Electronic Supplementary Information

Matrix-degrading soft-nanoplatform with enhanced tissue penetration for amplifying photodynamic therapeutic efficacy of breast cancer

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Experimental section

Materials

Cetyltrimethylammonium bromide (CTAB), anhydrous ethanol, tetraethoxysilane (TEOS) concentrated ammonia aqueous solution (25 wt%), hydrofluoric acid (HF, 48 wt% in H_2O), and glutaraldehyde (GA, 25%) were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). N-hydroxysulfosuccinimide (NHS), N, N-Dimethylformamide (DMF), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), and chlorin e6 (Ce6) were purchased from RHAWN Chemical Technology Co., Ltd. (Shanghai, China). Poly(ethylene imine) (PEI, Mw: 25000), hyaluronidase (HAase), human serum albumin (HSA), and Cy5.5-Maleimide (Cy5.5-Mal) were obtained from Sigma-Aldrich (St. Louis, MO, USA). 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) and Calcein/PI cell viability/cytotoxicity assay kit were purchased from Beyotime Biotechnology (Shanghai, China). Phosphate buffer saline (PBS), 4'-6-diamidino-2-phenylindole (DAPI), cell counting kit-8 (CCK-8), and Annexin V-FITC/PI apoptosis detection kits were obtained from Nanjing Keygen Biotech. Co., Ltd. (Nanjing, China). Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco Laboratories (NY, USA). Deionized water with a resistivity of 18 MΩ·cm was used in the experiments.

Preparation

The matrix-degrading soft-nanoplatform was prepared by a hard-templating method. Mesoporous silica nanoparticles (MSNs) synthesized according to our group's previously reported method were selected as the templets in the experiments [1]. In brief, 10 mL aqueous solution of MSNs (4 mg·mL^{-1}) was mixed with 10 mL aqueous solution of PEI (2 mg·mL^{-1}). After 20 min, the products were washed with deionized water for three times, and PEI-modified MSNs (MSNs-PEI) were obtained. The MSNs-PEI was then mixed with 5 mL aqueous solution of GA (25 %), and the mixture was shaken under dark for 12 h (200 times·min^{-1}). After washing with deionized water three times, MSNs-PEI-GA was formed, which was dispersed in 10 mL of

deionized water for further use. Afterwards, 30 mg of HSA and 20 mg of HAase were added to the aqueous solution of MSNs-PEI-GA, and shaken under dark at room temperature for 12 h (200 times·min^{-1}). After being collected by centrifugation at a speed of 10000 rpm for 8 min and washed with deionized water three times, HSA/HAase-coated MSNs (MSNs-HSA/HAase) were formed and dispersed in 10 mL of deionized water. The MSNs-HSA/HAase dispersed in deionized water was etched with HF for 1 min to remove the MSN hard templates, and the matrix-degrading HSA/HAase soft-nanocapsules (HSA/HAase SNCs) were finally obtained.

In order to prepare the matrix-degrading soft-nanoplatform, the HSA/HAase SNCs were modified with photosensitizer Ce6. Typically, 20 mg of Ce6 was mixed with 10 mg of EDC and 10 mg of NHS in 10 mL of DMF under dark at 25 °C for 12 h (200 times·min^{-1}) to form carboxyl-activated Ce6 [2]. 10 mL aqueous solution of the HSA/HAase SNCs (1 mg·mL^{-1}) was mixed with 1 mL DMF solution of the carboxyl-activated Ce6 (2 mg·mL^{-1}) under dark at 25 °C for 12 h (200 times·min^{-1}). Afterward, the products were collected by centrifugation and washed with DMF and deionized water, respectively. Finally, the matrix-degrading soft-nanoplatforms HSA/HAase@Ce6 were obtained, which were dispersed in deionized water for further use.

As a counterpart of the above-prepared soft-nanoplatforms, MSNs-HSA/HAase@Ce6 was synthesized following the same procedure of HSA/HAase@Ce6 without etching the hard MSN cores. In addition, HSA@Ce6 nanocapsules without HAase were prepared using similar procedures.

In order to observe the biological behaviors of the nanoplatforms, the HSA/HAase and MSNs-HSA/HAase were modified with Cy5.5. In brief, the MSNs-HSA/HAase (50 mg) or HSA/HAase etched from the 50 mg of MSNs-HSA/HAase were dispersed in a mixture of 12 mL deionized water, 0.2 mg Cy5.5-Mal, and 1.2 mL DMF. After shaking at room temperature for 12 h, Cy5.5-modified nanoparticles, referred to as HSA/HAase-Cy5.5 and MSNs-HSA/HAase-Cy5.5, were centrifuged and washed three times with DMF and deionized water, respectively.

Cytotoxicity and hemolysis

Human umbilical vein endothelial cells (HUVECs), smooth muscle cells (SMCs), and mouse breast cancer (4T1) cells were seeded in a 96-well plate at a density of 1×10^4 cells per well. After the cells adhered to the plate, fresh DMEM containing HSA/HAase SNCs at concentrations of 0, 25, 50, or 200 μg mL^{-1} was added to each well. After incubation for 24, 48, or 72 h, 10 μL of CCK8 solution was added to each well and further incubated for 2 h. The wells added with HSA/HAase SNCs at equivalent concentrations were set as a control to eliminate the influence of materials on testing results.

HUVECs, SMCs, 4T1 cells, and human breast cancer (MDA/MB-231) cells were cultured according to the above-described method. The culture media containing different cells were added with 100 μL of DMEM with HSA/HAase@Ce6 concentrations of 0 or 5.0 μg·mL^{-1} (calculated as Ce6 concentration). After incubation for 24 or 48 h, 10 μL of CCK8 solution was added to each well. After 2 h, the absorbance at 450 nm was measured using a multifunctional enzyme marker.

Whole blood was obtained from healthy Balb/c mice. Afterward, 1mL of the blood was centrifugated at 2000 rpm for 5 min to collect the red blood cells (RBCs). The obtained RBCs were then suspended in 2 mL of phosphate buffer saline (PBS) solution. 0.2 mL RBCs suspension in PBS was mixed with 0.8 mL of PBS containing HSA/HAase or HSA/HAase@Ce6 with concentrations of 0, 50, 100, 200, 400, or 800 μg·mL^{-1}. RBCs suspended in H_2O or PBS at equal amounts were set as the positive or negative control. After incubation for 4 h, the mixtures were centrifugated at 2000 rpm for 5 min, and the absorbance of the supernatant was measured at 416 nm.

***In vitro* cell uptake**

4T1 cells were cultured in a confocal dish. After 24 h, 1mL of MSNs-HSA/HAase@Ce6 or HSA/HAase@Ce6 solution in PBS (2.5 μg·mL^{-1}, calculated as Ce6 concentration) was added to the cells and incubated for an additional 6 h, separately. The cells were then washed with PBS twice and stained with DAPI. After 5 min,

the Ce6 signals in the cells and the nuclear morphology were observed using a confocal laser scanning microscopy (CLSM). Furthermore, 4T1 cells were incubated with MSNs-HSA/HAase-Cy5.5 and HSA/HAase-Cy5.5, separately, for 1, 3, and 6 h. After washing with PBS for three times, the 4T1 cells were resuspended in 100 μL PBS, and the cellular uptake was analyzed using a flow cytometer.

In vitro cellular penetration

The penetration ability of the HSA/HAase@Ce6 was investigated using three-dimensional 4T1-multicellular spheroids [3-5]. In brief, 0.16 g agarose was dissolved in 10 mL of DMEM medium. 100 μL the agarose solution was then added to each well of a 96-well plate. After solidification, 4T1 cells were added at a density of 1×10^3 cells·well^{-1} and allowed to form clusters over a period of 7 days. Finally, 100 μL fresh DMEM of MSNs-HSA/HAase@Ce6, HSA@Ce6, or HSA/HAase@Ce6 (2.5 μg·mL^{-1}, calculated as Ce6 concentration) were added to the wells, respectively.

In addition, three-dimensional CT26- and $PanO_2$-multicellular spheroids were constructed to investigate the penetration of the HSA/HAase SNCs. In brief, the CT26 or $PanO_2$ cells were added to an ultra-low attachment plate at a density of 1×10^4 cells·well^{-1}. After allowing the cells to form clusters over a period of 10 days, 100 μL fresh DMEM of MSNs-HSA/HAase@Ce6, HSA@Ce6, or HSA/HAase@Ce6 (2.5 μg·mL^{-1}, calculated as Ce6 concentration) was added, respectively. After the multicellular spheroids were co-cultured with the nanoplatforms for 12 h, the distribution of the nanoplatforms was observed using CLSM.

In vitro antitumor

4T1 cells were seeded in a 96-well plate at a density of 1×10^4 cells per well. After incubating for 24 h, MSNs-HSA/HAase@Ce6, HSA@Ce6, and HSA/HAase@Ce6 at concentrations of 0, 0.5, 1.5, 2.0, 2.5, and 5.0 μg·mL^{-1} (calculated as Ce6 concentration) were added to each well, respectively. After incubation for an additional 24 h period, the cells were washed with PBS twice. Afterward, the cells were exposed to a laser irradiation of 660 nm at a power density of 1 W·cm^{-2} for 5 min. 10 μL CCK8 solution was added to each well.

After 2 h, the absorbance of each well at 450 nm was measured using a multifunctional enzyme marker. The wells added with nanoplatforms at the same concentrations were set as controls to eliminate the interference of added materials during tests.

4T1 cells were seeded in confocal dishes at a density of 1×10^5 cells per dish. After 12 h, the culture media were replaced with fresh DMEM, and Ce6, MSNs-HSA/HAase@Ce6, HSA@Ce6, and HSA/HAase@Ce6 were added at an equivalent Ce6 concentration of 2.5 μg mL^{-1}. After an additional 12 h incubation, the cells were rinsed with PBS twice. Subsequently, 1mL PBS solution of DCFH-DA (10 mM) was loaded into cells. After 30 min, the well of cells was rinsed with PBS twice and replaced with fresh DMEM, followed by exposure to a 660 nm laser irradiation at a power density of 1 W cm^{-2} for 3 min. Finally, the fluorescence of the cells was observed using CLSM.

In brief, 4T1 cells were seeded in confocal dishes at a density of 1×10^5 cells per dish. After 12 h, the culture medium was replaced with the fresh DMEM, and Ce6, MSNs-HSA/HAase@Ce6, HSA@Ce6, and HSA/HAase@Ce6 were added at an equivalent Ce6 concentration of 2.5 μg·mL^{-1}. After 12 h, the cells were rinsed with PBS twice, replaced with fresh DMEM, and irradiated with a 660 nm laser at a power density of 1 W·cm^{-2} for 5 min. The 4T1 were stained using Calcein/PI cell viability/cytotoxicity assay kit [6]. Finally, the cells were observed using CLSM.

4T1 cells were seeded in 6-well plates at a density of 1×10^5 cells per well. After 12 h, the culture medium was replaced with the fresh DMEM, and Ce6, MSNs-HSA/HAase@Ce6, HSA@Ce6, and HSA/HAase@Ce6 were added at an equivalent Ce6 concentration of 2.5 μg·mL^{-1}. After 12 h, the cells were rinsed with PBS twice, replaced with fresh DMEM, and irradiated with a 660 nm laser at a power density of 1 W·cm^{-2} for 5 min. After 12 h, the cells were collected and incubated with Annexin V-FITC (10 μg·mL^{-1}) and PI (5 μg·mL^{-1}) for 30 min. Finally, the fluorescence of FITC/PI was analyzed using a flow cytometer.

The colony formation assay was performed following the previously described protocol [7]. In brief, 4T1 cells (500 cells·$well^{-1}$) were seeded in a 10% FBS medium and subjected to various treatments. After a 2-week

incubation period, the cells were fixed with methanol and stained with Giemsa. Finally, the morphology of the colonies was observed.

Animal models

Balb/c mice (14 ~ 16 g) were purchased from Gempharmatech Co., Ltd. All animal experiments were conducted following the Guide for the Care and Use of Laboratory Animals and received approval from the Laboratory Animal Center at the Affiliated Hospital of Nanjing University of Chinese Medicine (https://dwzx.njucm.edu.cn/brc/3.htm, approval number 202303A057). Following a previously reported protocol, 1×10^6 4T1 cells were inoculated into the armpit of the mice to establish a 4T1 breast tumor-bearing mouse model. *In vivo* antitumor experiments were conducted when the sizes of tumors reached 80 ~ 120 mm^3 [8].

Biodistribution

The mice bearing 4T1 tumors with a volume of approximately 200 mm^3 were subjected to intravenous injections of 100 μL fresh DMEM containing MSNs-HSA/HAase@Ce6, HSA@Ce6, or HSA/HAase@Ce6 with an equivalent Ce6 concentration of 2.5 $mg \cdot kg^{-1}$. After a 24 h interval, the mice were humanely euthanized, and the tumors as well as major organs were excised for optical imaging (the IVIS® Spectrum *in vivo* imaging system provided by PerkinElmer, America).

***In vivo* systemic toxicity**

Healthy Balb/c mice were subjected to intravenous injections of 100 μL PBS or HSA/HAase@Ce6 with an equivalent Ce6 concentration of 2.5 $mg \cdot kg^{-1}$. After a 24 h interval, the mice were blood collected and humanely euthanized.

Major organs including the heart, liver, spleen, lung, and kidney were harvested after treatment. The organs were subsequently fixed in 4% paraformaldehyde, embedded in paraffin, stained with hematoxylin and eosin (H&E), and examined using an optical microscope [9].

In vivo antitumor

Tumor-bearing mice were randomly divided into four groups (n = 5) and administered 100 μL of PBS or various therapeutic agents dissolved in PBS (MSNs-HSA/HAase@Ce6, HSA@Ce6, or HSA/HAase@Ce6 at a Ce6 concentration of 2.5 $mg \cdot kg^{-1}$) *via* tail-vein injection. Following the injections, the mice were kept in darkness for 24 h. A 660 nm laser with an intensity of 1 $W \cdot cm^{-2}$ was used to irradiate the tumor for 5 min, ensuring that the entire tumor was covered by the irradiation spot. Tumor volume and the weight of the tumor-bearing mice were measured and recorded every 3 days for a period of 24 days post-treatment. The tumor volume was determined using a vernier caliper and the formula: tumor volume V (mm^3) = $W^2 \times H/2$, where W represents the shortest diameter of the tumor and H stands for the longest diameter.

Following the completion of the treatment, tumors were harvested. The tumors were subsequently fixed in 4% paraformaldehyde, embedded in paraffin, stained with hematoxylin and eosin (H&E) or Ki67, and examined using an optical microscope.

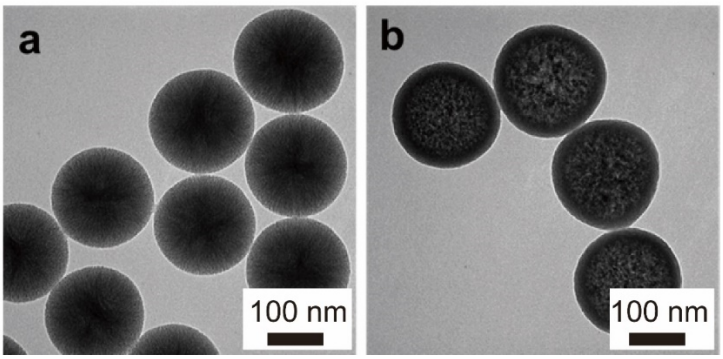

Figure S1. (a) TEM images of the MSNs. (b) TEM images of the MSNs-HSA/HAase.

TEM images of the MSNs showed a uniform diameter, excellent dispersion, and a well-defined spherical shape with a mesoporous structure (Figure S1a). The surface of the MSNs was coated with the HSA/HAase layer, resulting in a core-shell structure (Figure S1b). Upon coating with HSA and HAase, a slight deformation in the spherical shape was observed, attributed to the etching effect of GA.

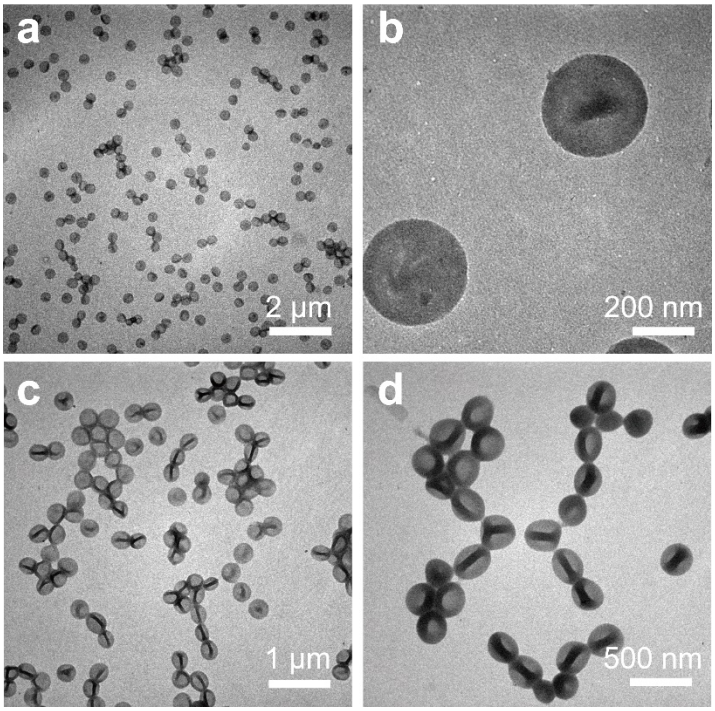

Figure S2. (a, b) TEM images of HSA/HAase at different magnifications after 30 days of storage in PBS. TEM images of HSA/HAase@Ce6 after 6 days of storage in (c) PBS and (d) DMEM.

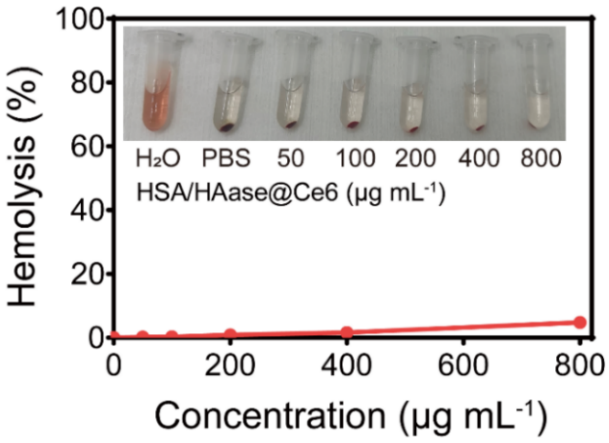

Figure S3. Hemolysis of RBCs co-incubated with different concentrations of HSA/HAase@Ce6 and corresponding optical photograph.

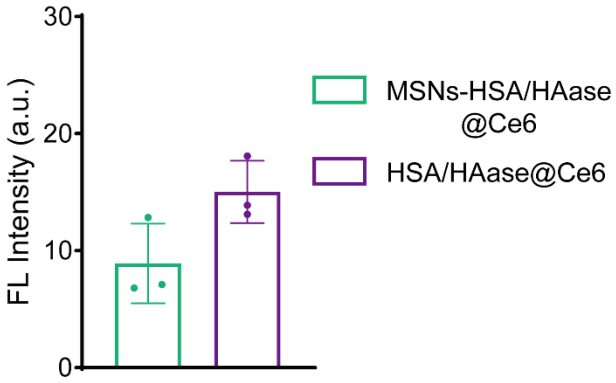

Figure S4. The statistical analysis of the fluorescence quantification from the CLSM images of 4T1 cells co-incubated with MSNs-HSA/HAase@Ce6 or HSA/HAase@Ce6 for 6 h.

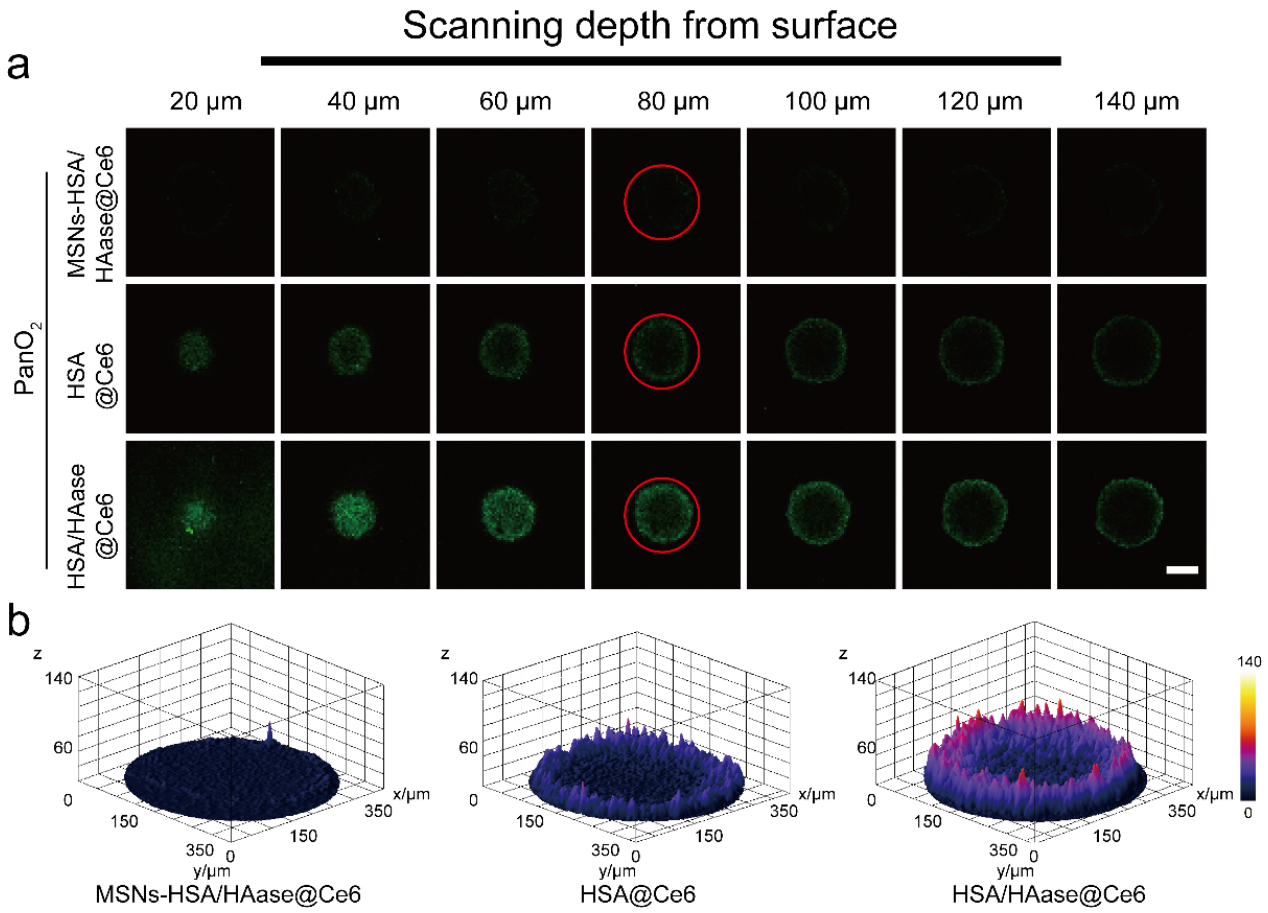

Figure S5. (a) CLSM Z-stack scanning images of $PanO_2$-multicellular spheroids incubated with the MSNs-HSA/HAase@Ce6, HSA@Ce6 and HSA/HAase@Ce6. Scale bars, 200 μm. (b) Surface plot images of multicellular spheroids along the arrow region at the Z-axis distance of 80 μm.

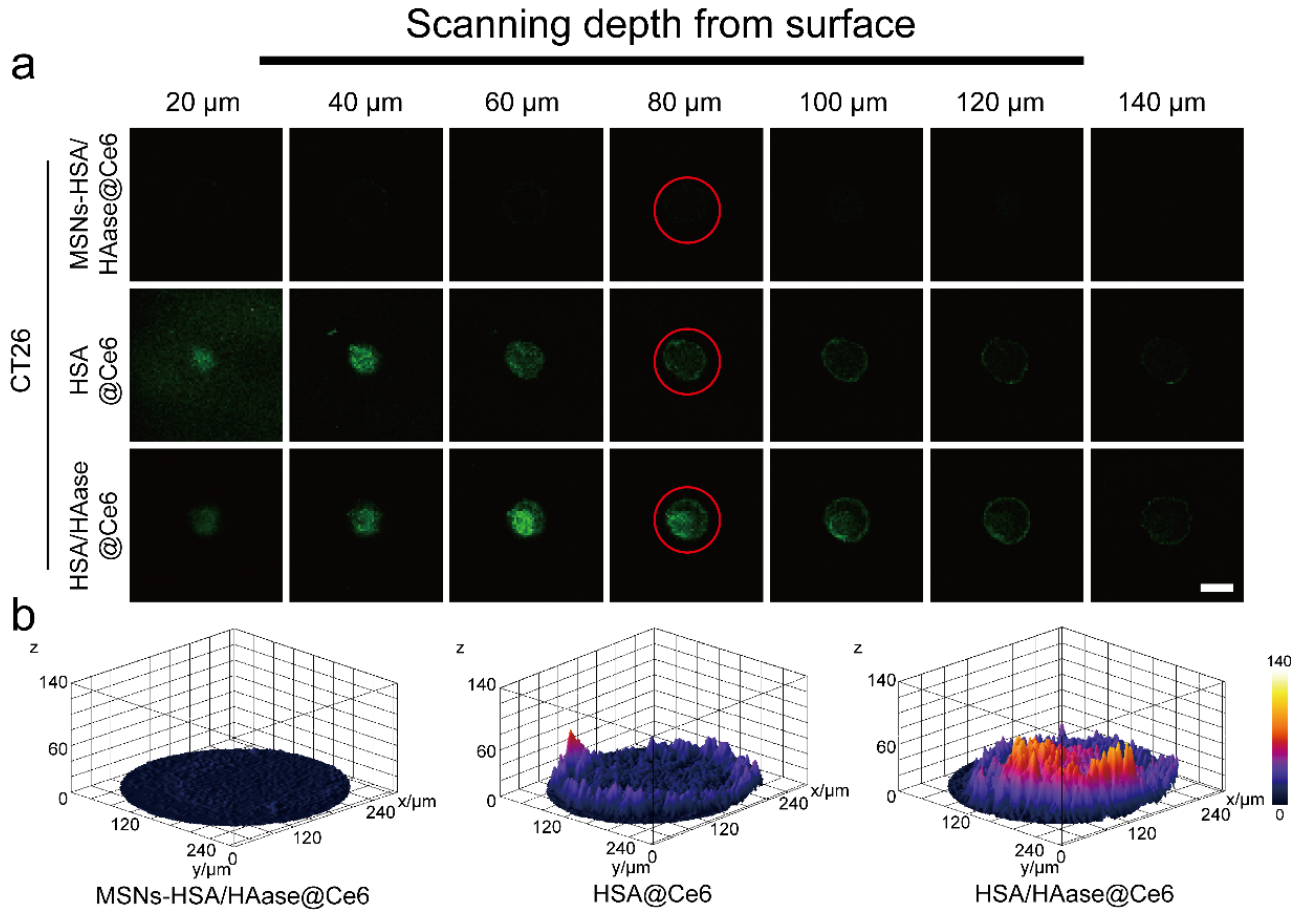

Figure S6. (a) CLSM Z-stack scanning images of CT26-multicellular spheroids incubated with the MSNs-HSA/HAase@Ce6, HSA@Ce6 and HSA/HAase@Ce6. Scale bars, 200 μm. (b) Surface plot images of multicellular spheroids along the arrow region at the Z-axis distance of 80 μm.

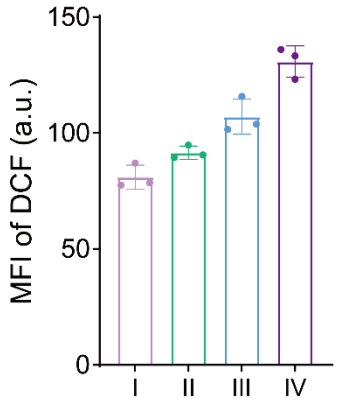

Figure S7. Semi-quantification of ROS fluorescence in 4T1 cells using DCFH-DA after incubating with different materials and exposing to laser irradiation. I: Ce6+Laser, II: MSNs-HSA/HAase@Ce6+Laser, III: HSA@Ce6+Laser, and IV: HSA/HAase@Ce6+Laser.

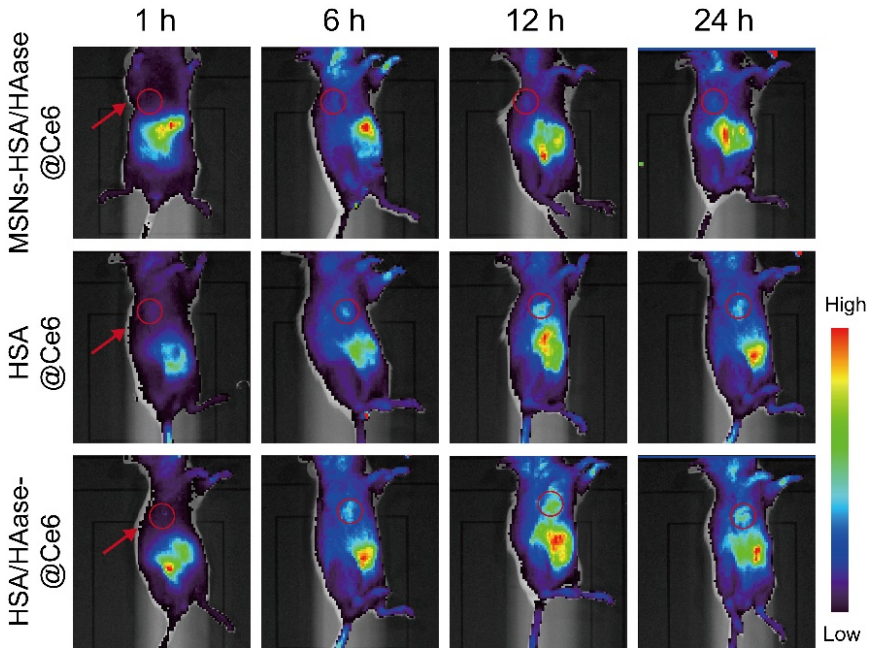

Figure S8 Fluorescence images of 4T1 tumor-bearing mice after intravenous injection of MSNs-HSA/HAase@Ce6, HSA@Ce6, and HSA/HAase@Ce6 with an equivalent Ce6 concentration of 2.5 mg·kg^{-1}.

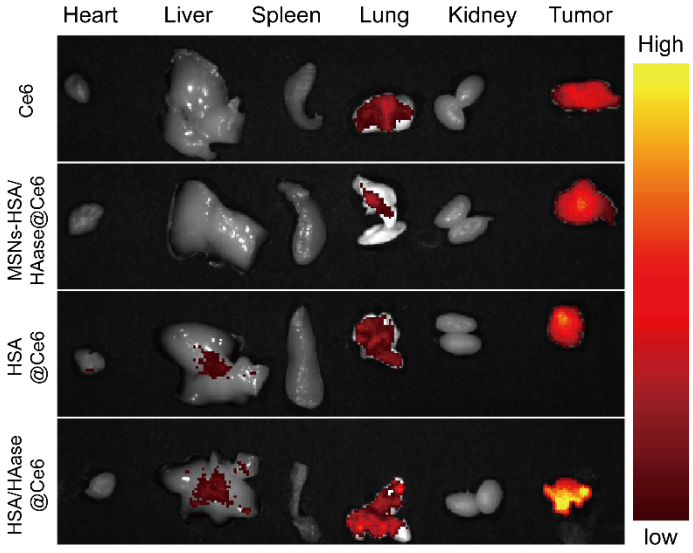

Figure S9. The *ex vivo* fluorescence distribution of harvested major organs and tumors from the mice with 4T1 tumor after the injection of free Ce6, MSNs-HSA/HAase@Ce6, HSA@Ce6, and HSA/HAase@Ce6 with an equivalent Ce6 concentration of 2.5 mg·kg^{-1}.

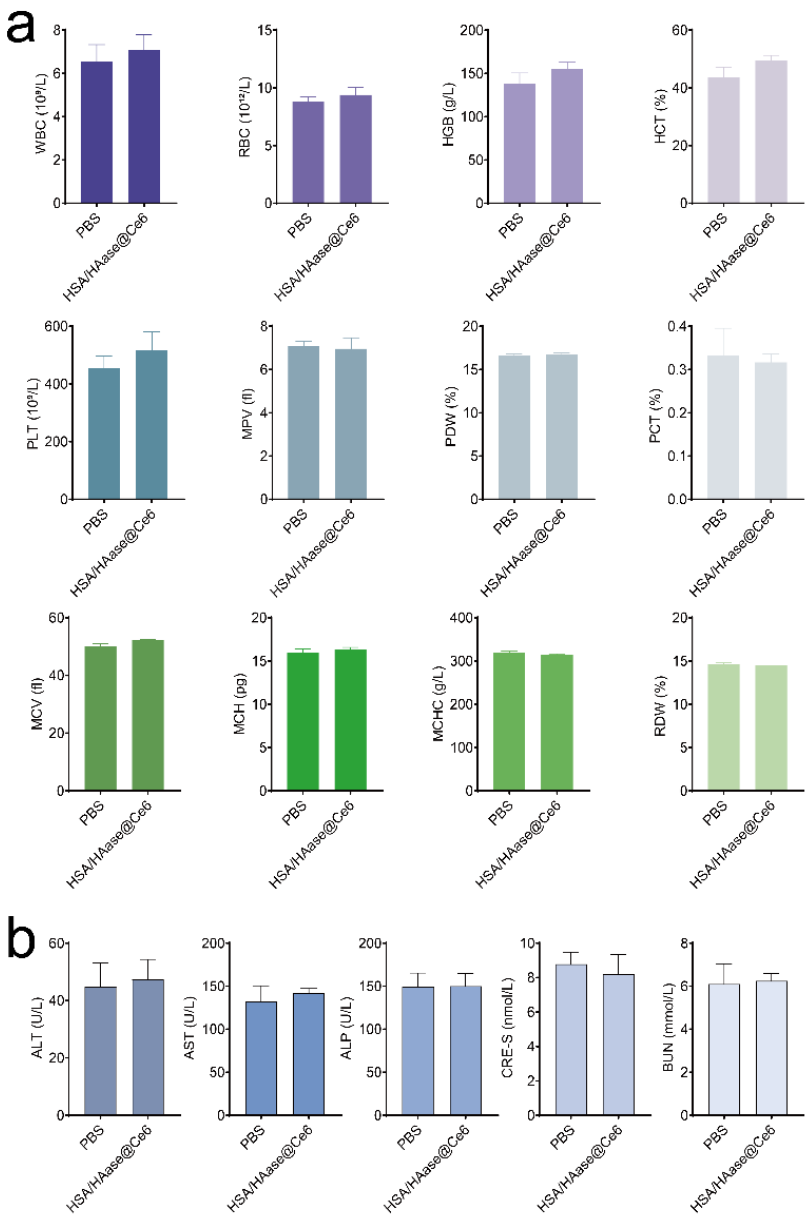

Figure S10. (a) Hematology analysis and (b) biochemical assay from mice treated with PBS or HSA/HAase@Ce6 (n = 3). Tested indicators of hematology analysis: white blood cell (WBC), red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), platelet (PLT), mean platelet volume (MPV), platelet distribution width (PDW), plateletcrit (PCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and red blood cell distribution width (RDW). Tested indicators of biochemical assay: aspartate aminotransferase (ALT), aspartate aminotransferas (AST), alkaline phosphatase (ALP), creatinine-serum (CRE-S), and blood urea nitrogen (BUN)

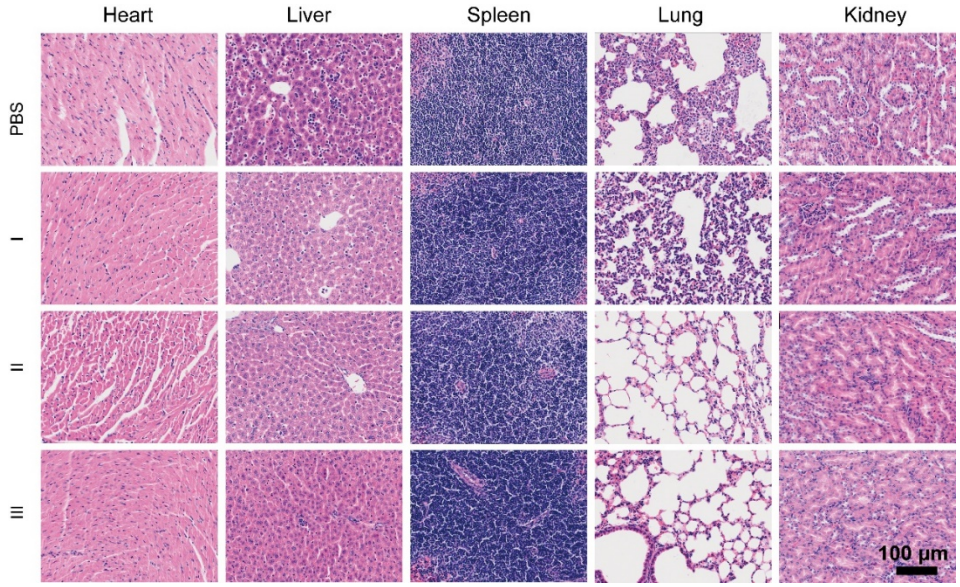

Figure S11. H&E staining of the major organs of mice (n = 5) intravenously injected with PBS, MSNs-HSA/HAase@Ce6, HSA@Ce6, and HSA/HAase@Ce6. Scale bars: 100 μm. I: MSNs-HSA/HAase@Ce6, II: HSA@Ce6, and III: HSA/HAase@Ce6.

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