Supporting Information

pH-sensitive loaded retinal/indocyanine green micelles as a "all-in-one" theranostic agent for multi-modal imaging *in vivo* guided cellular senescence-photothermal synergistic therapy

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Materials and methods

1.1. Materials

Indocyanine green (ICG), 4, 6-diamidino-2-phenylindole (DAPI), BMS493, and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (USA). Alexa Fluor 488 Annexin V/PI Cell Apoptosis Kit and Calcein-AM/Propidium Iodide (PI) Stained Kit were purchased from Invitrogen (USA). All other chemicals used were analytical reagent grade and used without further purification.

1.2. Preparation of pH-sensitive ICG loaded DR (DRI) micelles

DR conjugates were synthesized using a similar procedure as reported.[1] DRI micelles were obtained by a dialysis method.[2] Both of ICG and DR conjugates were dissolved into anhydrous DMSO at 10 mg/ml. ICG (100 ul) was then added into the DR solution (900 ul) for 3 h at room temperature, and the resulted solution was then dialyzed against distilled water with a dialysis membrane (MW = 3500 Da) for 24 h to obtain ICG/DR (DRI) micelles.

1.3. Characterization of DRI micelles

The particle size and zeta potential of DRI micelles could be measured by dynamic light scattering (DLS) using Nano-ZS ZEN3600 (Malvern) at 25 °C. UV data was determined free ICG, DR, and DRI micelles by using PerkinElmer Lambda UV spectrophotometer. Fluorescence intensity curves of free ICG, DR, and DRI micelles were measured using fluorescence spectrometer (Hitachi). The morphologies of DRI micelles were determined using the FEI Tecnai transmission microscope. To measure ICG loading efficiency of DRI micelles, DRI micelles were dissolved in DMSO and the content of ICG was measured by UV spectrophotometer. The loading efficiency of ICG by DRI micelles was calculated by the following equations:

$$\text{LC}\ (\%) = \left(\frac{weight\ of\ loaded\ ICG}{weight\ of\ DRI}\right) \times 100$$

1.4. Stability

To assess the stability of DRI micelles *in vitro*, the size and zeta potential were measured in different time in various solutions including water, and phosphate buffered saline (PBS), DMEM medium, and DMEM medium containing 10% FBS. Meanwhile, photos of DRI micelles dispersed in various solutions after 4 days.

1.5. *In vitro* drug release

To measure pH-dependent release of retinal, DRI micelles suspension in PBS buffer was transferred in a dialysis bag and immersed in 10-20 ml of PBS buffer (pH = 5.0 6.4, or 7.4). The solution was harvested at different time points, and the equal volume of fresh solution was added. The release content of retinal was calculated by UV spectrophotometer.

1.6. The photothermal properties of DRI micelles

The photothermal properties of DRI micelles were determined by observing temperature changes under laser irradiation. All tested agents including PBS, DR, free ICG, and DRI micelles would be used to irradiate for 6 min under 1.0 W/cm^2 808 nm laser, which temperature changes of all groups were measured using a thermal imaging camera (Fluke). Meanwhile, the temperature changes of different

concentrations of DRI micelles and different laser powers were measured.

1.7. Cell culture

A549 (adenocarcinomic human alveolar basal epithelial cells), MCF7 (human breast cancer cell), and 4T1 (mouse mammary carcinoma) were purchased from the American Type Culture Collection and were cultured in DMEM medium containing 10% FBS without antibiotics and with 1% Glutamine at 37 °C in a 5% CO_2 humidified atmosphere.

1.8. *In vitro* cellular uptake

Flow cytometry would be used to quantitatively assess the cellular uptake. 4T1 cells were cultured in 24-well plates overnight. Then the original medium was removed and new medium with ICG and DRI micelles was added. After 6 h incubation, cells were obtained by trypsin digestion, and tested using flow cytometry.

Confocal laser scanning microscopy (CLSM) could be used to observe the cell internalization and distribution of micelles. 4T1 cells were cultured in 24-well plates overnight. The medium was replaced by the medium containing DRI micelles and free ICG. After 6 h incubation, the cells were washed and fixed with paraformaldehyde (4%), and stained with DAPI before CLSM observation.

1.9. *In vitro* therapy

The cells were cultured in 96-well plates overnight. The medium was replaced with PBS, DR, free ICG, and DRI micelles with different concentrations of ICG. After 24 h, the cells were washed with PBS and fresh medium was added, and then irradiated for 6 min with 1.0 W/cm^2 808 nm laser. Finally, the cell viability could be used to measure by the MTT assay. Meanwhile, cells were stained with calcein-AM/PI stained kit for visualizing live and dead/late apoptotic cells after illumination, according to the manufacturer's instruction, and observed using fluorescence microscope by excluding the interference of ICG fluorescence background.

The cytotoxicity of cellular senescence-photothermal combinational therapy was measured using quantitatively Analyzing cells apoptosis. The cells were cultured in 24-well plates overnight, and treated with DRI micelles with/without BMS493 under laser irradiation. Then cells were obtained by trypsin digestion, and detected using flow cytometer after the Alexa Fluor 488 Annexin V/PI Cell Apoptosis Kit stained. Annexin V-positive and PI-negative cells were considered as apoptotic. Double-stained cells were considered as necrotic/late apoptotic cells.

1.10. Tumor model

BALB/c nude mice (~ 20 g) were cultured in SPF room, four to a cage. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee, Macau University. Tumor models were obtained by injecting 2.5 × 10^6 4T1 cells into the mice by subcutaneous injection. The tumor size could be used to measure with vernier calipers and then tumor volume was calculated using equations $V = [(\text{length}) \times (\text{width})^2]/2$. All experiments *in vivo* were conducted after the tumor size achieved ~ 100 mm^3.

1.11. Multi-modal tumor imaging *in vivo*

Mice were intravenously injected with Free ICG and DRI micelles (n = 3). After all mice were euthanized at different time points (6, 12, and 24 h), acquired fluorescence

images were performed on CRI maestro system, and unmixed by the Maestro software.

Additionally, *in vivo* photoacoustic imaging (PAI) experiments were conducted at 6, 12, and 24 h using the commercial photoacoustic equipment (Endra Nexus 128, MI). The photoacoustic images were conducted with mice keeping in a water system. The power density of laser on the skin surface of mice was about 9 mJ/cm^2 using a 750 nm wavelength.

Meanwhile, photothermal imaging was conducted to evaluate the maximum temperatures of tumor region after laser irradiation. Then the temperature changes and thermographic maps of tumor were determined at the 6 h post-injection by a thermal imaging camera when the tumor was irradiated for 6 min under 1.0 W/cm^2 808 nm laser.

1.12. *In vivo* combinational therapy

To evaluate the efficiency of cellular senescence-photothermal combinational therapy, mice containing tumor were randomly divided into five groups. The treatment schemeis as follows: (1) PBS; (2) free ICG; (3) DR; (4) DR plus ICG mixed solution (DR/ICG); (5) DRI micelles. All experiment groups were intravenously injected the concentrations of ICG were 2 mg/kg and irradiated for 6 min under 1.0 W/cm^2 808 nm laser. The mice weight and tumor volume were monitored every 3 days. According to the animal protocol, tumor volume reaching 1400 mm^3 were euthanized. Additionally, to evaluate the efficiency of cellular senescence-photothermal synergistic treatment *in vivo*, the tumor was obtained and sectioned into 10 μm slices to stain by hematoxylin and eosin (H&E) after therapy.

1.13. Biosafety evaluation

Mice bearing tumor were intravenously injected with PBS, free ICG, DR, DR/ICG, and DRI micelles. Serum levels of aspartate aminotransferase (AST) and alanine aminotranferease (ALT) were measured after post-injection by commercial kits. Meanwhile, mice were euthanized and main organs were obtained, and fixed with paraformaldehyde (4%) and stained by H&E before fluorescence microscope observation. Additionally, to evaluate the blood biocompatibility of the DRI micelles, fresh blood was centrifuged and then diluted with PBS. The red blood cells were mixed with DRI micelles at different concentrations and incubated at 37 °C for 4 h, and then centrifuged. The release of hemoglobin was determined at 540 nm by a microplate reader. Red blood cells with PBS treatment and Triton-X were considered as the negative control and positive control, respectively. Percentage of hemolysis was calculated using the following equations:

$$\text{Hemolytic (\%)} = \frac{(absorbance\ for\ polyplexes\ treatment - absorb\square ance\ for}{(absorbance\ for\ Triton - X\ treatment - absorbance\ for} \times 100$$

1.14. Statistical analysis

Statistical analysis was conducted by the Student's t-test, and differences were set at $p < 0.05$ (*$p < 0.05$, **$p < 0.01$).

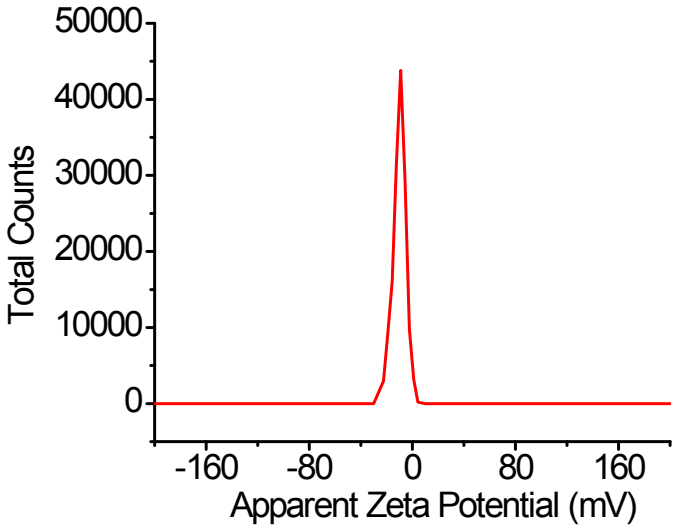

Fig. S1 Zeta potential of DRI micelles by DLS measurement.

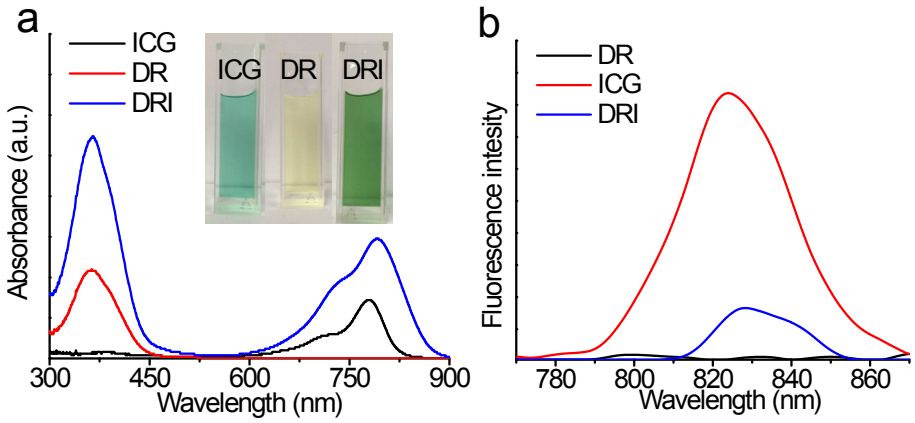

Fig. S2 Vis-NIR absorption (a) and fluorescence (b) spectrum of DRI micelles.

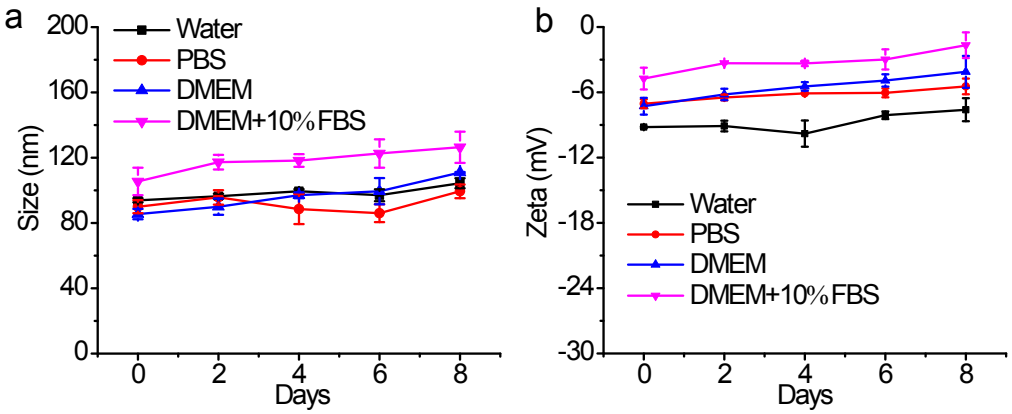

Fig. S3 The size (a) and zeta potential (b) of DRI micelles in Water, PBS, DMEM, and DMEM+10% FBS for 8 days.

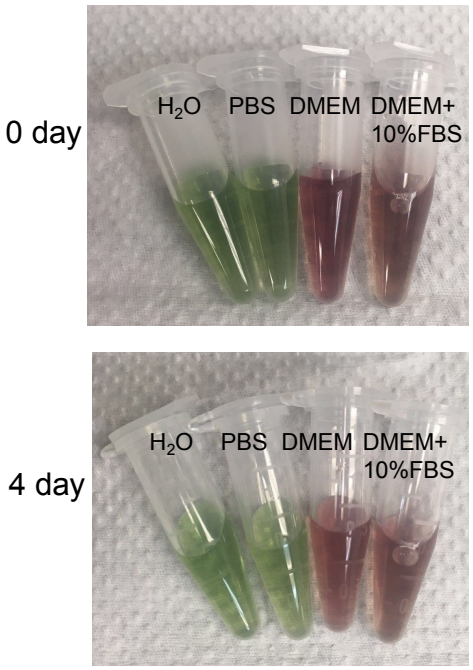

Fig. S4 A photo of the DRI micelles in various solutions: water, PBS, DMEM medium, and DMEM medium containing 10% FBS at 0 and 4 days.

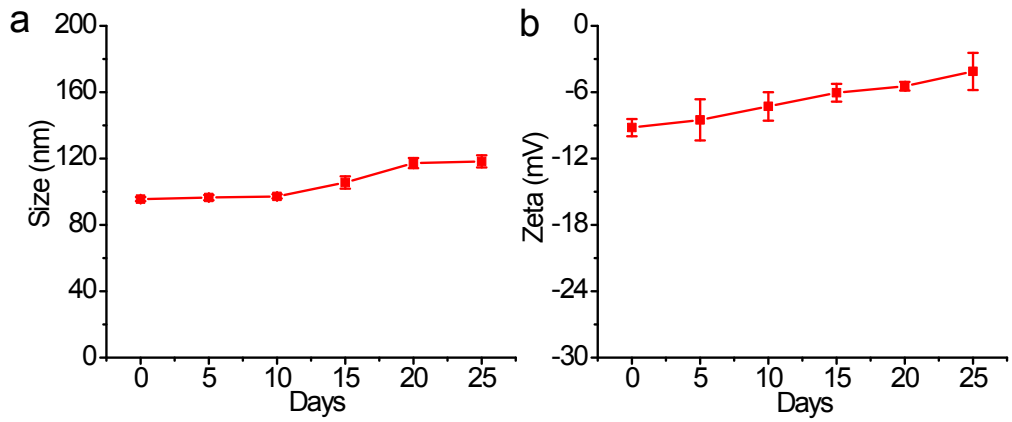

Fig. S5 Stability test of particles size (a) and zeta potential (b) in water for 25 days.

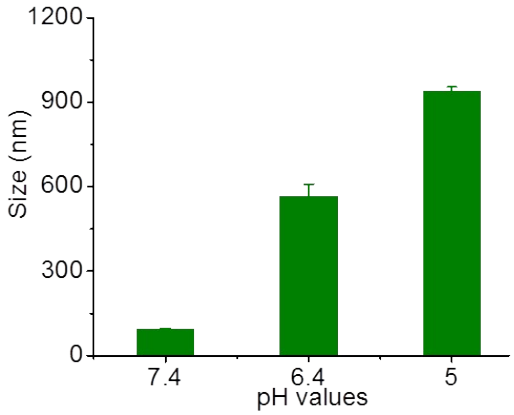

Fig. S6 Size of DRI micelles in different pH conditions.

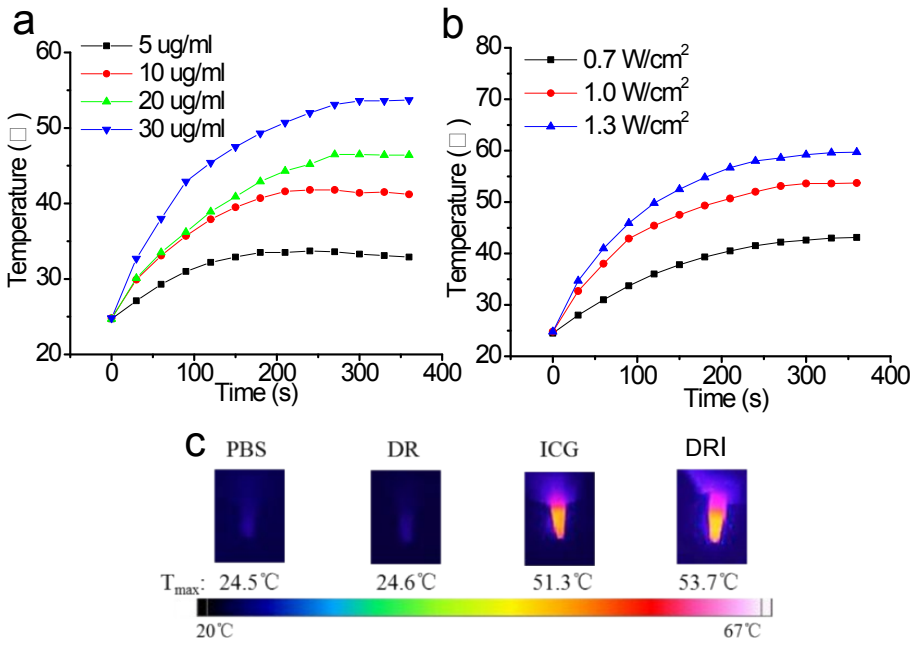

Fig. S7 (a) The temperature curves of the different concentrations of the DRI micelles (5, 10, 20, and 30 μg/ml) under laser irradiation. (b) The temperature curves of DRI micelles different laser power intensities (0.7, 1.0, and 1.3 W/cm^2). (c) Infrared thermographic maps of centrifuge tubes with PBS, ICG, DR, and DRI micelles were measured at 6 min with an infrared thermal imaging camera.

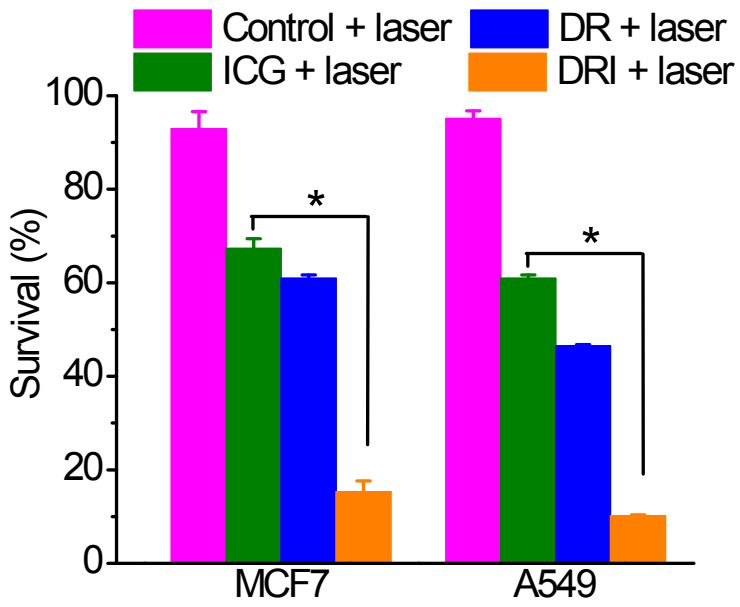

Fig. S8 Quantitative detection of MCF7 and A549 cells viability after different treatments (n = 4).

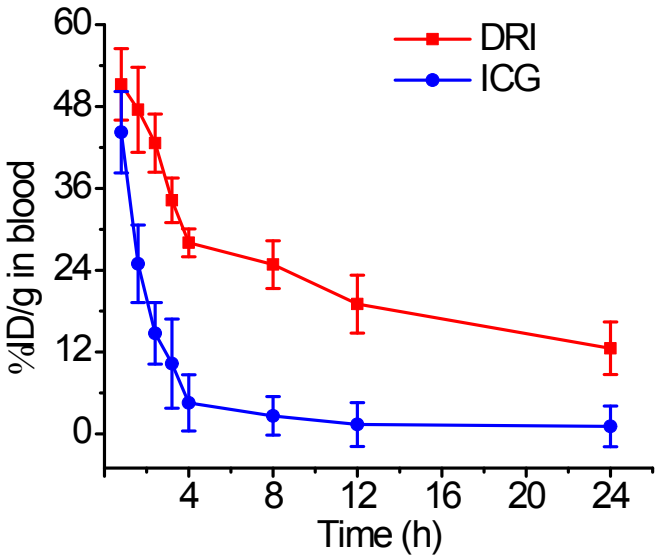

Fig. S9 Blood circulation curves of free ICG and DRI micelles in mice after intravenous injection determined based on ICG fluorescence in the blood lysates (n = 3).

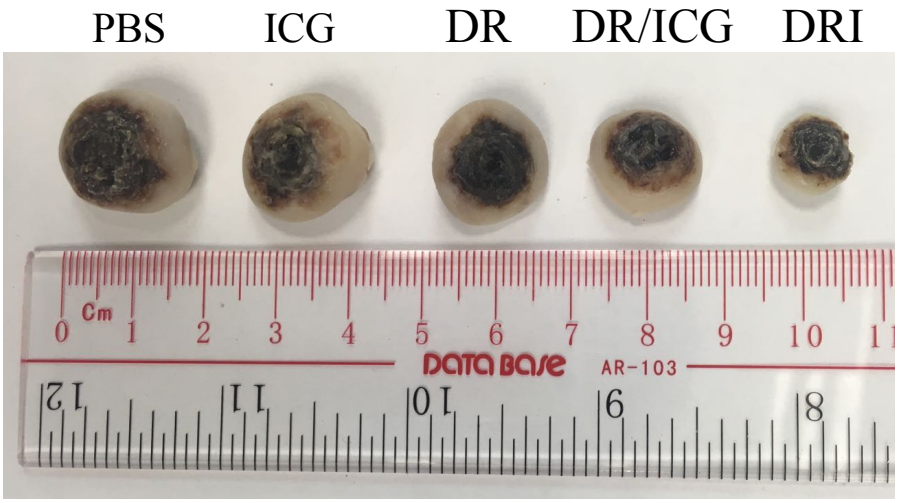

Fig. S10 The digital photographs of tumors collected from different groups of mice at the end of various treatments.

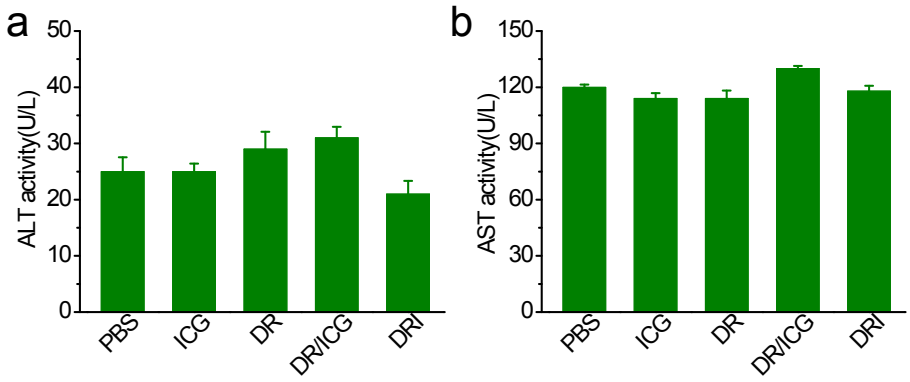

Fig. S11 Serum levels of ALT (a) and AST (b) were measured using commercial assay kits after injection (n = 3).

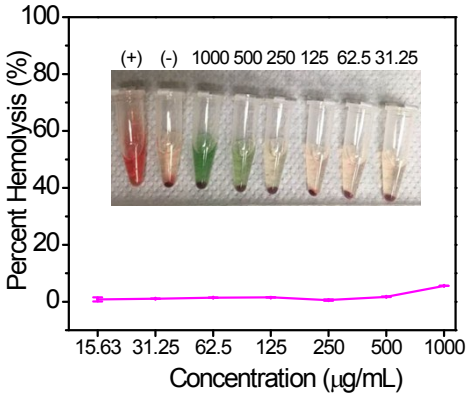

Fig. S12 Hemolytic percent of red blood cells incubated with DRI micelles at various concentrations (n = 4).

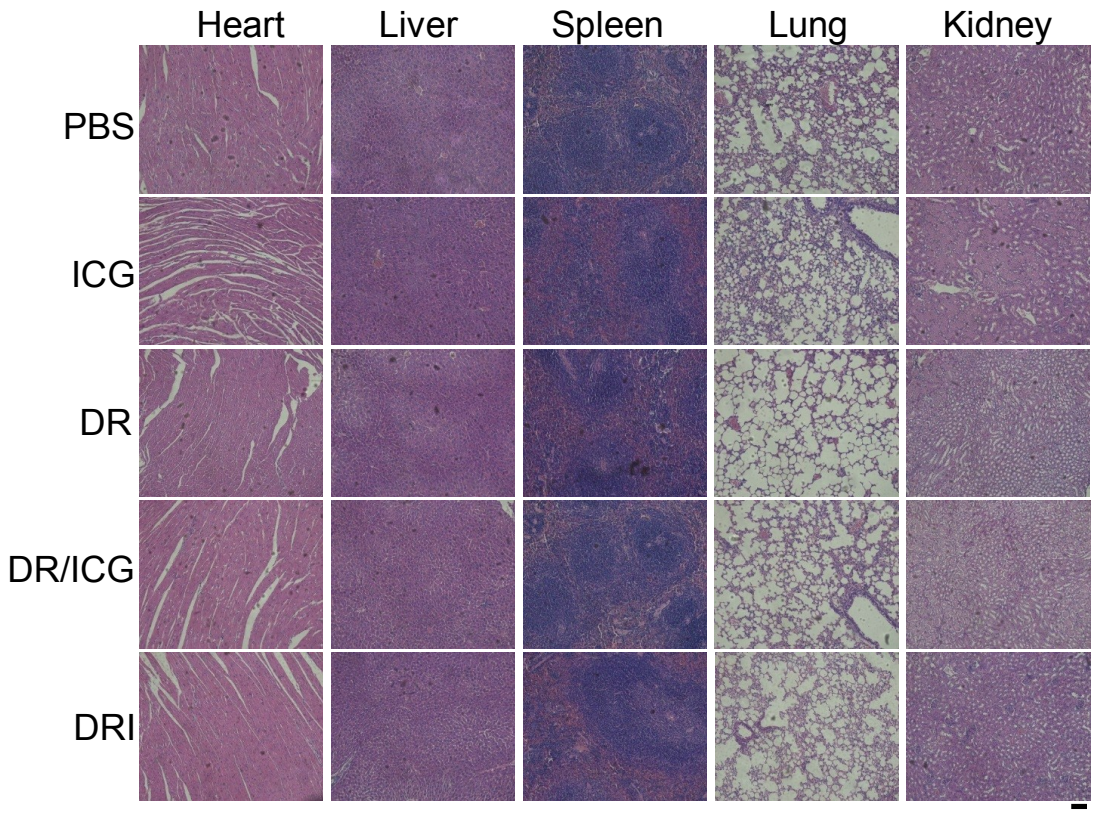

Fig. S13 H&E staining images of major organs including heart, liver, spleen, lung and kidney after the mice were sacrificed post intravenous injection with PBS, ICG, DR, DR/ICG and DRI micelles. Bar represents 50 μm.

References

1 Y. Zhang, P. Li, H. Pan, L. Liu, M. Ji, N. Sheng, C. Wang, L. Cai, Y. Ma, *Biomaterials,* 2016, *83*, 219-232.

2 Q. Xiong, M. Zhang, Z. Zhang, W. Shen, L. Liu, Q. Zhang, *Int. J. Pharm.,* 2014, *474*, 232-240.