime Biotechnology (Shanghai, China). Thiol Tracker^{TM} Violet was purchased from Thermo Fisher Scientific (Shanghai, China).

Preparation and characterization of polyaniline (PANI) nanofibers

The polyaniline nanofibers (PANI NF) were prepared *via* chemical oxidative polymerization method.[1] Briefly, AN (365 μL, 4.0 mmol) and APS (0.609 g, 2.7 mmol) were dissolved in 40 ml of perchloric acid solution ($HClO_4$, 0.1 mol/L) with the aid of ultrasound with a frequency of 37 kHz at 100% power (P30H, Elma, Germany) at room temperature for 10 min. Then, the reaction mixture was stirred for 24 h in an ice bath. The resulted PANI nanofibers were obtained by centrifugation and washed by ethanol and ultrapure water for three times. The PANI nanofibers were characterized by transmission electron microscopy (TEM, H-7500, Hitachi Co., Tokyo, Japan) and ultraviolet and visible spectroscopy (UV-vis, Lambada 35, Perkinemer).

Preparation of alginate hydrogel and nanocomposite hydrogel

The injectable Ca^{2+} cross-linked alginate hydrogel was obtained by mixing $CaCl_2$ solution and the sodium alginate solution.[2] Specifically, about 350 μL ALG solution (4 mg/mL) was mixed with $CaCl_2$ solution (100 μL, 18 mmol) to produce the ALG hydrogel (HG). Different concentrations for ALG and $CaCl_2$ solutions were used to explore the gel formation. The melittin and polyaniline nanofibers were incorporated into the hydrogel under the same process to prepare the nanocomposite hydrogel (MP@HG). The interior microstructures of ALG and MP@HG hydrogels in the freeze-dried stated were observed by SEM.

In vitro melittin and Ca^{2+} release study

For the *in vitro* drug release study, the MP@HG (900 μL) was infused into a dialysis bag with a molecular weight cutoff of 3 kDa. The dialysis bags were immersed into PBS solutions with pH 6.0 and 7.4 containing Ca^{2+} (1.8 mM). At predetermined time intervals, the amount of released Ca^{2+} was determined by the Ca^{2+} assay kit using a microplate reader (SpectraMax M2, Shanghai, China), and the concentration of released melittin was measured using a fluorospectrophotometer (FluoroMax, HORIBA Scientific, Tokyo, Japan) with an excitation wavelength of 280 nm and an emission wavelength of 356 nm.[3] The release profiles of melittin under NIR light irradiation were also investigated. At predetermined time intervals, the dialysis bags were exposed to NIR light (1.0 W/cm^2) for 5 min and then incubated for another 5 min. The released amount of melittin were determined before and after NIR light irradiation.

Hemolysis assay of melittin and melittin-incorporated hydrogels

The hemolysis activities of melittin and melittin-incorporated hydrogels were evaluated according to the reported method.[4] Fresh mouse blood was collected from Balb/c mouse for red blood cells (RBCs) isolation by centrifugation and washed with saline for three times. Then, the RBCs were suspended at a density of 5×10^7 cells/mL in PBS buffer. Subsequently, 0.1 mL of RBCs was incubated with free melittin, melittin-incorporated hydrogel (M@HG) and melittin-incorporated nanocomposite hydrogel (MP@HG) with

different melittin concentration at 37 °C for 1 h. The Triton X-100 and PBS solution were used as positive and negative control, respectively. The incubated solutions were centrifuged at 10000 rpm for 10 min. The absorbances at 540 nm for the supernatants in each group were analyzed by a microplate reader. The hemolysis ratio was calculated by the following equation:

$$\text{Hemolysis ratio (\%)} = (A_p - A_b)/(A_t - A_b) \times 100\%$$

Where A_p represents the absorbance of RBC exposed to melittin and melittin-incorporated hydrogels; A_b and A_t represent the absorbances of RBC incubated with PBS and Triton X-100, respectively. All assays were carried out as triplicates.

Photothermal properties of PANI nanofibers and nanocomposite hydrogel

To evaluation the photothermal properties, the PANI nanofibers solutions and nanocomposite hydrogel were exposed to the NIR laser irradiation at 1064 nm with a power density of 1.0 W/cm^2 for 10 min (LWIRL808-2W-F, Beijing Laserwave Optoelectronics Technology Co., Beijing, China). The temperature changing profiles were recorded using an infrared (IR) thermal camera (Fortic 225, IRS Systems Inc, Shanghai, China).

Glutathione (GSH) depletion properties

The glutathione depletion property of PANI nanofibers were explored using Ellman's assay.[5] Typically, GSH bicarbonate buffer solution (GSH: 10 mM) was mixed with the PANI nanofiber solution at different PANI concentrations. After incubation at room temperature, a Tris solution of DTNB (100 mM) were added into the mixtures. The PANI nanofibers were removed by centrifugation at 10000 rpm for 10 min. Then, the absorbance of supernatant was monitored at a wavelength of 412 nm by a microplate reader. Meanwhile, ultrapure water was mixed with the GSH solution as the negative control. The loss of GSH was calculated according to the following equation:

$$\text{Loss of GSH (\%)} = ((A_n - A_s)/A_n) \times 100\%$$

where A_n represented the average absorbance of the negative control, while A_s

represented of the samples. All assays were performed as triplicates. The GSH depletion effect of MP@HG was also performed in the same way as that of PANI nanofibers.

Cell lines and animals

Human breast adenocarcinoma cells (MCF-7), murine breast cancer cells (4T1) and normal 3T3 cells were purchased from Keygen Biotech (Nanjing, China) and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and 1% antibiotics (penicillin and streptomycin) under an atmosphere of 5% CO_2 at 37℃. BALB/c mice (6-8 weeks old) were purchased from Qinglong Shan Co., Ltd (Nanjing, China). All animal experiments in this work were approved by the Ethic Committee of Nanjing University of Posts and Telecommunications (Reference number: 2023004) followed the guidelines of National Experimental Animal Management Regulations (China, 2017).

In vitro cell experiments

The cytocompatibility of the hydrogels and synergistic therapeutic effects of the nanocomposite hydrogels were investigated by the *in vitro* cell experiments. Firstly, MCF-7 cells (5×10^3 cells/well) were seeded in a 96-well plate for 24 h of culturing. Then, the cells were treated with different materials with/without NIR irradiation (1064 nm, 1.0 W/cm^2, 10 min). After incubation for a predetermined time, standard methyl thiazolyl tetrazolium (MTT) method was used to quantify the cell viabilities.[6] Apart from MTT assay, the therapeutic efficacies of the nanocomposite hydrogels were also evaluated by the lived/dead dual staining assay as below. After incubation with different materials, the cells were rinsed twice and co-stained with calcein-AM and PI. The cells were then washed with PBS twice and imaged by a confocal laser scanning microscope (CLSM, FV1000-IX81, Olympus, Japan). In addition, the analysis of apoptosis and necrosis on cells were studied with Annexin FITC and PI staining using a flow cytometry (Amnis Flowsight, USA).

In order to evaluate the ability for membrane disruption from melittin, the cell

membrane integrity assay was performed by a commercial membrane detection probe Dil. The intracellular Ca^{2+} levels of the cells were determined by a commercial fluorescence Ca^{2+} indicator, Fluo-4 AM and Honchest 33342 probe. The mitochondrial membrane changes were measured by utilizing a commercial detection reagent of JC-10, which could form aggregates with red fluorescence in the normal mitochondrial membrane but exist as monomers with green fluorescence in the damaged mitochondrial membrane. The intracellular GSH levels were detected by using a commercial glutathione detection probe of Thiol TrackerTM Violet and DAPI probe. All the staining cells were observed by the confocal laser scanning microscope. The ATP levels of cells were studied by utilizing a commercial detection reagent of ATP assay kit.

In vivo antitumor assays

The *in vivo* antitumor efficacies of nanocomposite hydrogels were studied using the male mice (6-8 weeks) inoculated with 4TI cells subcutaneously in the right anterior limb of the mice. When the tumor volume reached around 110 mm^3, the mice were divided into three groups for the treatment with different materials, including PBS (100 μL), M@HG (80 μL, melittin: 50 μg/mL), MP@HG+L (80 μL, melittin: 50 μg/mL; PANI nanofibers: 0.6 mg/mL; NIR irradiation: 1064 nm, 1.2 W/cm^2, 10 min). The mice were treated by intra-tumoral injection. The diameter of the laser spot was set as 1 cm, which could fully cover the tumor areas. The tumor volume and mice weight were measured every 2 days. The tumor volume was defined as $V=L\times W^2/2$, where L is the longest tumor diameter and W is the shortest tumor diameter perpendicular to L. The major organs of the mice were removed at the end of the treatment for subsequent hematoxylin and eosin (H&E) staining for histological analysis.

Supplementary Figures

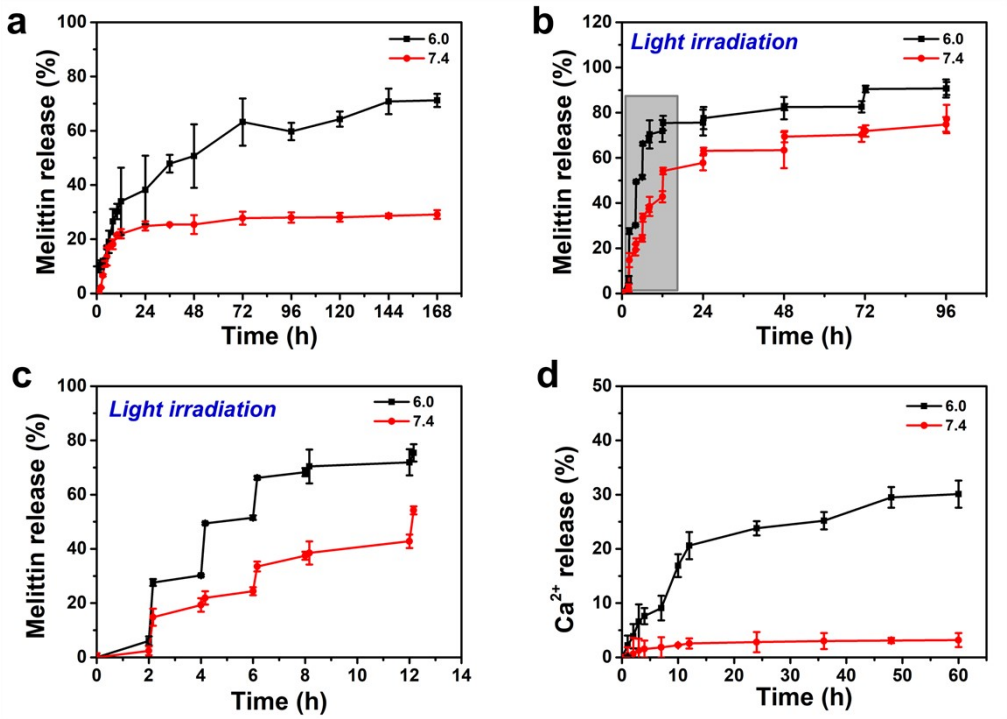

Fig. S1. (a) Melittin release profiles from MP@HG hydrogel in PBS containing 1.8 mM of Ca^{2+} at pH 7.4 and 6.0. (b and c) Melittin release profiles from MP@HG hydrogel in PBS containing 1.8 mM of Ca^{2+} at pH 7.4 and 6.0 under NIR light irradiation (1.0 W/cm^2, 5 min) for 96 h and 14 h, respectively. (d) Ca^{2+} release profiles from MP@HG hydrogel in PBS containing 1.8 mM of Ca^{2+} at pH 7.4 and 6.0.

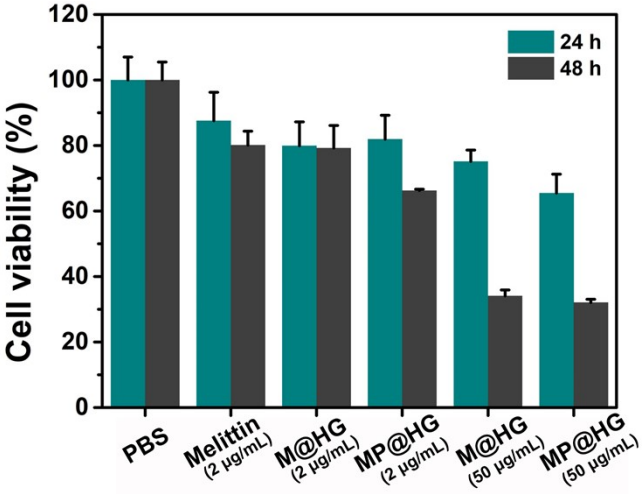

Fig. S2 The cell viabilities of 3T3 cells treated by free melittin and melittin-incorporated hydrogels at different melittin concentrations.

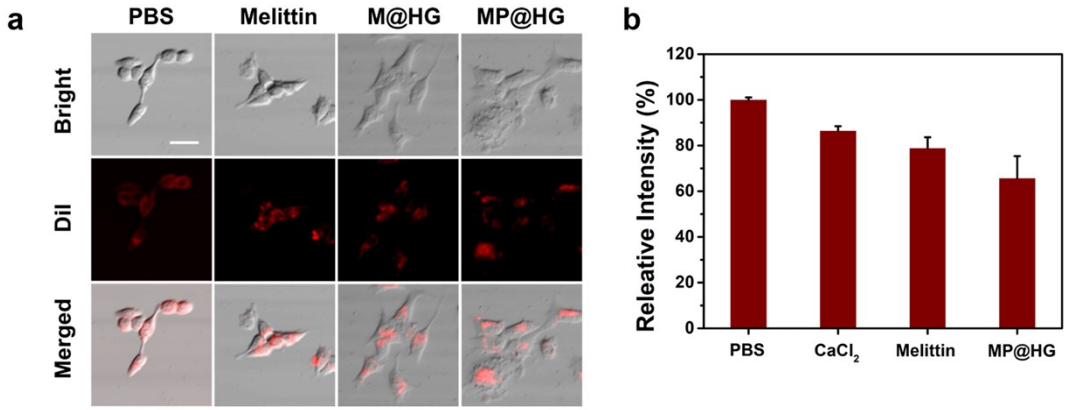

Fig. S3. (a) MCF-7 Cell membrane disruption treated with melittin, M@HG, MP@HG (scale bar: 10 μm). (b) Changes of intracellular ATP contents of MCF-7 cells after treated with Ca^{2+}, melittin with Ca^{2+} (Ca^{2+} concentration: 1.8 mM) and MP@HG.

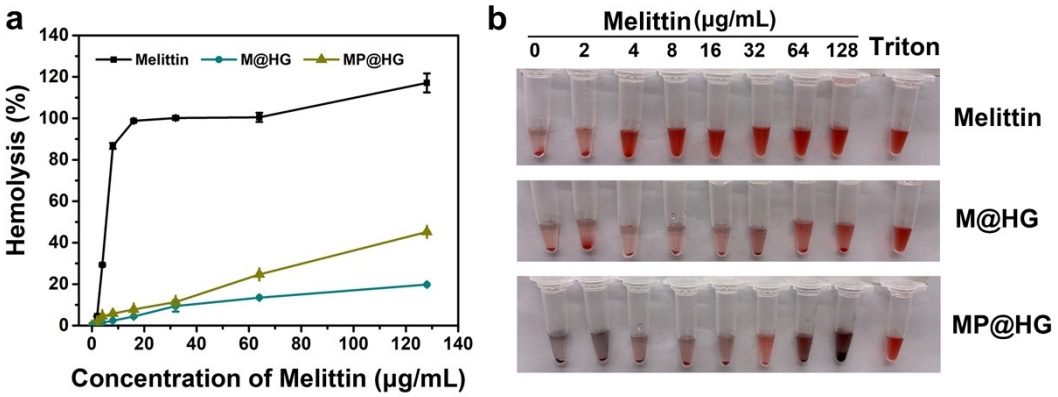

Fig. S4 The hemolysis activities of melittin, M@HG and MP@HG hydrogels at

different melittin concentrations.

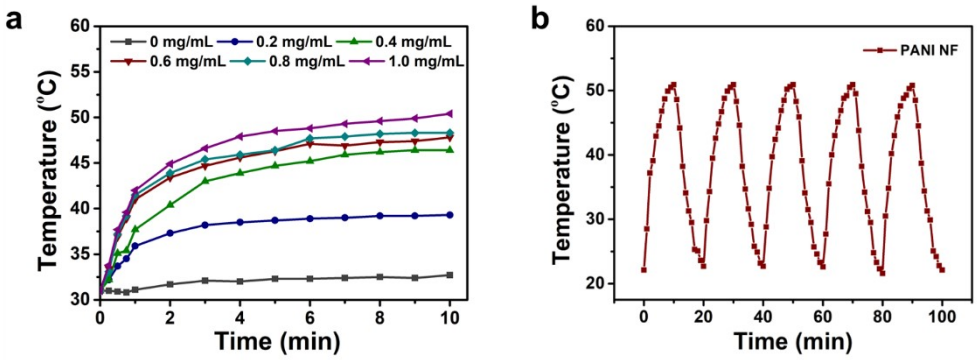

Fig. S5. (a) The temperature changes of PANI NF under NIR light irradiation. (b) The photothermal cycling stability of PANI NF.

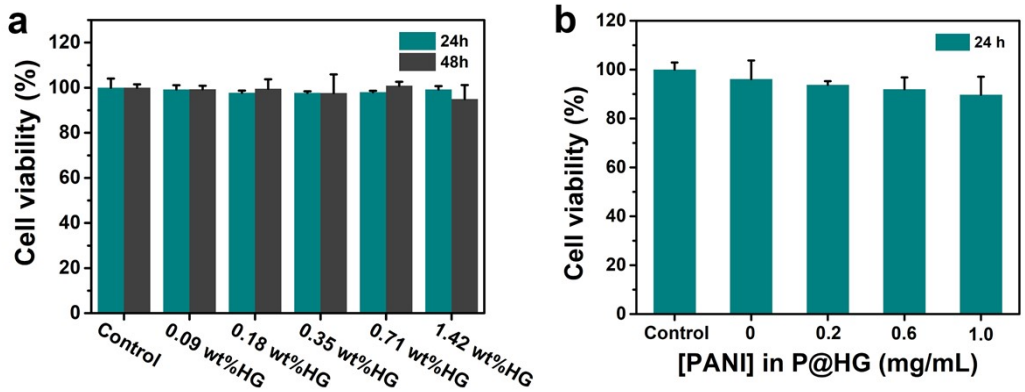

Fig. S6. (a) Cell cytocompatibility of MCF-7 cells incubated with HG for 24 h and 48 h. (b) Cell cytocompatibility of MCF-7 cells incubated with P@HG for 24 h.

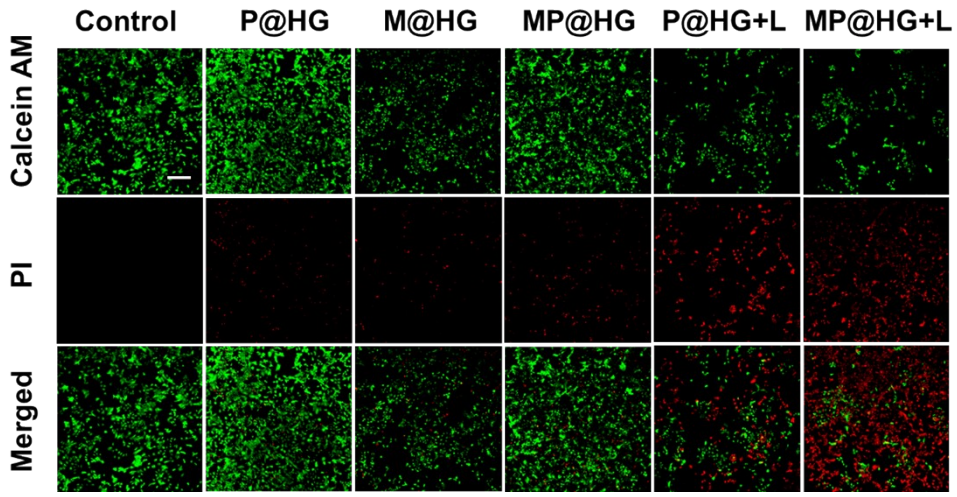

Fig. S7. Confocal fluorescence images of MCF-7 cells treated by different materials

and stained by calcein-AM and PI (scale bar: 200 μm).

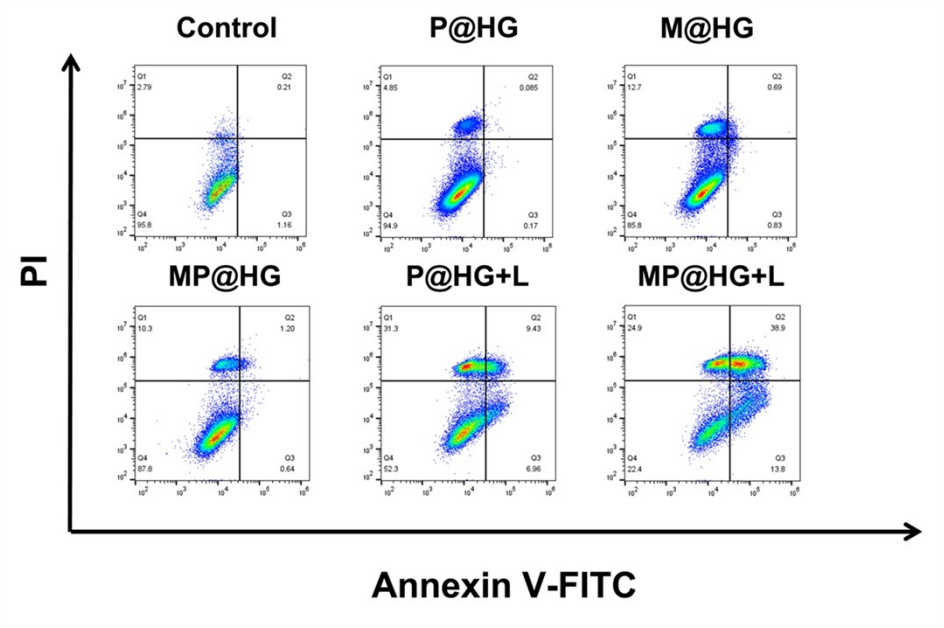

Fig. S8. Flow cytometry analysis of MCF-7 cells apoptosis treated by different materials with/without NIR irradiation for 24 h.

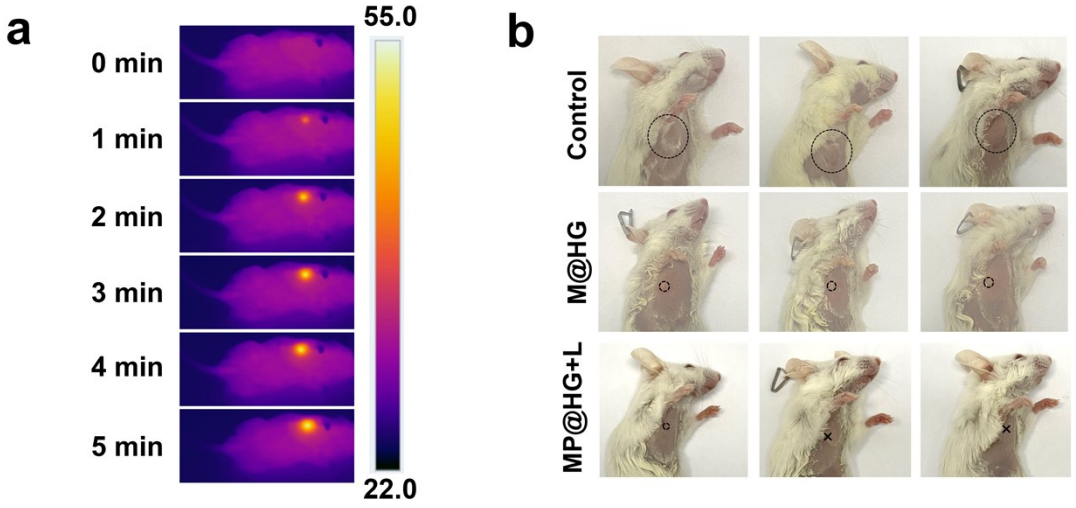

Fig. S9. (a) Thermal images of mice treated by MP@HG with NIR laser irradiation. (b) Photographs of mice at the end of treatment.

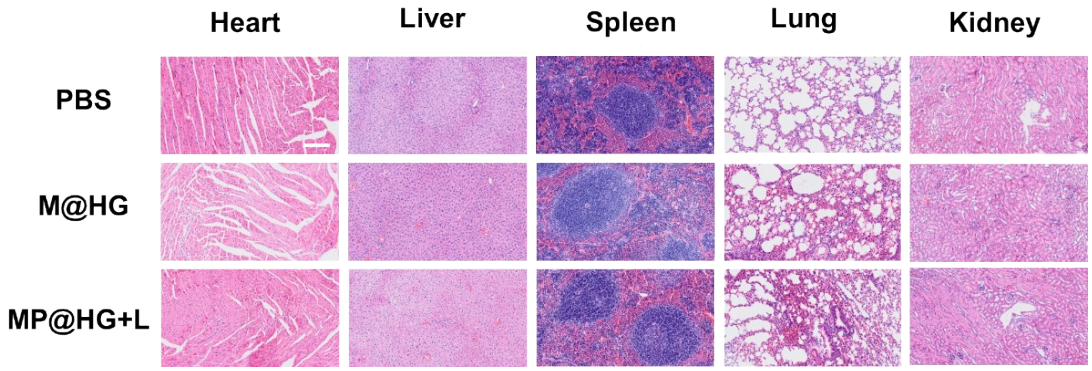

Fig. S10. Histologic analyses of major organs (heart, liver, spleen, lung and kidney) with H&E staining in tumor-bearing mice treated by PBS, M@HG and MP@HG+L hydrogels (scale bar: 100 μm).

References:

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