Supporting Information

A Tumor-targeting and Mitochondria-anchorable Probe for NIR/MRI Dual-modal Imaging-guided Photodynamic Therapy of Colorectal Cancer

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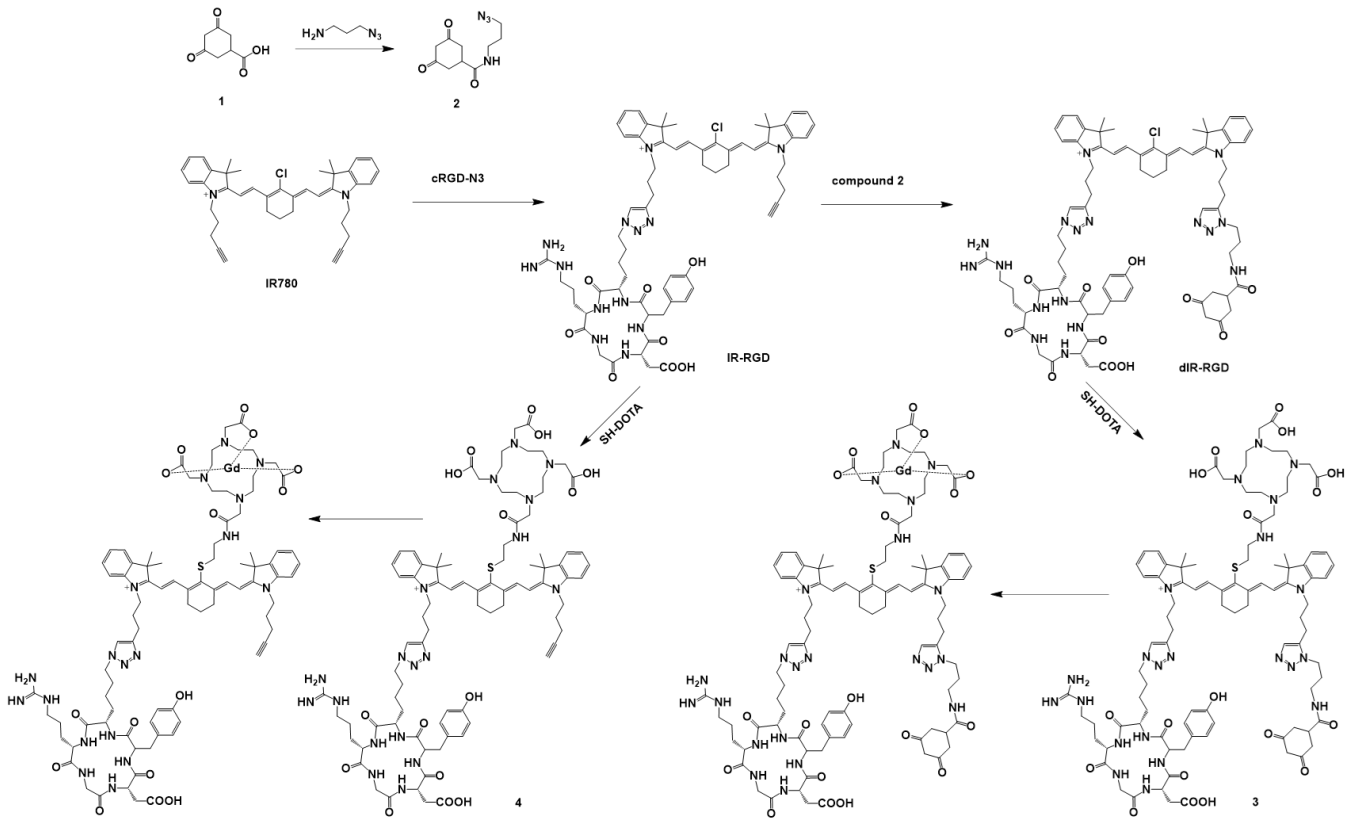

Scheme S1. Synthetic route of probe dIR-Gd and control IR-Gd.

Synthesis of compound 2. 3,5-dioxocyclohexane carboxylic acid (20 mg, 0.128 mmol), 1-ethyl-3-(3-dimet-hylaminopropyl) carbodiimide (EDC) hydrochloride (25 mg, 0.13 mmol) and N-hydroxy succinimide (NHS) (15 mg, 0.14 mmol) were dissolved in 5 mL of anhydrous DMF solution under stirring at 0 ℃ for 10 min, and then stirred at room temperature for 2 h. 3-azidopropylamine (15 mg, 0.14 mmol) and 10 μL DIPEA were added into above solutions and stirred for another 4 h. The solvent was removed under vacuum and the crude product was purified by silica gel chromatography (eluent: dichloromethane/methanol = 20:1, v/v) to afford compound **2** (19 mg, 62.3%). MS (MALDI-TOF) Calcd for: $C_{10}H_{14}N_4O_3$([M-H]$^-$): 237.11, found: 237.10.

Synthesis of IR-RGD. IR780 (10 mg, 0.02 mmol) and RGD-N_3 (10.4 mg, 0.02 mmol) was dissolved in 4 mL of DMSO, into which 1 mL of aqueous solution containing $CuSO_4$ (0.48 mg, 0.003 mmol), and sodium ascorbate (1.19 mg, 0.006 mmol) were introduced. After reacting overnight at room temperature, The reaction mixture was purified by preparative HPLC on a C18 reversed-phase column with a gradient elution of H_2O/CH_3CN (0.1% TFA)(The elution was performed with a linear gradient of solvent A (water containing 0.1% trifluoroacetic acid) and solvent B (acetonitrile containing 0.1% trifluoroacetic acid): 5% – 90% B in 10 min, then 90% – 100% B in 15 min) to afford the IR-RGD (12 mg, 60% yield). MS (MALDI-TOF) Calcd for: $C_{67}H_{83}ClN_{13}O_8$ ([M]$^+$): 1232.62, found: 1232.813.

Synthesis of dIR-RGD. Compound IR-RGD (15 mg, 0.02 mmol) was dissolved in 2 mL of DMSO, into which 2 mL of aqueous solution containing Compound2 (3 mg, 0.02 mmol), $CuSO_4$ (0.48 mg, 0.003 mmol), and sodium ascorbate (1.19 mg, 0.006 mmol) were introduced. After reacting overnight at room temperature, the reaction mixture was purified by preparative HPLC on a C18 reversed-phase column with a gradient elution of H_2O/CH_3CN (0.1% TFA) to obtain the compound dIR-RGD. MS (MALDI-TOF) $C_{77}H_{97}ClN_{17}O_{11}$ ([M]$^+$): 1470.72, found: 1470.797.

Synthesis of compound 4. Compound IR-RGD (20 mg, 0.02 mmol) and DOTA（1,4,7,10-tetraazacy-clododecane-1,4,7,10-tetraacetic acid）(4.8 mg, 0.02 mmol) was dissolved in 5 mL of DMSO, into which 1 uL triethylamine were introduced. After reacting overnight at room temperature, the reaction mixture was purified by preparative HPLC on a C18 reversed-phase column

with a gradient elution of H_2O/CH_3CN (0.1% TFA) to obtain the compound **4** (18.2 mg, 73%). MS (MALDI-TOF) Calcd for: $C_{85}H_{115}N_{18}O_{15}S^+$ ($[M+H]^+$): 1660.85, found: 1660.2626.

Synthesis of compound IR-Gd. Compound **4** (10 mg, 0.38 mmol) was dissolved in 10 mL 50% H_2O/DMF (v/v). To which, a solution of $GdCl_3$•$6H_2O$ (100 mg, 0.38 mmol) in water was added. After stirring at room temperature for 5 min, the pH value of the reaction solution was adjusted to 6-7 using NH_3 H_2O, and the reaction mixture was stirred at room temperature overnight. After the reaction, the reaction mixture was purified by preparative HPLC on a C18 reversed-phase column with a gradient elution of H_2O/CH_3CN (0.1% TFA) to give desired product as a green solid (8 mg, 67%). MS (MALDI-TOF) Calcd for: $C_{85}H_{112}GdN_{18}O_{15}$ S^+ ($[M]^+$): 1814.75, found: 1814.761.

Synthesis of compound 3. Compound dIR-RGD (20 mg, 0.02 mmol) and DOTA（1,4,7,10-tetraazacy-clododecane-1,4,7,10-tetraacetic acid）(4.8 mg, 0.02 mmol) was dissolved in 5 mL of DMSO, into which 1 uL triethylamine were introduced. After reacting overnight at room temperature, the reaction mixture was purified by preparative HPLC on a C18 reversed-phase column with a gradient elution of H_2O/CH_3CN (0.1% TFA) to obtain the compound **3** (16 mg, 62%). MS (MALDI-TOF) Calcd for: $C_{95}H_{129}N_{22}O_{18}S^+$ ($[M+H]^+$): 1898.96, found: 1898.839.

Synthesis of compound dIR-Gd. Compound **3** (10 mg, 0.38 mmol) was dissolved in 10 mL 50% H_2O/DMF (v/v). To which, a solution of $GdCl_3$•$6H_2O$ (100 mg, 0.38 mmol) in water was added. After stirring at room temperature for 5 min, the pH value of the reaction solution was adjusted to 6-7 using NH_3 H_2O, and the reaction mixture was stirred at room temperature overnight. After the reaction, the reaction mixture was purified by preparative HPLC on a C18 reversed-phase column with a gradient elution of H_2O/CH_3CN (0.1% TFA) to give desired product as a green solid (8.6 mg, 70%). MS (MALDI-TOF) Calcd for: $C_{95}H_{126}GdN_{22}O_{18}$ S+ ($[M+H]^+$): 2053.86, found: 2053.715.

MATERIALS AND METHOD

***In vitro* stability study.** The aqueous solutions of the experimental probe dIR-Gd and control probe IR-Gd (10 μM) were separately diluted in PBS buffer. The samples were placed at room temperature for different time points and their UV-vis spectra were recorded by using a UV spectrometer from 0 to 10 days. Both probes were stored at room temperature, and their particle sizes were measured by DLS at 1, 3, 5, 7, and 10 days with consistent sample preparation to evaluate nano size stability.

Hemolysis assay. The blood samples from healthy adult BABL/C mice were used to evaluate the biocompatibility of dIR-Gd and IR-Gd (10 μM). The red blood cells (RBCs) were collected by removing the serum from the blood after centrifugation and suction. RBCs were then purified by washing with PBS five times, and diluted to 10 times of their initial volume with PBS buffer. A 0.5 mL of diluted RBCs suspension was subsequently mixed with 0.5 mL 0.9% NaCl as a negative control, 0.5 mL Mili-Q water is a positive control, The concentrations of dIR-Gd and IR-Gd (0.5 mL, 0.9% NaCl) are ranging from 5 to 50 μM. The mixtures were shaken slightly and kept for 2 h at room temperature. The samples were centrifuged (10000 rpm, 5 min) and photographed, the supernatant was eventually subjected to absorption measurement at 570 nm.

Colocalization assay. CT26 cells were treated with dIR-Gd or IR-Gd for 12 h respectively, and then washed by PBS for three times to remove excessive probes followed by incubation with Mito-tracker as well as Lyso-tracker. After washing with PBS, the cells were visualized on confocal laser scanning microscopy (CLSM). The corresponding pearson correlation coefficient was calculated by the ImageJ software.

Cell viability assays. CT26 cells and 3T3 cells were seeded into 96 well plates at a density of 8×10^3 cells per well and grown overnight. The cells were then incubated with different concentrations (0, 2.5, 5, 10, 25, and 50 μM) of experimental probe dIR-Gd and control probe IR-Gd (0, 2.5, 5, 10, 25, and 50 μM) for 24 h. Finally, the relative survival rate of the cells was determined by standard CCK-8 method.

Cytotoxicity assays. CT26 cells and 3T3 cells were seeded into 96 well plates at a density of 8×10^3 cells per well and grown overnight.

The cells were then incubated with different concentrations (0, 2.5, 5, 10, 25, and 50 μM) of experimental probe dIR-Gd and control probe IR-Gd (0, 2.5, 5, 10, 25, and 50 μM) for 12 h followed by three times of PBS wash to remove excessive probes. Fresh medium was added, and the cells were incubated for 12 h. Then the cells were irradiated by 808 nm laser (0.5 W/cm ², 5 min). After additional 48 h incubation, the relative survival rate of the cells was determined by a standard CCK-8 method.

Apoptosis analysis. CT26 cells were seeded in 6-well plates (~ 2×10^5 cells/well) and cultured to 80% confluence. The cells were treated with 1 mL of probe-containing solutions (10 μM) for 12 h followed by three times of PBS wash to remove excessive probes. Fresh medium was added, and the cells were incubated for 12 h. Then the cells were irradiated by 808 nm irradiation (0.5 W/cm ², 5 min). After 24 h of post-irradiation incubation, the cells were harvested and stained with Annexin V-FITC and propidium iodide for 15 min in dark. The cells were resuspended in 1 mL PBS and analyzed by flow cytometry.

Mice tumor model. Female BALB/c mice with body weights of 15-17 g purchased from Changzhou Cavensla Experimental Animal Technology Co. Ltd were housed under standard conditions (25 ± 2 ℃/60 ± 10% relative humidity) with 12 h light/dark cycle. The tumors were grafted by subcutaneous inoculation of 1×10^6 CT26 cells in about 50 μL PBS into the back of each mouse. Fluorescence imaging studies were carried out when the tumor size reached about 50 mm^3. All animal experiment protocols were compliant with the animal ethics committee of Soochow University Laboratory Animal Center.

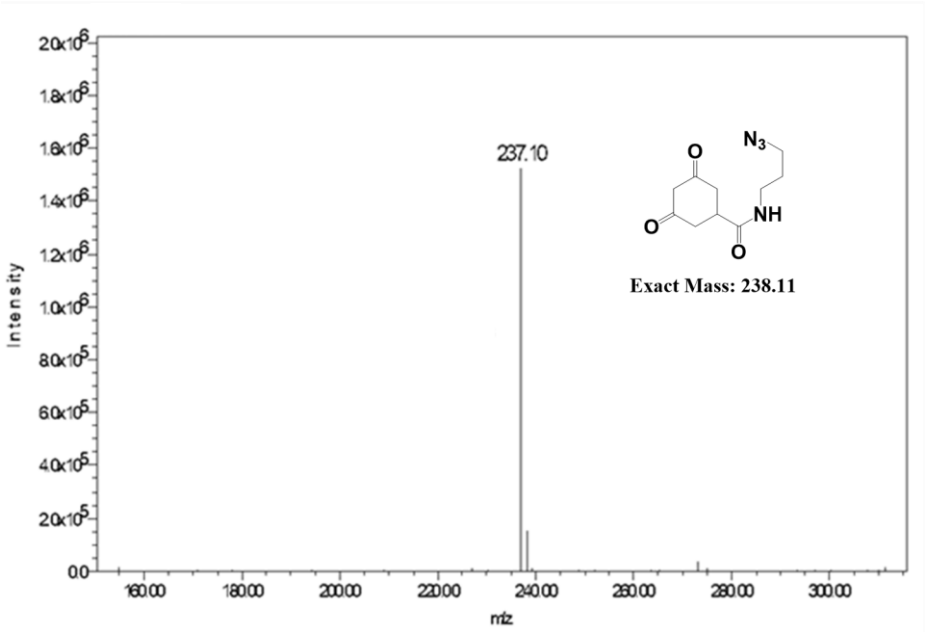

Figure S1. HR-MS spectrum of compound **2**.

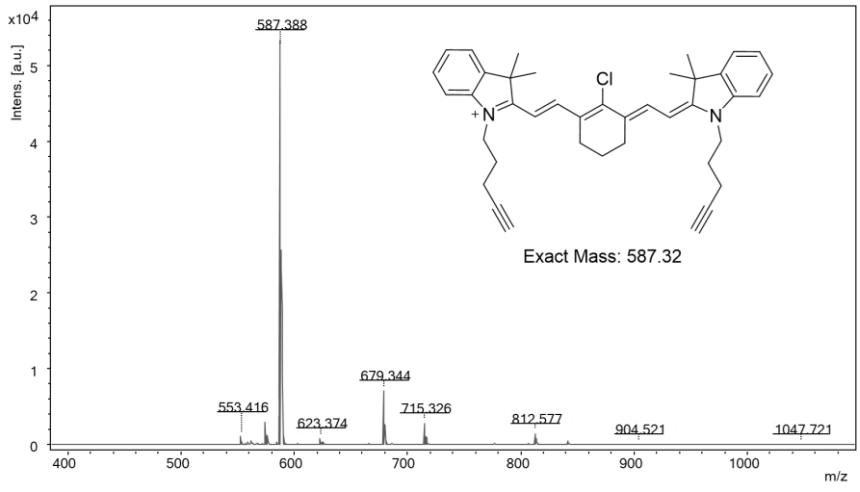

Figure S2. HR-MS spectrum of IR780.

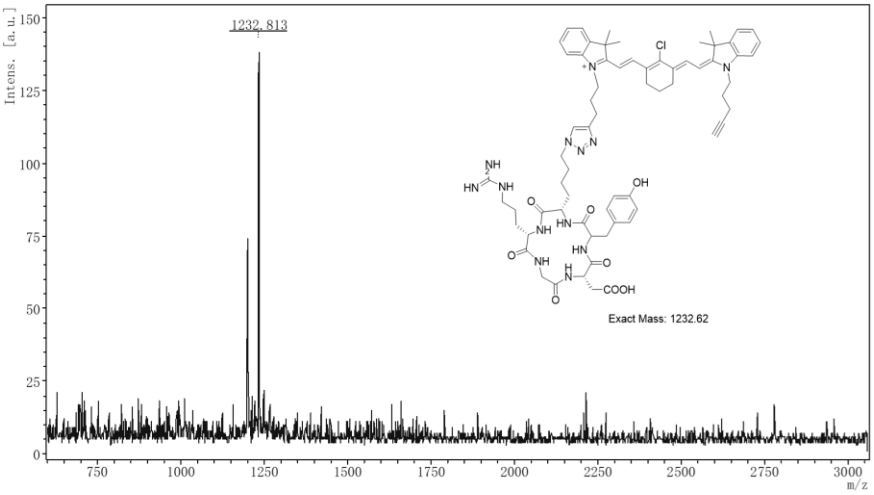

Figure S3. HR-MS spectrum of compound IR-RGD.

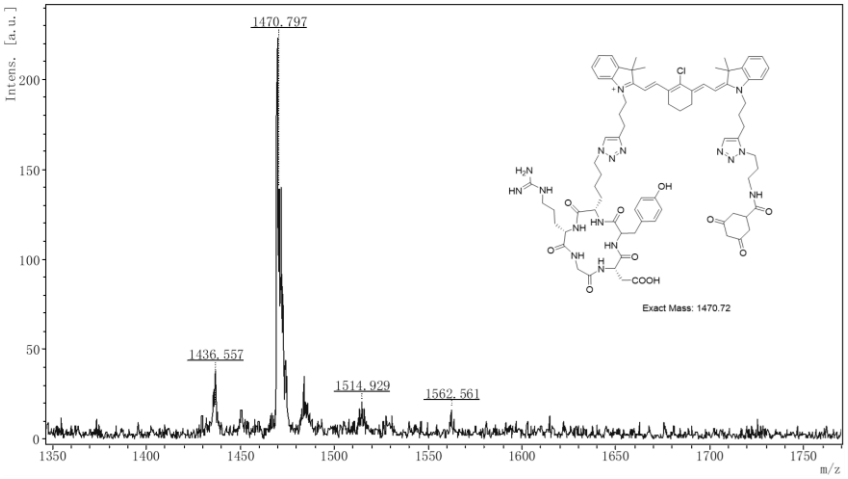

Figure S4. HR-MS spectrum of compound dIR-RGD.

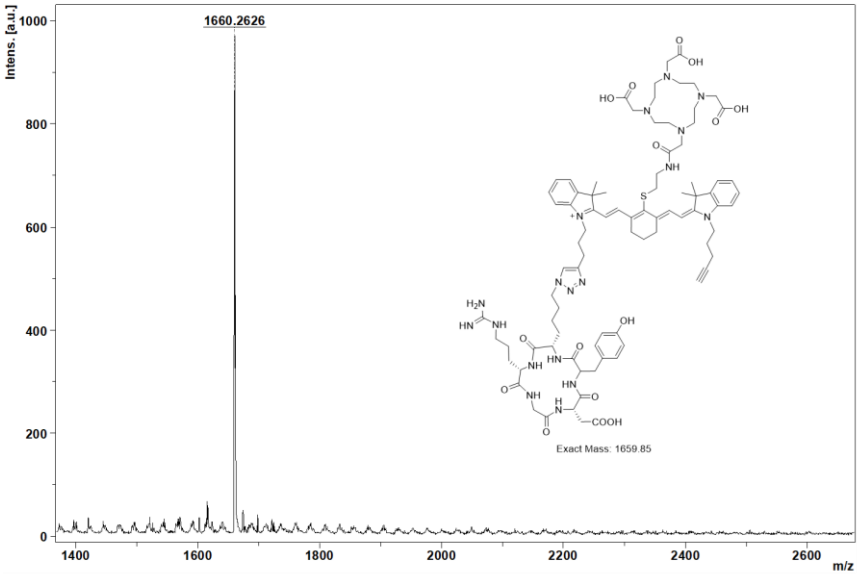

Figure S5. HR-MS spectrum of compound **4**.

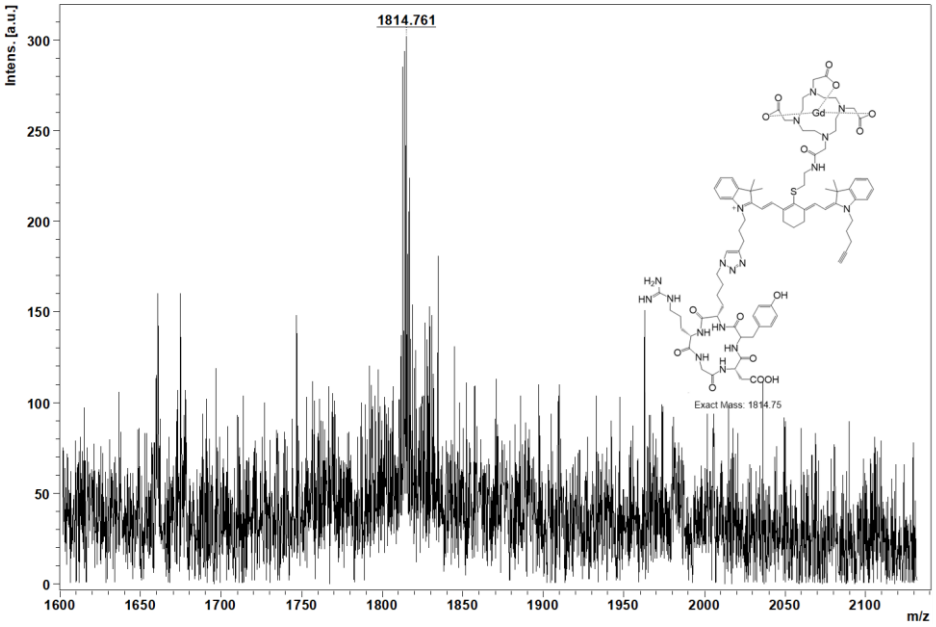

Figure S6. HR-MS spectrum of compound IR-Gd.

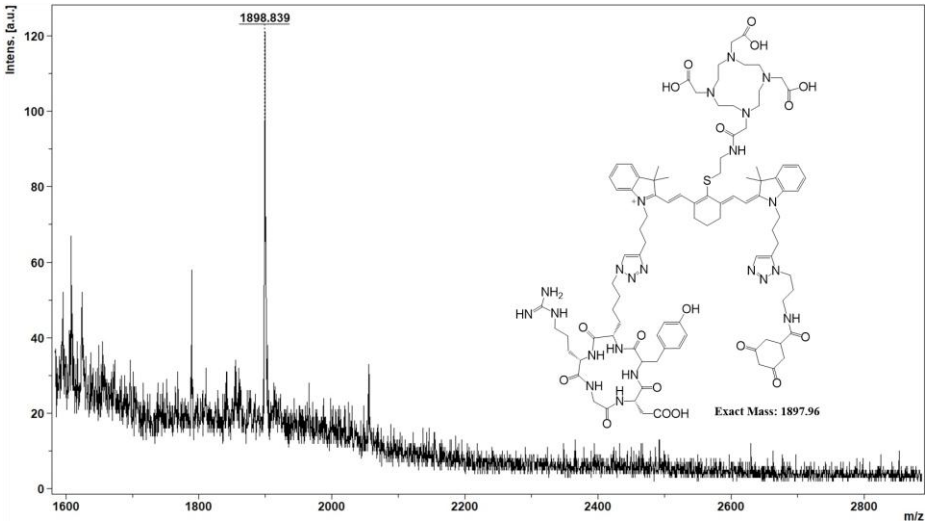

Figure S7. HR-MS spectrum of compound **3**.

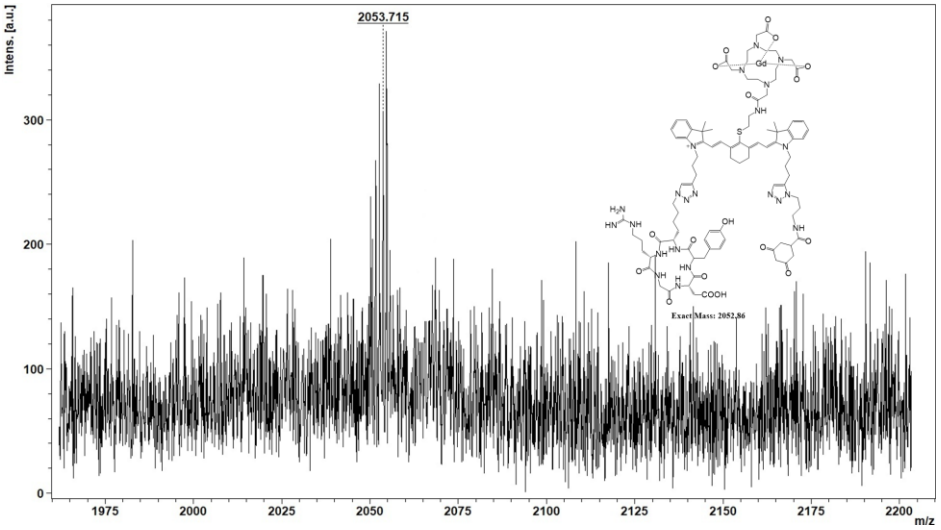

Figure S8. HR-MS spectrum of compound dIR-Gd.

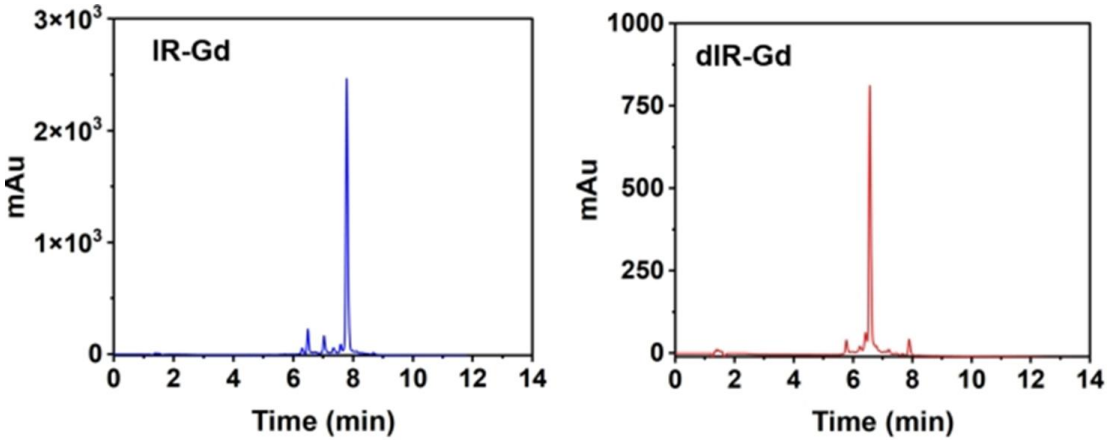

Figure S9. HPLC of compound dIR-Gd and IR-Gd.

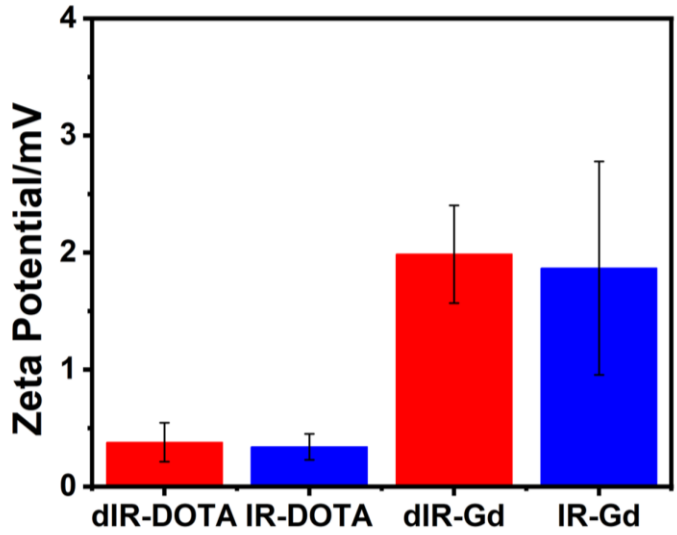

Figure S10. Zeta potential of probe dIR-Gd and IR-Gd.

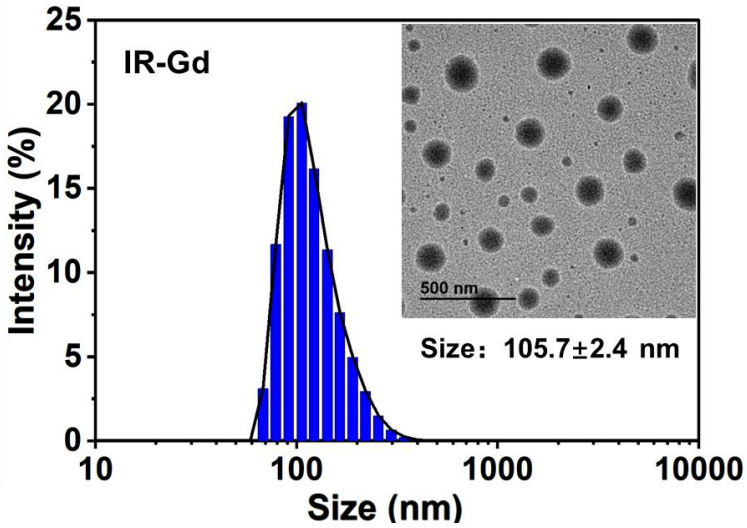

Figure S11. DLS results of IR-Gd. Size distribution profiles of IR-Gd in H_2O. The inset shows the TEM image. Scale bar: 500 nm.

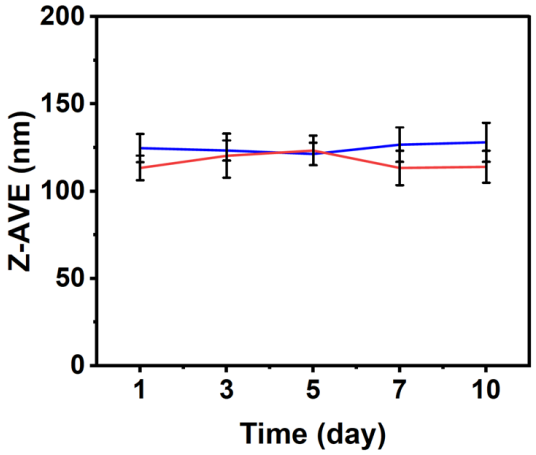

Figure S12. DLS of probe dIR-Gd and IR-Gd in H_2O at different time points (1, 3, 5, 7, and 10 days).

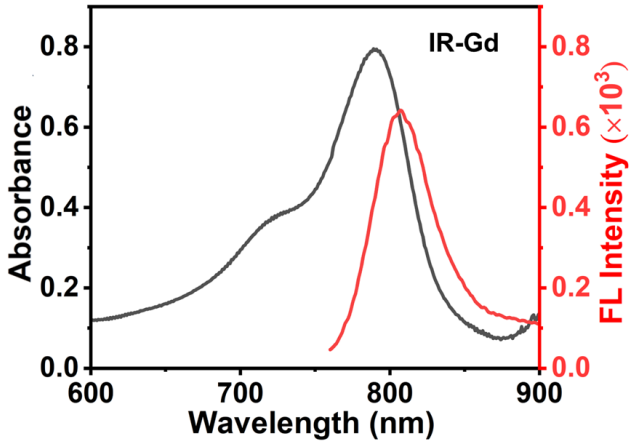

Figure S13. Absorption and fluorescence spectra of IR-Gd (10 μM) in a DMSO/water (5:95 by vol) mixture.

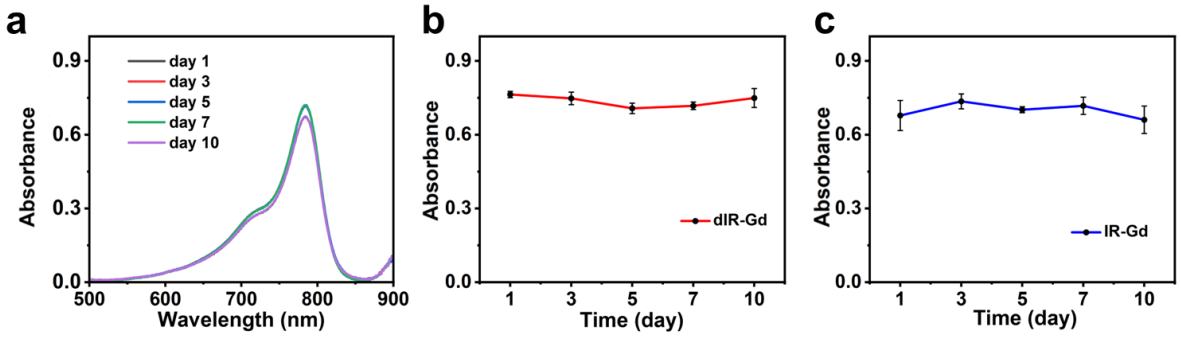

Figure S14. Chemical stability of probe dIR-Gd and IR-Gd. The UV-Vis absorption spectra of IR-Gd (a). The absorbance at 784 nm of dIR-Gd (b) and IR-Gd (c) in a PBS at different time points (1, 3, 5, 7, and 10 days).

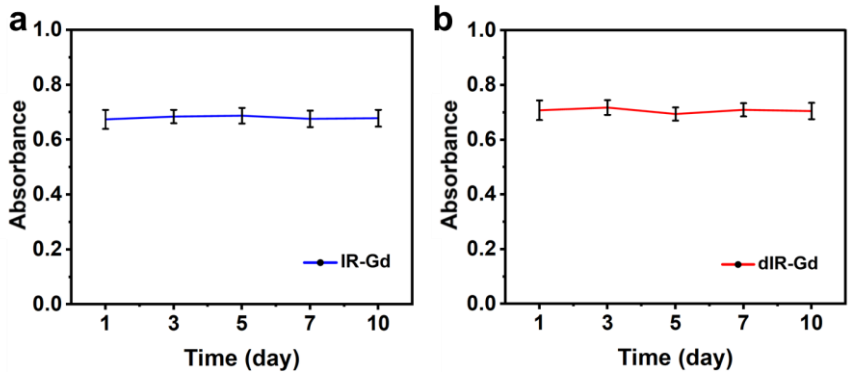

Figure S15. The absorbance at 784 nm of dIR-Gd (b) and IR-Gd (c) in serum at different time points (1, 3, 5, 7, and 10 days).

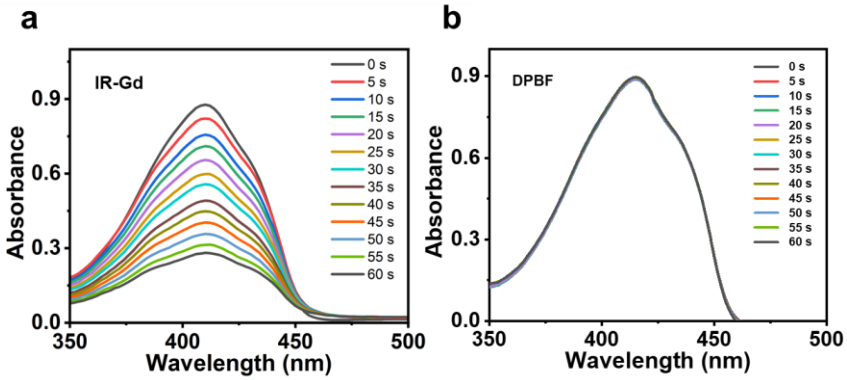

Figure S16. Absorbance change of DPBF with IR-Gd (a) upon 808 nm irradiation for different times, and absorbance change of DPBF upon 808 nm irradiation for different times.

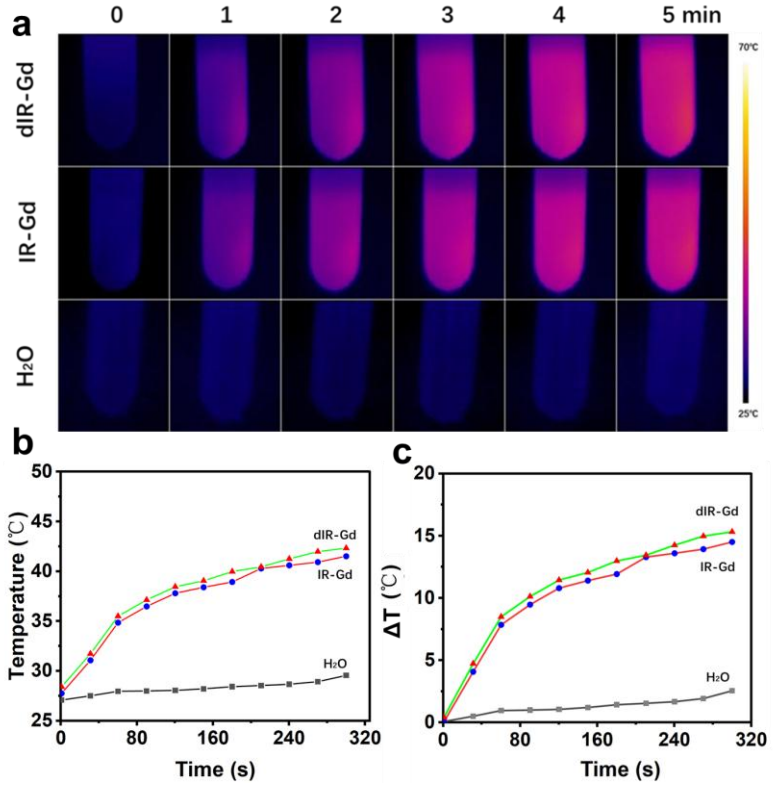

Figure S17. The temperature change of pure water, IR-Gd, and dIR-Gd (10 μM) at different time under 808 nm laser irradiation (500 mW cm^{-2})

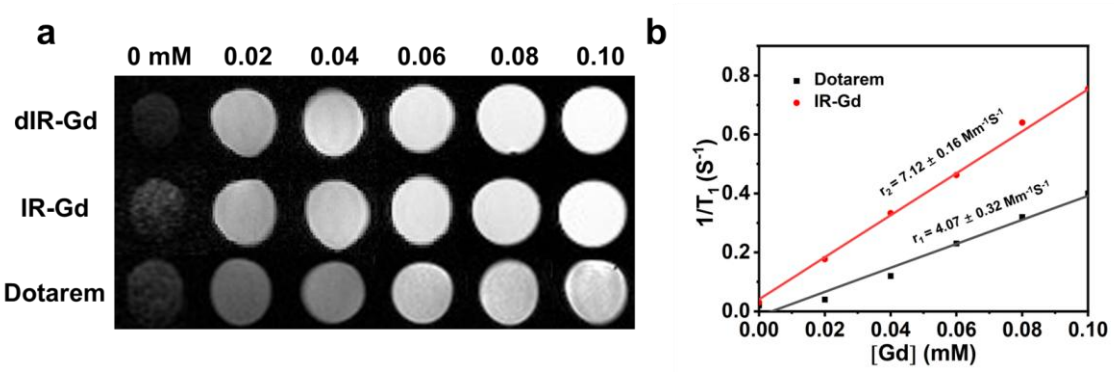

Figure S18. (a) T1-weighted phantom MRI images of IR-Gd, dIR-Gd, and Dotarem at 3.0 T at different Gd(III) concentrations (mM). (b) Determination of r1 relaxivities of IR-Gd at 3.0 T.

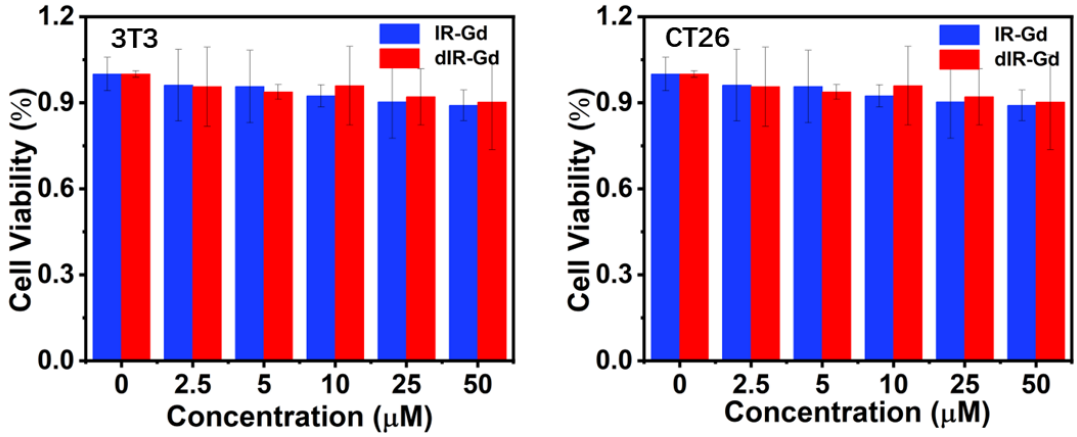

Figure S19. Cell viability of normal cells 3T3 and colorectal cancer cells CT26 after 12 h of treatment with dIR-Gd or IR-Gd at different concentrations ranging from 2.5 to 50 μM.

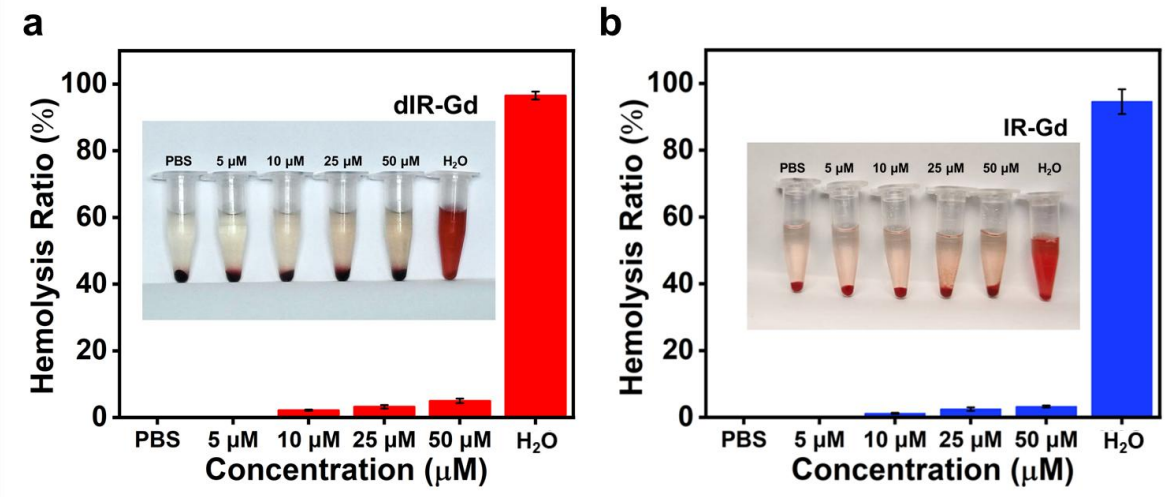

Figure S20. Hemolysis assays of dIR-Gd (a) and IR-Gd (b) at different concentrations ranging from 5 to 50 μM.

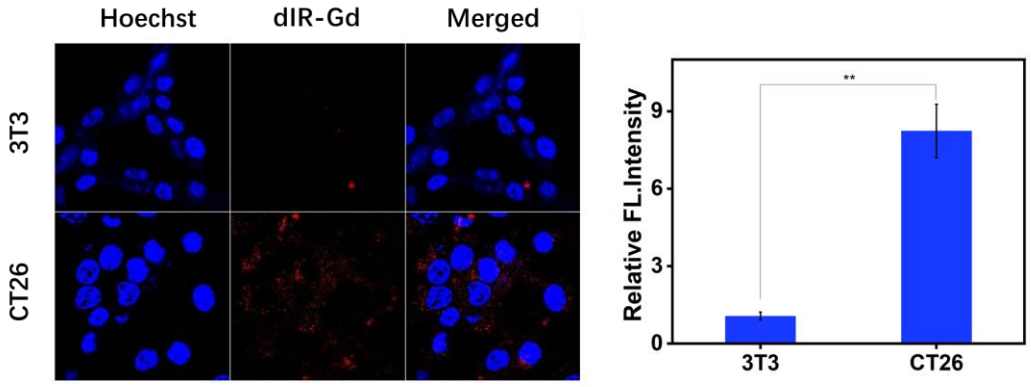

Figure S21. CLSM images of CT26 cells and 3T3 cells treated with dIR-Gd (10 μM). Data were presented as means ± SD. The significance of the data was evaluated according to one-way ANOVA Tukey's multiple comparisons test. **P <0.01.

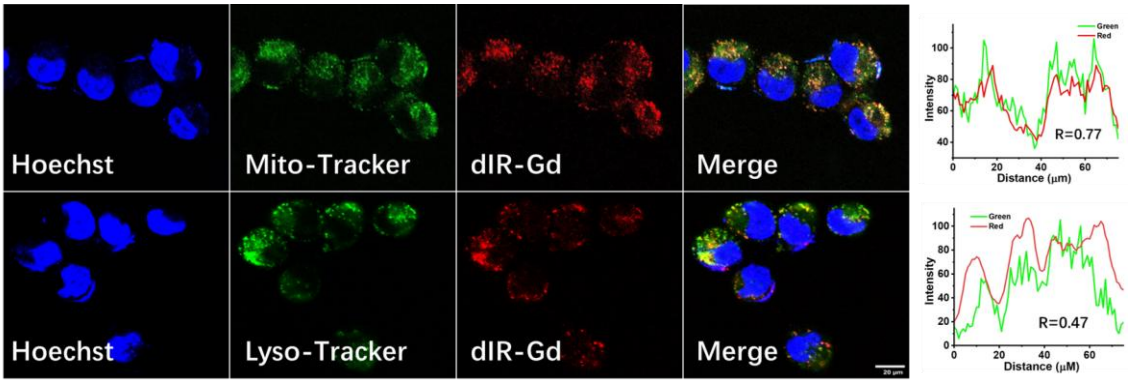

Figure S22. CLSM images of CT26 cells incubated with dIR-Gd (10 μM) for 12 h followed by staining with Mito-tracker green or Lyso-tracker green (scale bar = 20 μm).

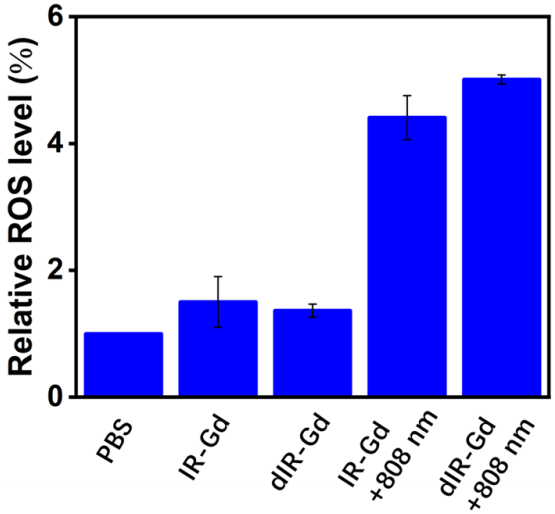

Figure S23. Quantitative analysis of the cellular ROS level of the CT26 cells treated with PBS, IR-Gd, dIR-Gd, IR-Gd+808 nm, and dIR-Gd+808 nm.

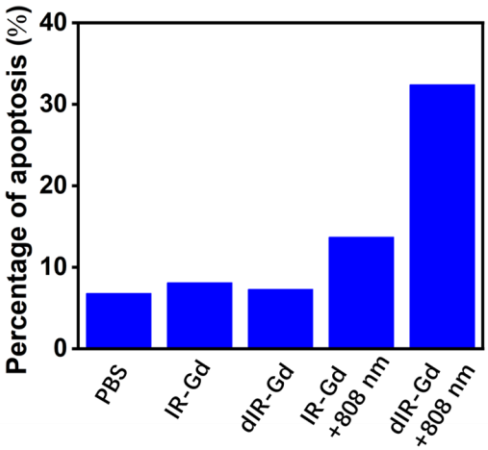

Figure S24. The cell apoptosis analysis of CT26 cells treated with PBS, IR-Gd, dIR-Gd, IR-Gd+808 nm, and dIR-Gd+808 nm.

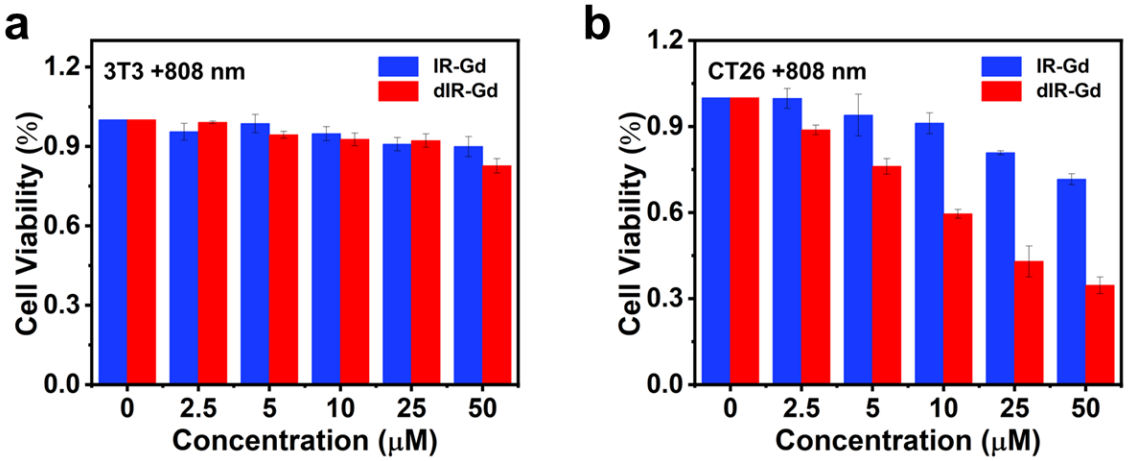

Figure S25. Cell viability of colorectal cancer cells CT26 and normal cells 3T3 treated with dIR-Gd and IR-Gd, respectively, for 12 h followed by three PBS washes to remove excessive probes. Fresh medium was added, and cells were incubated for 12 h.

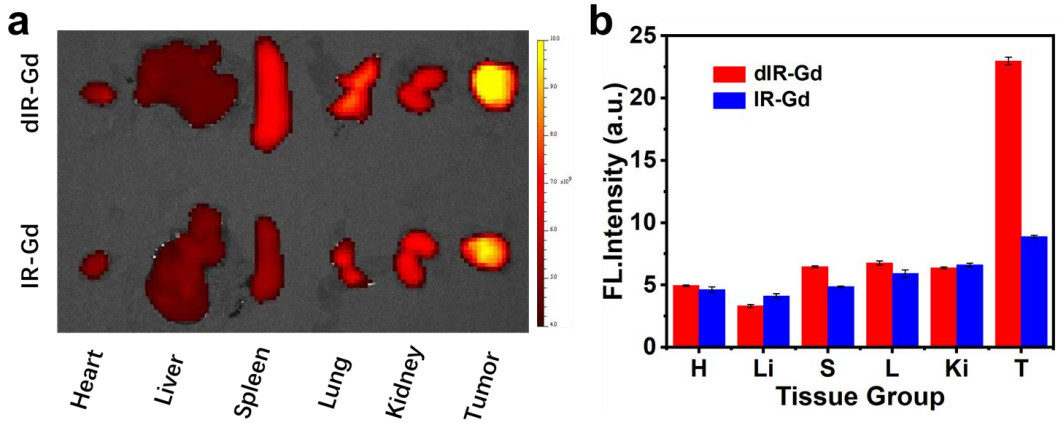

Figure S26. Biodistribution of dIR-Gd and IR-Gd in major organs and tumors at 24 h post-injection. (H: Heart; Li: Liver; S: Spleen; L: Lung; Ki: Kidney; T: Tumor).

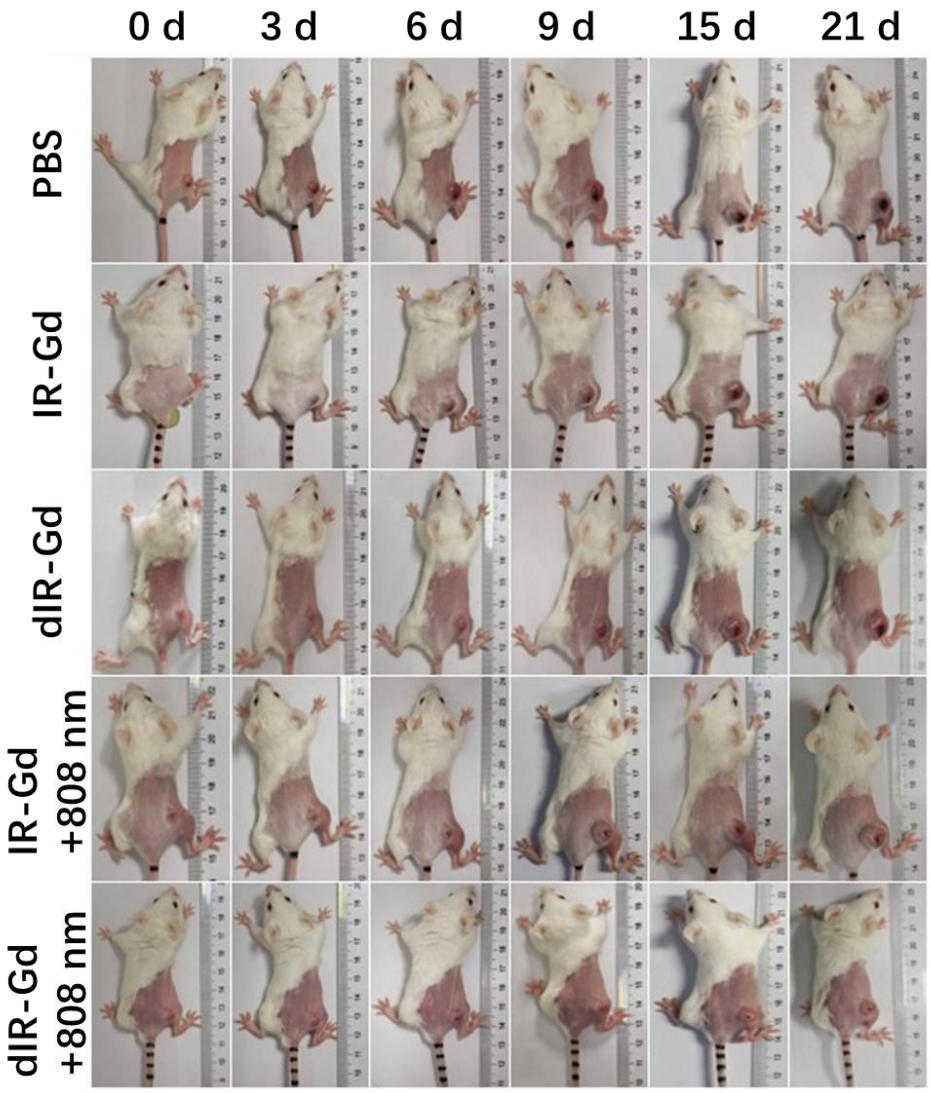

Figure S27. The photographs of the mice with different treatments.

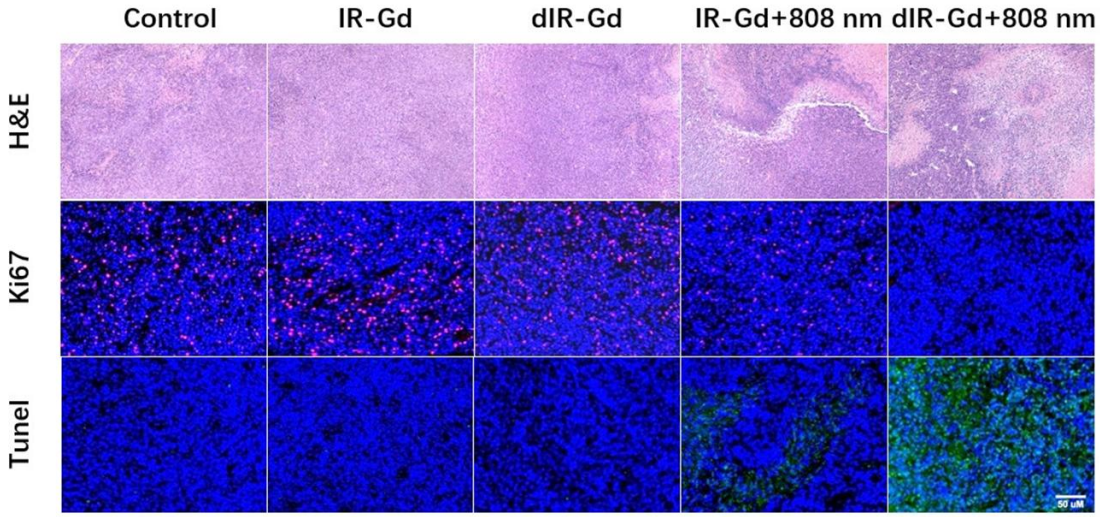

Figure S28. H&E, Ki-67, and TUNEL immunofluorescence staining of tumorous tissues for the mice with different combination treatments.

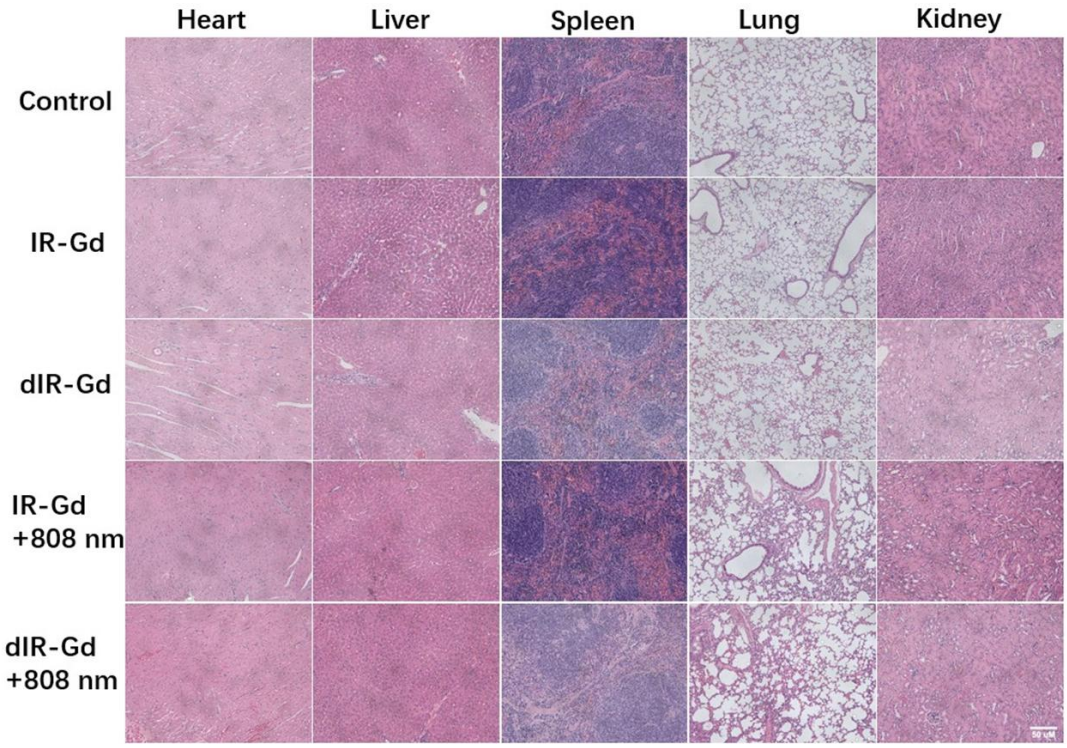

Figure S29. H&E staining of the major organ tissues (heart, liver, spleen, lungs, kidneys) from the mice with different treatments. Organs were harvested on the 21th day after treatment.