Supporting Information

Biomimetic Dual-Responsive Bioengineered Nanotheranostics for Intracellular Cascade-Synthesizing Chemo-Drugs and Efficient Oncotherapy

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1. Materials and methods

1.1. Materials

Indocyanine green (ICG) was purchased from Haoyun Chemical Technology Co, Ltd. 1,5-dihydroxynaphthalene (1,5-DHN) was obtained from Aladdin. 5-hydroxy 1,4-naphthoquinone was bought from Bidpharm. Anhydrous methanol and Tetrahydrofuran (THF) were provided from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Calcein-acetoxymethyl ester (Calcein AM)/propidium iodide (PI) double staining reagent was provided by KeyGEN BioTECH. Annexin V-FITC

apoptosis detection kit, 4, 6-diamidino-2-phenylindole (DAPI), 2, 7-dichlorofluorescein diacetate (DCFH-DA), and 3-[4,5-dimethylth-iazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Beyotime. Dulbecco's modified eagle's medium (DMEM), roswell park memorial institute (RPMI) 1640, fetal bovine serum (FBS), singlet oxygen sensor green (SOSG), penicillin-streptomycin, and trypsin were supplied by Thermo Fisher Scientific. Dulbecco's phosphate-buffered saline (PBS) purchased from Servicebio. 4T1 cells were supplied by American Type Culture Collection (ATCC). BALB/c nude mice were provided by Xiamen University Laboratory Animal Center. All animal procedures were complied with the guidelines of the Xiamen University Institutional Animal Care and Use Committee.

1.2. Cell culture

4T1 cells were provided from the cell bank of Chinese Academy of Sciences and cultured in DMEM supplemented with 10% of FBS and 1% of penicillin/streptomycin at 37 °C and 5% of CO_2 until the stationary phase of growth was realized.

1.3. Animals and their tumor models

BALB/c mice were obtained from the Laboratory Animal Center of Xiamen University. All animal experiments were carried out based on a protocol approved *via* the Institutional Animal Care and Use Committee of Xiamen University, and the assigned approval/accreditation number is XMU-LAC20170109. 4T1 tumor BALB/c mice were built via subcutaneously inoculating 5×10^6 4T1 cells on both the right lower limb of each mouse.

1.4. Construction of ICG-DHN

ICG-DHN was fabricated by a simple supramolecular co-assembly method between ICG and DHN. Briefly, a methanol solution of ICG (200 μL and 2 mg/mL) was mixture with a THF solution of DHN (400 μL and 2 mg/mL). After stir in the dark environment for 2 h at 25 ℃, the mixed organic solutions containing ICG and DHN were injected dropwise into 4 mL of deionized (DI) water and then stirred overnight in the dark environment at 25 ℃. Next, the crude product was dialyzed *via* DI water for 12 h (molecular weight cut off (MWCO) = 1,000 DA, Slide-A-Lyzer, Thermo Scientific, USA) and meanwhile DI water was replaced every 2 h. ICG-DHN was formed by co-assembly of ICG and DHN. Lastly, the resultant ICG-DHN was stored at 4 °C environment for future application.

1.5. Characterization of ICG-DHN

Molecular docking was performed on the AutoDock Vina program (http://cloud.yinfo tek.com). Fourier transform infrared (FT-IR) spectroscopy was performed on a Bruker IFS-55 infrared spectrometer (Bruker, Zurich, Switzerland). Ultraviolet-visible (UV-vis) absorbance was conducted on the Shimadzu UV spectrophotometer (UV-2550/2450, Shimadzu, Japan). The morphology was observed with the transmission electron microscope (TEM, JEM 2100, JEOL, Tokyo, Japan) at 200 kV. Hydrodynamic diameter and polydispersity index (PDI) were determined *via* dynamic light scattering (DLS) by a Malvern Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, U.K.). Zeta potential was determined by electrophoretic light scattering (ELS) using the same equipment. Proton nuclear magnetic resonance (^{1}H NMR) spectra were measured with the Bruker AV400 MHz NMR spectrometer (Bruker, Billerica, MA, USA). X-ray diffraction (XRD) spectra were obtained by the X-ray diffractometer (Phillips X 0 pert Pro Super, Panalytical, Almelo, Netherlands).

1.6. Construction of red blood cell membranes (RBCs)-cloaked ICG-DHN

RBCs@ICG-DHN was constructed by coating RBCs onto ICG-DHN surface *via* a multiple-step extrusion. In brief, the RBCs-derived vesicles were obtained by a well-established procedure and redispersed in PBS for further application[1]. After that, 200 μL of RBCs-derived vesicles (1 mg/mL) was added into the 3 mL of ICG-DHN dispersions (0.25 mg/mL). After sonication for 10 min at 4 ℃ and then extrusion through 0.80, 0.45, and 0.20 μm polycarbonate porous membranes (Nuclepore Track-Etch Membrane Filter, USA). Next, the abovementioned dispersions were ultracentrifuged at 15000 rpm for 10 min at 4 ℃, washed with cold PBS (1×). After that, the abovementioned dispersions were ultracentrifuged at 15000 rpm for 10 min at 4 ℃ and then redispersed in 10 mL of DI water *via* ultrasonication at 400 W at an ice bath for 5 min. The resultant RBCs@ICG-DHN dispersions were stored at 4 ℃ for further application.

1.7. Characterization of RBCs@ICG-DHN

Morphology was collected by TEM (JEM 2100, JEOL, Tokyo, Japan) at 200 kV. Hydrodynamic diameter and PDI were determined via DLS by a Malvern Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, U.K.). Zeta potential was determined by ELS using the same equipment. UV-vis absorption was conducted on the Shimadzu UV spectrophotometer (UV-2550/2450, Shimadzu, Japan). Drug encapsulation efficiency and drug payload rate were measured by UV-vis absorption

and then calculated with the following equations: drug encapsulation efficiency (%) = ($weight_{drug}$ of encapsulation)/(initial $weight_{drug}$); drug payload rate (%) = ($weight_{drug}$ of encapsulation)/(total weight of RBCs@ICG-DHN) × 100.

1.8. *In vitro* stability

In vitro physiological stability of RBCs@ICG-DHN was estimated by diameter change at predesigned time intervals. Briefly, the diameter change of RBCs@ICG-DHN in DI water, PBS (pH 7.4), DMEM, and DMEM containing FBS was measured by DLS by a Malvern Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, U.K.) at 0, 24, 48, 72, 96, and 120 h.

In vitro photostability of RBCs@ICG-DHN was evaluated by fluorescence intensity and UV-vis absorption changes at predesigned time intervals. In brief, changes in both fluorescence intensity and UV-vis absorption of RBCs@ICG-DHN were determined by the FluoroMax-4 Spectrofluorometer (HORIBA Jobin Yvon, USA) and the Shimadzu UV spectrophotometer (UV-2550/2450, Shimadzu, Japan) at 0, 24, 48, 72, 96, and 120 h, respectively.

1.9. *In vitro* $^1O_2$ generation

The $^1O_2$ generation was determined by a commercial SOSG assay according to the manufacturer's suggested instruction. In a typical procedure, ICG and RBCs@ICG-DHN at the equivalent ICG concentration of 25 μg/mL were mixed with 10 μM of SOSG, respectively. After 808 nm laser irradiation (100 mW/cm^2) for 0 s, 50 s, 100 s, 150 s, 200 s, 250 s, and 300 s, the fluorescence intensity of SOSG from the abovementioned samples was determined by the FluoroMax-4 Spectrofluorometer (HORIBA Jobin Yvon, USA) at emission wavelength of 488 nm.

1.10. *In vitro* photooxidation

In vitro photooxidation of RBCs@ICG-DHN was evaluated by UV-vis absorption changes at predesigned time points. In brief, 10 mL of DHN (10×10^{-4} M) and 1 mL of RBCs@ICG-DHN (0.5 mg/mL) were added into a round bottom flask and then stirred until completely mixed. Next, after 808 nm laser irradiation (100 mW/cm^2) for 30 min and these changes in UV-Vis absorption at 298 and 419 nm were measured by the Shimadzu UV spectrophotometer (UV-2550/2450, Shimadzu, Japan) at 5, 10, 15, 20, 25, and 30 min. After that, the decrease in absorbance at 298 nm was employed to detect the depletion of DHN, whose concentration was calculated by the molar extinction coefficient (ε = 7664 $M^{-1}\cdot cm^{-1}$). The increase in absorbance at 419 nm was used to detect the production of juglone, whose

concentration was calculated by the molar extinction coefficient (ε =3500 $M^{-1}\cdot cm^{-1}$). The final reaction yield is calculated by the ratio of the juglone production concentration and the initial DHN concentration.

1.11. *In vitro* drug release and disassembly

In vitro release behaviors of DHN and ICG from RBCs@ICG-DHN were evaluated by various simulated physiological conditions. In a typical procedure, 3 mL of RBCs@ICG-DHN was transferred into a dialysis bag (MWCO = 1,000 DA, Slide-A-Lyzer, Thermo Scientific, USA) with/without 808 nm laser irradiation (100 mW/cm^2 and 5 min), which was immersed into 20 mL of PBS at different pH values (7.4, 6.5, and 5.0). After that, 3 mL of the external PBS was withdrawn and then replaced with 3 mL of fresh PBS quickly based on the predesigned time points. Next, the release amount of DHN and ICG was measured by the Shimadzu UV spectrophotometer (UV-2550/2450, Shimadzu, Japan). Besides, the morphology changes of RBCs@ICG-DHN at pH 6.5 and 5.0 with 808 nm laser irradiation (100 mW/cm^2 and 5 min) were observed by TEM.

1.12. *In vitro* cellular uptake

In vitro cellular uptake of RBCs@ICG-DHN was evaluated by confocal laser scanning microscopy (CLSM) and flow cytometry analysis. In a typical procedure, for CLSM analysis, 4T1 cells were seeded into 6-well plates at a density of 2.0×10^5 cells/well and then incubated at 37°C for 24 h. After that, 4T1 cells were incubated with ICG, ICG-DHN, and RBCs@ICG-DHN at the equivalent ICG concentration of 25 μg/mL based on the predesigned incubation time points (1 h and 4 h) at 37 ℃, respectively. Next, culture medium was removed. 4T1 cells were washed thrice by cold PBS and then fixed by 4% of paraformaldehyde for 30 min. Nuclei were stained using DAPI for 20 min. Finally, 4T1 cells were imaged by a Leica SP8-STED CLSM with excitation at 638 nm for ICG and 360 nm for DAPI.

For flow cytometry analysis, the previous steps were the same as the CLSM experiments that 4T1 cells were cultured with different formulations based on the predesigned incubation time points (1 h and 4 h) at 37 ℃. After that, 4T1 cells were detached with trypsin/EDTA, suspended in PBS with 10% of FBS, centrifuged at 2, 000 rpm for 5 min at 4 °C, and resuspended in fluorescence-activated cell sorting (FACS) buffer. Finally, the cellular fluorescence intensity of ICG was recorded by an Attune NXT ultrahigh speed flow cytometer (Thermo Scientific, USA) and then the experimental results were further analyzed by flowjo V10.

1.13. Detection of termination of intracellular $^1O_2$ generation

Intracellular $^1O_2$ generation of RBCs@ICG-DHN was estimated by CLSM and flow cytometry analysis. In brief, Intracellular generation of $^1O_2$ in 4T1 cells was measured by using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). For CLSM analysis, 4T1 cells were seeded in 6-well plates at a density of 3.0×10^5 cells/well and incubated for 24 h. Next, culture medium was removed and then 4T1 cells were washed thrice with cold PBS. In addition, 4T1 cells were incubated with ICG, ICG-DHN, and RBCs@ICG-DHN at equivalent ICG concentration (25 μg/mL) with 808 nm laser irradiation (100 mW/cm^2 and 5 min) for 8 h, respectively. Lastly, the supernatant was removed and then 4T1 cells were washed thrice with cold PBS. After incubation with DCFH-DA for 30 min, 4T1 cells were washed thrice by cold PBS and then fixed by 4% of paraformaldehyde for 30 min. Nuclei were stained by using DAPI for 20 min. Finally, 4T1 cells were imaged by a Leica SP8-STED CLSM with excitation at 488 nm for DCFH and 360 nm for DAPI.

For flow cytometry analysis, the previous steps were the same as the CLSM experiments that 4T1 cells were cultured with different formulations at 8 h at 37 ℃. After that, 4T1 cells were detached with trypsin/EDTA, suspended in PBS with 10% of FBS, centrifuged at 2, 000 rpm for 5 min at 4 °C, and resuspended in FACS buffer. Finally, the cellular fluorescence intensity of DCFH was recorded by an Attune NXT ultrahigh speed flow cytometer (Thermo Scientific, USA) and then the experimental results were further analyzed by flowjo V10.

1.14. *In vitro* cytotoxicity

4T1 cells were seeded in 96-well plates at a density of 2.0×10^4 cells/well. After culture for 24 h, 4T1 cells were incubated with ICG, DHN, ICG-DHN, and RBCs@ICG-DHN at the equivalent DHN concentration (2, 5, 10, 20, and 40 μg/mL) with 808 nm laser irradiation (100 mW/cm^2 and 5 min) for 24 h, respectively. Next, the cell viability was determined by a commercial MTT assay based on the manufacturer's suggested instruction. Every determination groups had three parallel samples.

1.15. *In vitro* live/dead cell staining

4T1 cells were seeded in 12-well plates at a density of 5.0×10^5 cells/well. After incubation for 24 h, 4T1 cells were treated with ICG, DHN, ICG-DHN, and RBCs@ICG-DHN at the equivalent DHN concentration of 20 μg/mL with 808 nm laser irradiation (100 mW/cm^2 and 5 min) for 16 h, respectively. Finally, the live/dead

cell staining was estimated by a calcein-AM/PI staining Kit according to the manufacturer's suggested protocol and imaged by a Leica DM6000B fluorescence microscope in USA.

1.16. *In vitro* apoptosis assay

4T1 cells were seeded in 6-well plates at a density of 2.0×10^5 cells per well. After incubation for 24 h, 4T1 cells were incubated with ICG, DHN, ICG-DHN, and RBCs@ICG-DHN at the equivalent DHN concentration of 20 μg/mL with 808 nm laser irradiation (100 mW/cm^2 and 5 min) for 16 h. The cell apoptosis was estimated by an Annexin V-FITC/PI apoptosis detection Kit based on the manufacturer's suggested instruction and then the experimental results were further analyzed by flowjo V10.

1.17. *In vivo* NIR fluorescence self-imaging

4T1 tumor-bearing mice were intravenously injected with 200 μL of ICG, ICG-DHN, and RBCs@ICG-DHN at the equivalent ICG concentration of 100 μg/mL through the tail vein. Finally, the NIR fluorescence self-imaging was collected on an IVIS Lumina imaging system in USA at 0, 3, 6, 9, 12, and 24 h post-injection. Every determination groups had three mice.

1.18. In vivo synergistic therapy

When the tumor volume reached 80 mm^3, mice were divided into 8 groups (n = 6): PBS group as a control, ICG group, ICG/laser group, DHN group, ICG-DHN group, ICG-DHN/laser group, RBCs@ICG-DHN group, and RBCs@ICG-DHN/laser group. After the intravenous injection through the tail vein for 24 h, the tumor regions of mice were exposed to the 808 nm laser irradiation (100 mW/cm^2 and 5 min). After that, the tumor volume and body weight were recorded every 2 d for weeks. The tumor size was determined by a vernier caliper. Next, the tumor volume (V) was calculated as V = length ×width^2/2.

Next, after observation for 14 d, the 4T1 tumor-bearing mice were euthanized, the main organs and tumors were dissected to conduct hematoxylin and eosin (H&E) staining according to our previous method[2]. In addition, the tumor tissues were also employed to perform DCFH-DA and Ki 67 staining based on our previous method[3].

1.19. Statistical analysis

All data were expressed as means ± standard deviation (SD). Statistical differences among groups are analyzed by using one-way ANOVA analysis, followed by Tukey's post-test. $P < 0.05$ was considered to be statistically significant with

notation by * (** and *** indicate $P < 0.01$ and $P < 0.001$, respectively).

2. References

1. Y. Chen, Y. Li, J. Liu, Q. Zhu, J. Ma and X. Zhu, *J. Controlled Release*, 2021, **335**, 345-358.
2. Z. Fan, B. Jiang, Q. Zhu, S. Xiang, L. Tu, Y. Yang, Q. Zhao, D. Huang, J. Han, G. Su, D. Ge and Z. Hou, *ACS Applied Materials & Interfaces*, 2020, **12**, 14884-14904.
3. Z. Fan, D. Shi, W. Zuo, J. Feng, D. Ge, G. Su, L. Yang and Z. Hou, *ACS Applied Materials & Interfaces*, 2022, **14**, 5033-5052.

3. Supplementary data

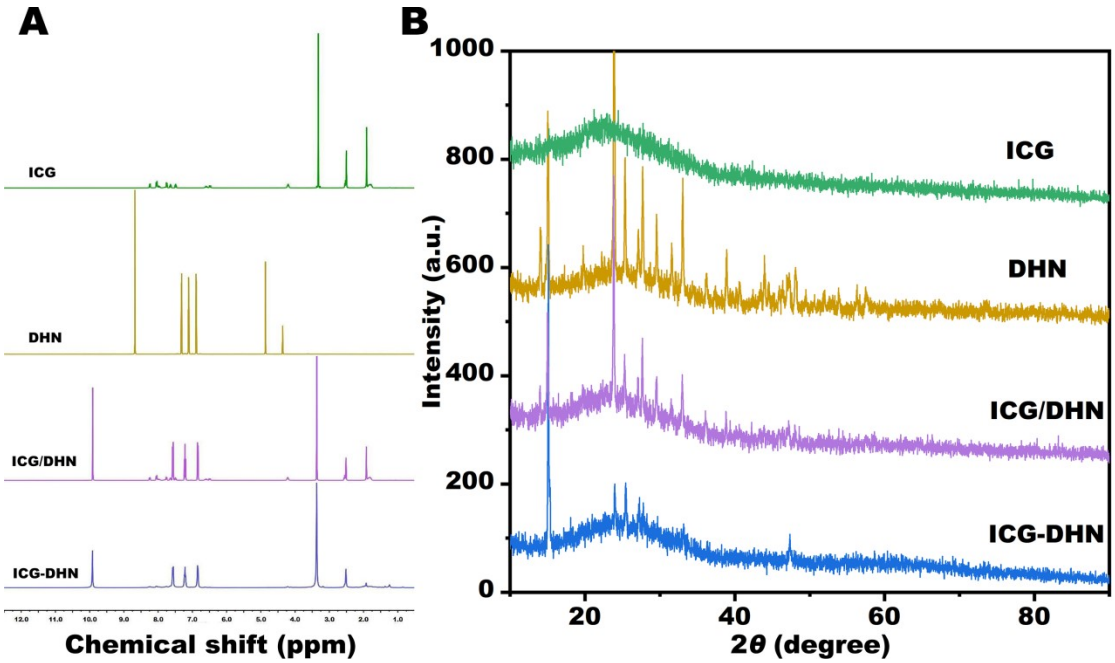

Figure S1. (A) ^{1}H NMR and (B) XRD spectra of ICG, DHN, ICG/DHN mixture, and ICG-DHN.

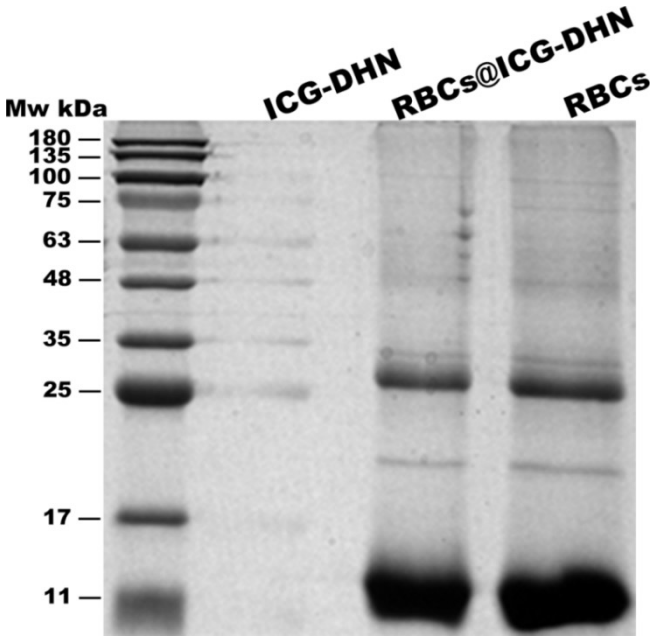

Figure S2. SDS-PAGE analysis of ICG-DHN, RBCs@ICG-DHN, and RBCs.

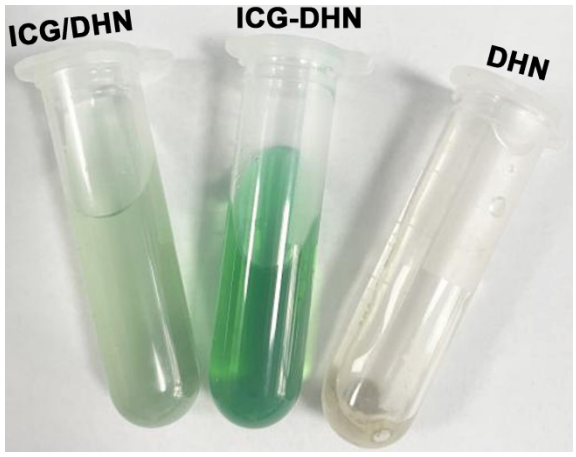

Figure S3. Optical images of ICG/DHN, ICG-DHN, and DHN dissolved in PBS for 24 h.

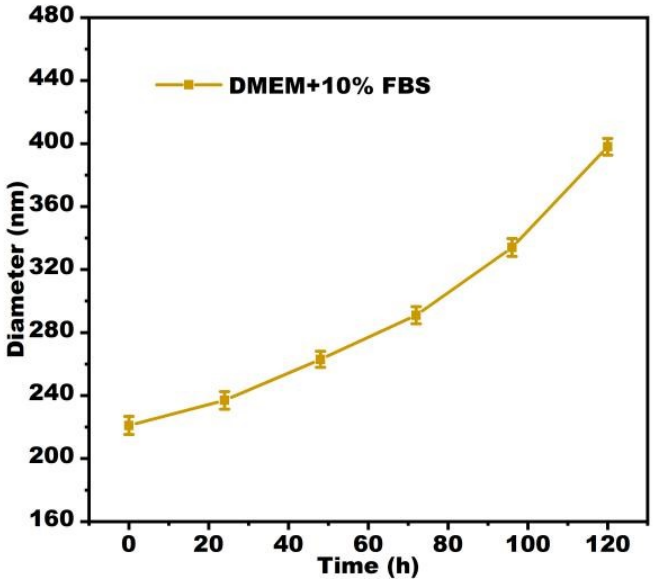

Figure S4. Change in the hydrodynamic diameter of ICG-DHN in DMEM containing 10% FBS for 120 h. Data are presented as the means ± SD (n = 3).

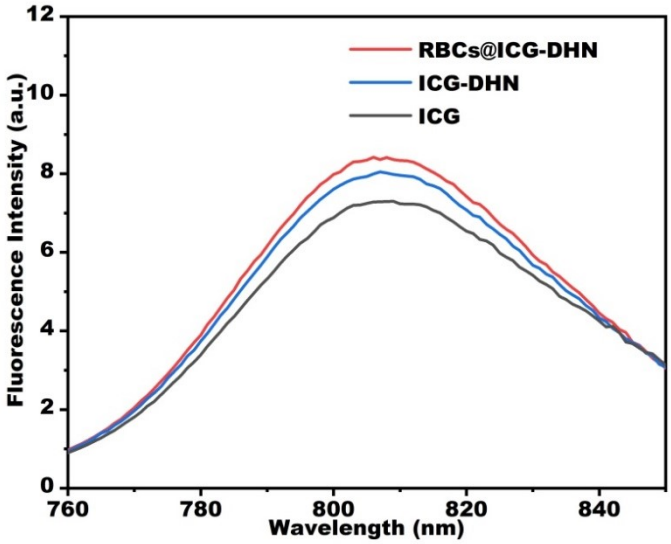

Figure S5. Fluorescence spectra of ICG, ICG-DHN, and RBCs@ICG-DHN with the same ICG concentration in aqueous solution for 120 h.

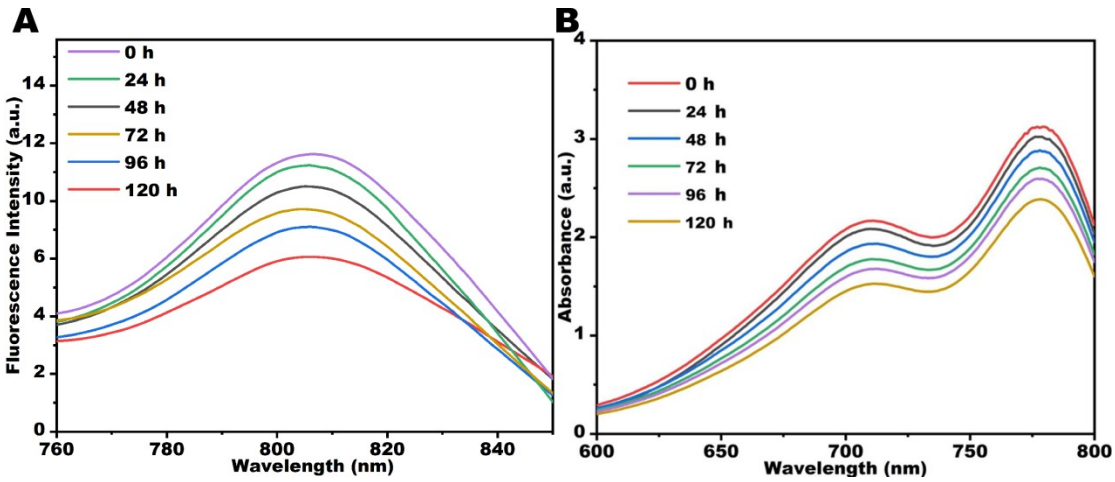

Figure S6. (A) Fluorescence and (B) UV-vis absorption spectra of ICG-DHN in aqueous solution for 120 h.

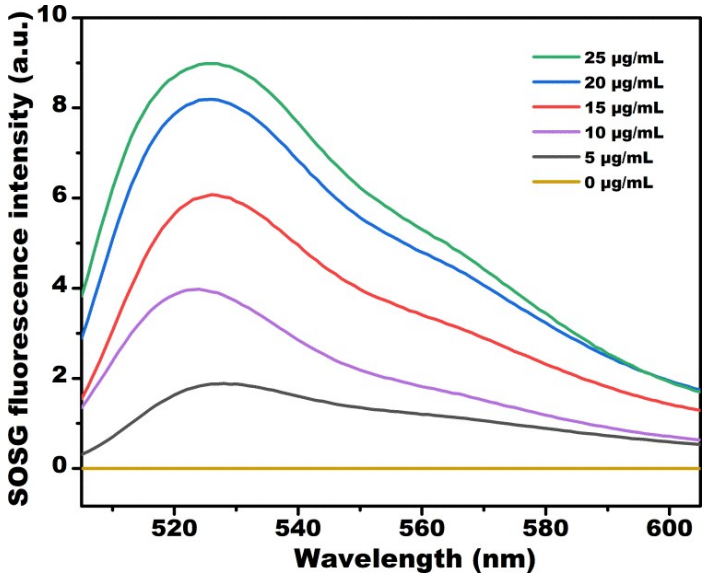

Figure S7. Fluorescence intensity of SOSG generated by RBCs@ICG-DHN at different ICG concentrations under 808 nm laser irradiation (100 mW/cm^2) for 5 min.

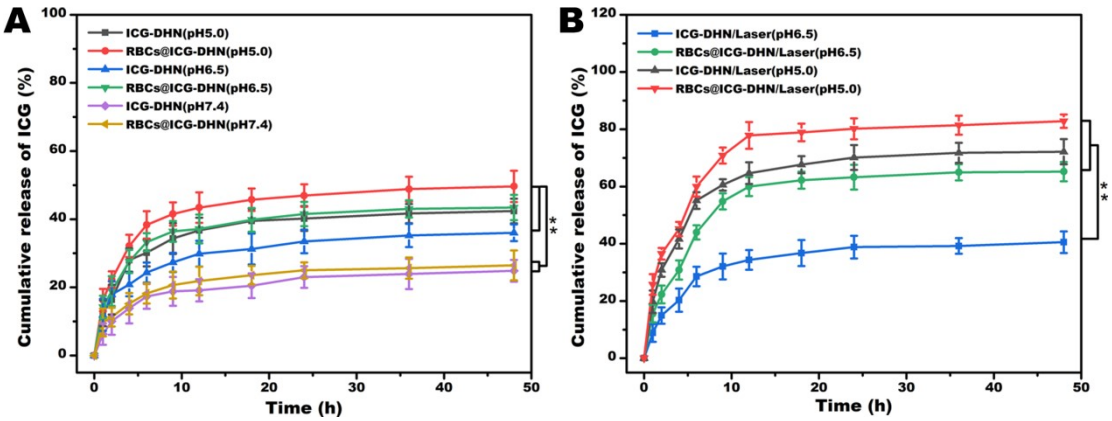

Figure S8. (A) Cumulative release of ICG from ICG-DHN and RBCs@ICG-DHN at pH 7.4, 6.5, and 5.0. (B) Cumulative release of ICG from ICG-DHN and RBCs@ICG-DHN at pH 6.5 and 5.0 with 808 nm laser irradiation (100 mW/cm^2) for 5 min. Data are presented as the means ± SD (n = 3) and $^{**}P < 0.01$.

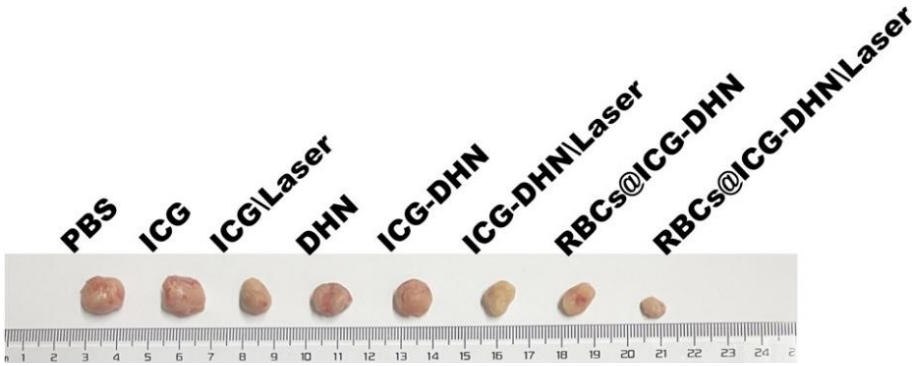

Figure S9. Photographs of representative tumor tissues after different therapies.

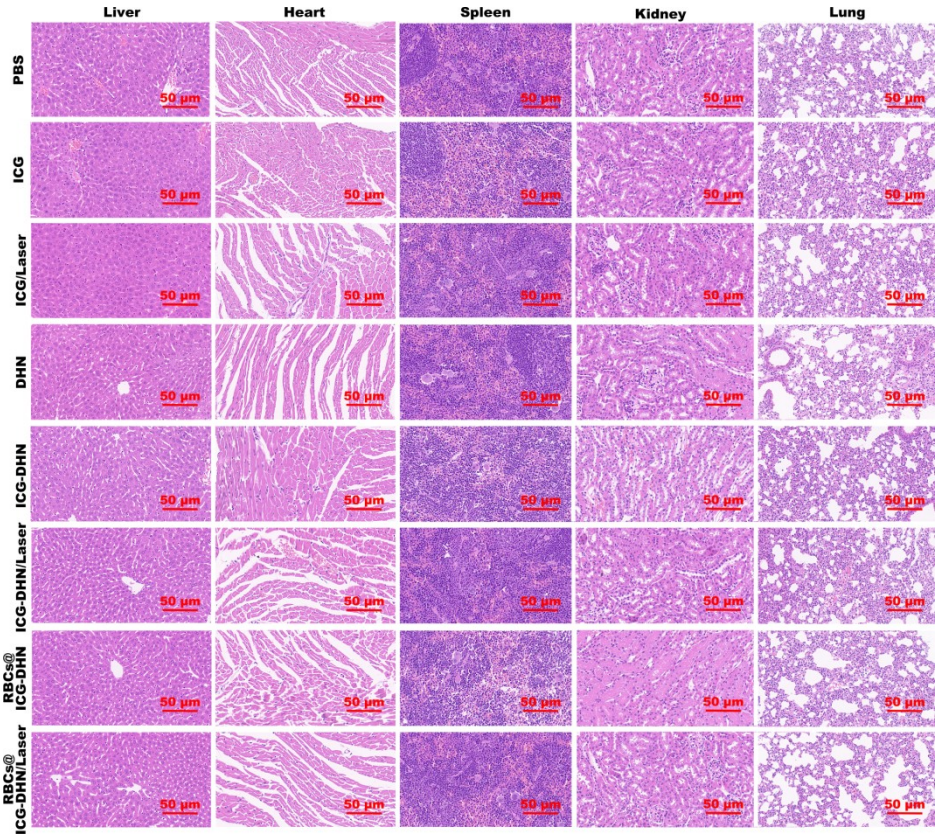

Figure S10. H&E stained images of normal tissues (heart, liver, spleen, lung, and kidney) after different therapies.

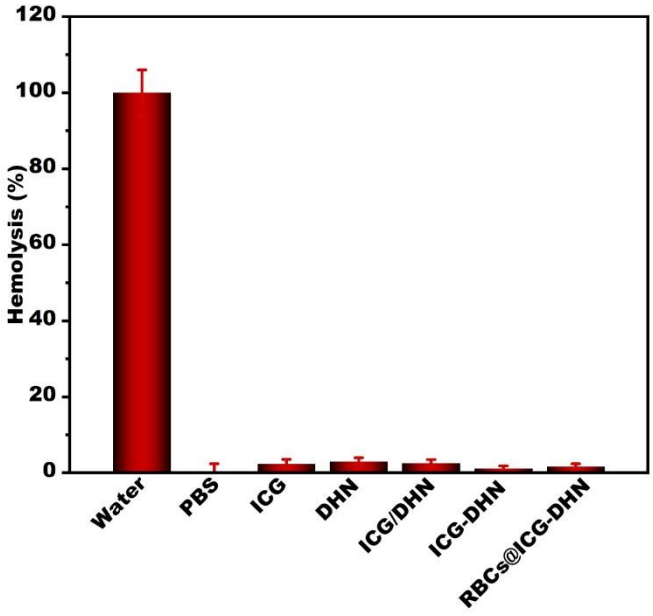

Figure S11. Hemolysis rate of water, PBS (pH 7.4), ICG, DHN, ICG/DHN mixture, ICG-DHN, and RBCs@ICG-DHN. Data are presented as the means ± SD (n = 3).