Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.
This journal is © The Royal Society of Chemistry 2021

Supporting Information

A novel self-targeting theranostic nanoplatform for photoacoustic imaging-monitored and enhanced chemo-sonodynamic therapy

Yifan Yang[1‡], Zhongxiong Fan[1‡], Kaili Zheng[2], Dao Shi[1], Guanghao Su[3], Qingliang Zhao[2]*, Xu Fu[4]*, Zhenqing Hou[1]*

[1] *Department of Biomaterials, College of Materials, Research Center of Biomedical Engineering of Xiamen & Key Laboratory of Biomedical Engineering of Fujian Province & Fujian Provincial Key Laboratory for Soft Functional Materials Research, Xiamen University, Xiamen 361005, China.*

[2] *State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics & Center for Molecular Imaging and Translational Medicine, School of Public Health, Xiamen University, Xiamen 361102, China*

[3] *Children's Hospital of Soochow University, Suzhou 215025, China*

[4] *Lanzhou University Second Hospital, Lanzhou 730000, China*

***Corresponding authors**

E-mail addresses: zhaoql@xmu.edu.cn (Q. Zhao), ery_fux@lzu.edu.cn (X. Fu), houzhenqing@xmu.edu.cn (Z. Hou)

‡Y. Yang and Z. Fan contributed equally to this work.

1. Experimental section

1.1 Materials and reagents

D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS), carbodiimide hydrochloride (EDC·HCl) and 4-dimethylaminopyridine (DMAP) were purchased from Shanghai Macklin Biochemical Co, Ltd. (Shanghai, China). Pemetrexed（PEM）and folic acid (FA) were obtained from Abmole Bioscience Inc. Indocyanine green (ICG) and 2, 7-dichlorofluorescein diacetate (DCFH-DA) were provided from Sigma-Aldrich (USA). Pyrene, Cell Counting Kit-8 (CCK-8), Hoechst 33342, calcein-acetoxymethyl ester (calcein AM) and propidium iodide (PI) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Dimethylsulfoxide (DMSO) was obtained from Sinopharm Chemical Reagent Co, Ltd (Shanghai, China). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), trypsin/ethylenediamine tetra-acetic acid (EDTA) and penicillin/streptomycin were gained from HyClone (USA). Singlet oxygen sensor green (SOSG) was purchased from Thermo Fisher Scientific Inc. HeLa, A549 and L02 cells were supplied by American Type Culture Collection (ATCC). BALB/c 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 Synthesis of TPGS-PEM prodrug

The targeting TPGS-PEM prodrug was synthesized through a classical esterification reaction. In brief, 1.0 mmol of PEM, 1.2 mmol of EDC·HCl, 1.2 of mmol DMAP and 1.0 mmol of TPGS were dissolved in 1 mL of DMSO, respectively. Next, the mixture solution of 1.0 mmol of PEM and 1.2 mmol of EDC·HCl were stirred under N_2 protection for 2 h to activate the carboxyl group of PEM. Subsequently, DMAP and TPGS were added into the above solution. After continuous stirring for 48 h, the crude production was dialyzed in deionized (DI) water by a membrane (molecular weight cutoff (MWCO) = 1000 Da) to remove the excess PEM, TPGS, EDC·HCl, DMAP and DMSO. Finally, the purified TPGS-PEM was lyophilized and then stored at 4℃ for further exploitaion. The yield of TPGS-PEM prodrug was calculated in accordance with the following equation: the content of TPGS-PEM prodrug (%) = (the weight of purified TPGS-PEM prodrug after lyophilization)/(the weight of all the raw materials) × 100 %.

1.3 Fabrication of TPI

To fabrication the TPGS-PEM-ICG nanoplatform (TPI), 5 mg of synthesized TPGS-PEM prodrug and 1 mg of ICG were dissolved in 1 mL of DMSO, respectively. Then, the solution of TPGS-PEM prodrug and ICG were gradually dropwise added into the 2 mL of DI water and stirred for 10 min in the dark surrounding. After stirring 4 h and

maintaining for 2 h, the products were dialyzed in DI water to remove the organic solvent DMSO to obtain TPI. The synthetic process of TPGS-ICG was the same as that of TPI, except that TPGS-PEM prodrug was replaced by TPGS.

1.4 Characterization of TPGS-PEM prodrug and TPI

The morphology of TPGS-PEM and TPI were observed by using transmission electron microscopy (TEM, JEM 2100, JEOL, Tokyo, Japan). Hydrodynamic diameter, zeta potential and hydrodynamic diameter (PDI) were measured by dynamic light scattering and electrophoretic light scattering by the Malvern Nano-ZS (U. K.). The element mapping of TPI was conducted on a high-resolution transmission electron microscope (HRTEM, FEI, Talos 200s). Fourier transform infrared spectra (FT-IR) were performed on the Avatar 370 infrared spectrometer (Nicolet, USA) and ^{1}H nuclear magnetic resonance (^{1}H NMR) spectra were demonstrated by Bruker AVANCE III 400 MHz NMR spectrometer. The molecular weights of the PEM, TPGS, and TPGS-PEM were detected by electrospray ionization mass spectra (ESI-MS, Bruker Esquire 3000 plus spectrometer).

1.6 Measurement of the critical micellar concentration (CMC)

To measure the CMC of TPI, the pyrene dissolved in acetone was used as a fluorescence probe. In brief, 6.0×10^{-4} mg/mL of pyrene acetone solution was formulated and subsequently TPI was diluted to

different concentration from 0.5 × 10^{-4} to 0.5 mg/mL. After that, 20 μL of the pyrene solution was injected dropwise into TPI of a series of concentrations. Next, the resulting solution was placed on the fuming cupboard and left overnight at room temperature to volatilize the acetone. The emission spectra of all samples were determined using a FluoroMax-4 Spectrofluorometer (HORIBA Jobin Yvon, USA) at 335 nm excitation wavelength. The change in the fluorescence intensity ratio (I_{374} /I_{391}) of pyrene versus TPI concentration was plotted from the emission spectra. Subsequently, the CMC value of TPI was calculated as the intersecting point of two lines obtained by linear regression.

1.7 Drug loading content and entrapment efficiency

ICG loading content (LC%) and entrapment efficiency (EE%) of TPI could also be determined using an ultraviolet-visible (UV-vis) spectrophotometer (Perkin-Elmer, Norwalk CT) at 399.5 nm. The LC% and EE% of TPI were calculated according to the following formulas:

$$LC\% = \frac{weight\ of\ ICG\ in\ TPI}{weight\ of\ TPI} \times 100\%$$

$$EE\% = \frac{weight\ of\ ICG\ in\ TPI}{threoretical\ weight\ of\ ICG\ in\ TPI} \times 100\%$$

1.8 *In vitro* physical stability

To evaluate the *in vitro* physical stability of TPI, DI water, PBS and serum were employed as the physiological media. In brief, the *in vitro*

physical stability of TPI was determined in DI water, PBS (pH 7.4), DMEM, DMEM containing 10 of FBS and plasma. The physical stability of TPI in different physiological media for predesigned incubation time points was evaluated *via* determining the hydrodynamic diameter, morphology using DLS. In addition, we employed UV-vis-NIR spectrophotometer and spectrofluorometer to assess fluorescence stability of TPI and free ICG under continuous 5 min exposed to ultrasound (US) (1MHz, 1W/cm^2) irradiation at various times.

1.9 *In vitro* disassembly and drug release

The *in vitro* release profile of ICG from TPI was performed by dialysis using a membrane (MWCO = 1000 Da). In brief, 4 mL of TPI with the same concentration was dialyzed within 50 mL of PBS under different pH conditions (7.4, 6.5 and 5.5) with/without 10 mM of esterase and with/without US irradiation at 37°C under shaking at 100 rpm. Periodically, 4 mL of aliquots were collected and replaced with 4 mL of different pH of PBS. Amount of the released ICG was determined using a UV-Vis spectrophotometer. In addition, the morphology and hydrodynamic diameter changes of TPI at different conditions were analyzed by DLS and TEM.

1.10 *In vitro* $^1O_2$ generation

The generation of $^1O_2$ was measured by a commercial assay kit based on SOSG. ICG, TPGS-ICG and TPI containing different

concentrations of ICG (5, 10, 15, 20, 25 and 30 μg/mL) were mixed with 10 μM of SOSG, respectively. After irradiation with US for 5 min, the fluorescence intensity of these samples was detected by fluorescence spectrometer at excitation wavelength of 488 nm.

1.11 Cell culture

HeLa and 4T1 cells were selected for the following experiments due to their high-levels of folate receptor expression. A549 cells were chosen as a comparison due to their low expression of folate receptor. L02 cells were chosen as a blank control. Cells were cultured in DMEM medium containing 10% FBS and 1% penicillin/streptomycin within a water-jacketed CO_2 incubator (Thermo Fisher Scientific Inc., USA) with 5% CO_2 at 37^{o}C.

1.12 *In vitro* cellular uptake

Cellular uptake was evaluated by HeLa cells, A549 cells and L02 cells. For confocal laser scanning microscopy (CLSM), cells were seeded into 6-well plates at a density of 5.0×10^5 cells/well and incubated at 37°C for 24 h. Then, ICG, TPGS-ICG and TPI at equivalent ICG concentration (10 μg/mL) were added to each well. After the designed incubation time, cells were washed thrice with PBS, stained with Hoechst 33258 for 15 min, and then fixed with 4 % paraformaldehyde for 20 min. Finally, CLSM (Leica TCS SP8, USA) was applied to observe cellular uptake. For the flow cytometry, three kinds of cell lines were seeded in 6-

well plates with 2 × 10^5 cells per well, and cultured for 24 h until fully attached. Cells were then treated with ICG, TPGS-ICG and TPI at an equal ICG concentration (10 μg/mL) in accordance with the predesigned incubation time intervals at 37°C. After that, cells were harvested using trypsin-EDTA, then washed and suspended in PBS with 10% FBS, centrifuged at 2000 rpm for 5 min at 4°C, and resuspended in fluorescence-activated cell sorting (FACS) buffer. The cellular fluorescence intensity of ICG was investigated by a FACSCalibur flow cytometer (Becton Dickinson, USA) and subsequently, the final results were analyzed with Cell Quest software. To investigate the targeting mechanisms of TPI, competitive inhibition experiments were performed. Hela cells were seeded into 6-well plates at a density of 5.0 × 10^5 cells/well and incubated at 37°C for 24 h. And then cells were pretreated with or without free FA (200 μM) for 0.5 h. After that, they were then treated with TPGS-PEM/ICG and TPI at the same ICG concentration (5 μg/mL) for 3 h. After the predetermined time, the fluorescence intensity of cells was measured by flow cytometer and CLSM.

1.13 Detection of intracellular ROS

DCFH-DA could be enzymatically digested and converted into DCFH by cells. The DCFH would be oxidized into strong fluorescent DCF as the concentration of ROS increased. Firstly, HeLa cells were seeded in 6-well plates with 2 × 10^5 cells per well and cultured for 24 h.

Next, cells were washed with PBS and incubated with ICG, TPGS-ICG or TPI at the equivalent concentration of ICG (5 μg/mL) for 6 h. Subsequently, cells were incubated with DCFH-DA (10 μM) for another 30 min. At last, cells were washed for three times and exposed to US for 5 min, and the ability of producing $^1O_2$ was detected by CLSM and FACSCalibur flow cytometer.

1.14 *In vitro* cytotoxicity

A CCK-8 assay was conducted according to the manufacturer's suggested procedure to quantitatively assess the therapy efficiency. In brief, Hela and 4T1 cells were seeded in 96-well plates at a density of 5.0 × 10^3 cells per well and then cultured for 24 h. The cell culture medium was removed and then replaced with 200 μL of fresh medium containing ICG, TPGS-ICG and TPI at the equivalent ICG concentration. After incubation for 8 h, cells were irradiated with US for 5 min. Control cells were identically treated but not received US irradiation. Finally, the cell viability was measured *via* CCK-8 in accordance with the manufacturer's instructions. The absorbance was measured with a microplate reader (Bio Tek ELX800, USA). Moreover, the cell viability of cells treated with PEM, TPGS-PEM at the equivalent PEM concentrations were measured using the same method as well.

Meanwhile, live/dead cell staining assays were conducted to visually reveal the *in vitro* antitumor activity by Calcein AM/PI costaining assay.

Briefly, Hela cells were cultured by ICG, PEM, TPGS-ICG, TPGS-PEM and TPI within 6-well plates (5×10^5 cells per well) for 8 h. After that, these cells were irradiated with/without US for 5 min and incubated for another 16 h. At last, cells were stained using a live/dead staining kit in accordance with the manufacturer's instructions and subsequently observed with a fluorescence microscope (Leica Microsystems, Germany).

1.15 *In vitro* apoptosis assay

Cellular apoptosis was quantified by flow cytometry to reflect the apoptosis-inducing ability of TPI. The steps were similar as the CCK-8 experiments that Hela cells were seeded into 12-well plates with 2×10^5 cells per well and cultured for 24 h until all cells were fully attached. Next, cells were treated with ICG, PEM, TPGS-ICG, TPGS-PEM and TPI for 8 h, then applied for US irradiation for 5 min. After incubation for 24 h, cells were stained with Annexin V-FITC and PI complied with the manufacturer's suggested procedures. Finally, cell apoptosis was determined by FACSCalibur flow cytometer and analyzed by CellQuest/FlowJo (Tree Star, USA) software.

1.16 Animals

Study protocols involving animals were ratified by the Institutional Animal Care and Use Committee of Xiamen University. BALB/c mice (5

weeks, female, 18-20 g) came from the Experimental Animal Laboratory of Cancer Research Center of Xiamen University.

1.17 *In vivo* imaging

HeLa cells (5×10^6 cells in 100 μL of PBS) were subcutaneously injected into the right leg of each mouse to establish the tumor-bearing mouse model. When the tumor volume reached *ca.* 80 mm^3, 200 μL of ICG, TPGS-ICG and TPI at the same ICG concentration (20 μg/mL) were intravenously injected into the Hela tumor-bearing nude mice. After tail vein injection for 2, 4, 6, 12, 24 h *in vivo,* fluorescence images were acquired using the IVIS Lumina II System (Caliper Life Sciences, USA) and then semiquantitatively analyzed with Living Image Software. After that, the tumor-bearing mice were euthanized and primary organs (heart, liver, spleen, lung and kidney) and tumor tissues were reaped for *ex vivo* fluorescence imaging.

In vivo PA imaging studies were also performed on Hela tumor-bearing mice. The previous steps were the same as the fluorescence imaging studies that free ICG, TPGS-ICG and TPI (at an ICG-equivalent dosage of 20 μg/mL) were separately administered into the tumor-bearing mice *via* intravenous injection. Subsequently, the PA signals of tumor tissues were collected and analyzed at 1, 2, 4, 6, 12, 24 h using the Vevo 2100 LAZR system (FUJIFILM Visual Sonics, Toronto, Canada) and the Vevo LAZR software.

1.18 *In vivo* antitumor activity

Hela tumor-bearing mice were selected for experiments. When the tumors grew until reaching approximately 80 mm^3, the mice were randomly divided into eight groups (n = 6 per group): (a) PBS-treated group; (b) ICG-treated group; (c) ICG + US-treated group; (d) PEM-treated group; (e) TPGS-ICG + US-treated group; (f) TPGS-PEM-treated group; (g) TPI-treated group and (h) TPI + US-treated group. Then, the mice were treated intravenously with 200 μL of PBS, ICG, PEM, TPGS-PEM, TPGS-ICG, and TPI at an equal ICG dose (6 mg/kg) every 5 d. Subsequently, the tumor volume and body weight were recorded every second day for 14 d. The following formula was applied to calculate the tumor volume: $V = (\text{tumor length} \times \text{tumor width}^2)/2$.

After treatment for 14 d, mice were euthanized to perform the histological analysis. Briefly, the major organs (heart, liver, spleen, lung and kidney) and tumors were harvested and fixed in 4% paraformaldehyde. Afterwards, they were embedded in paraffin, cut into 8 mm slices and stained with hematoxylin and eosin (H&E). Finally, the tissue sections were observed with the optical microscope (DM5500B, Leica Microsystems, Germany).

1.20 *In vitro* hemolysis analysis

The blood was collected from retro orbital choroid plexus of rats and added in 0.1% heparin sodium. Red blood cells were obtained by

centrifugation at 3000 rpm for 10 min and washed thrice using PBS (pH 7.4). After that, ICG, PEM, TPGS-ICG, TPGS-PEM and TPI were added into the 2 % red blood cells, serving as the experimental groups. PBS and ultrapure water were prepared as negative and positive control, respectively. After incubation at 37^{o}C for 24 h, the samples were centrifuged to gain supernatant and the absorbance values of the supernatant were measured by microplate reader at 541 nm. The hemolysis percent of red blood cells was calculated in accordance with the following equation: hemolysis percentage (%) = $(A_{sample} - A_{PBS})/(A_{water} - A_{PBS}) \times 100$ %. In the above-mentioned equation, A_{sample}, A_{PBS}, and A_{water} are the 541 nm absorbance values of the experimental groups, the negative (PBS) and positive (ultrapure water) group, respectively.

1.21 Statistical analysis

The quantitative data were expressed as mean ± standard deviation (SD) values. Student's t-test was employed to compare two groups and one-way ANOVA analysis was applied to compare multiple groups. Statistical significance was set at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.005$.

2. Supplementary data

			Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm):	136.6	Peak 1:	157.6	100.0	61.31
PdI:	0.122	Peak 2:	0.000	0.0	0.000
Intercept:	0.954	Peak 3:	0.000	0.0	0.000
Result quality :	Good				

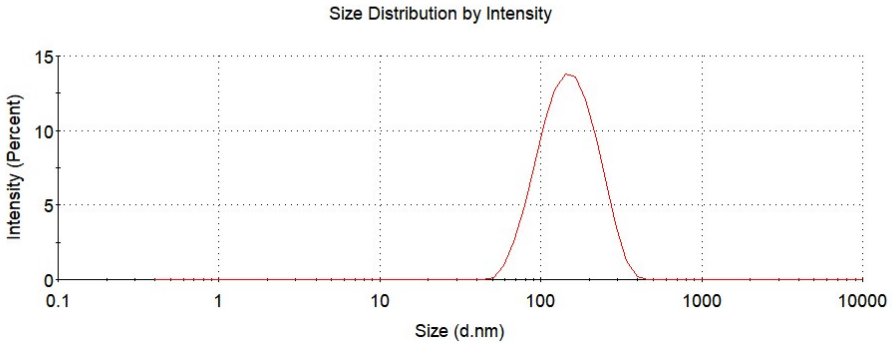

Figure S1. PDI of TPI dispersed within DI water.

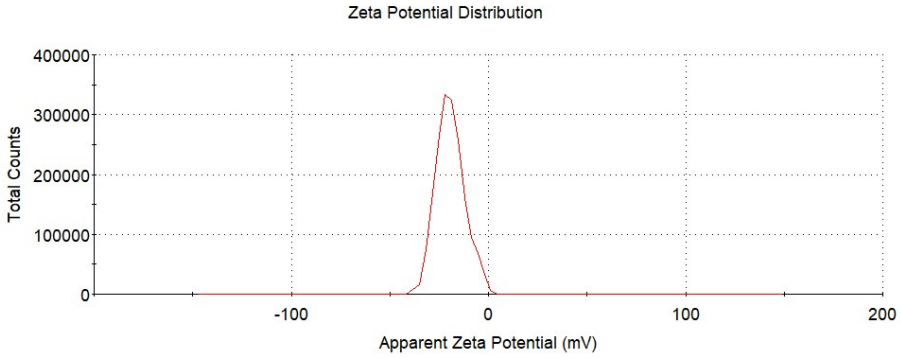

Figure S2. Zeta potential of TPI dispersed within DI water.

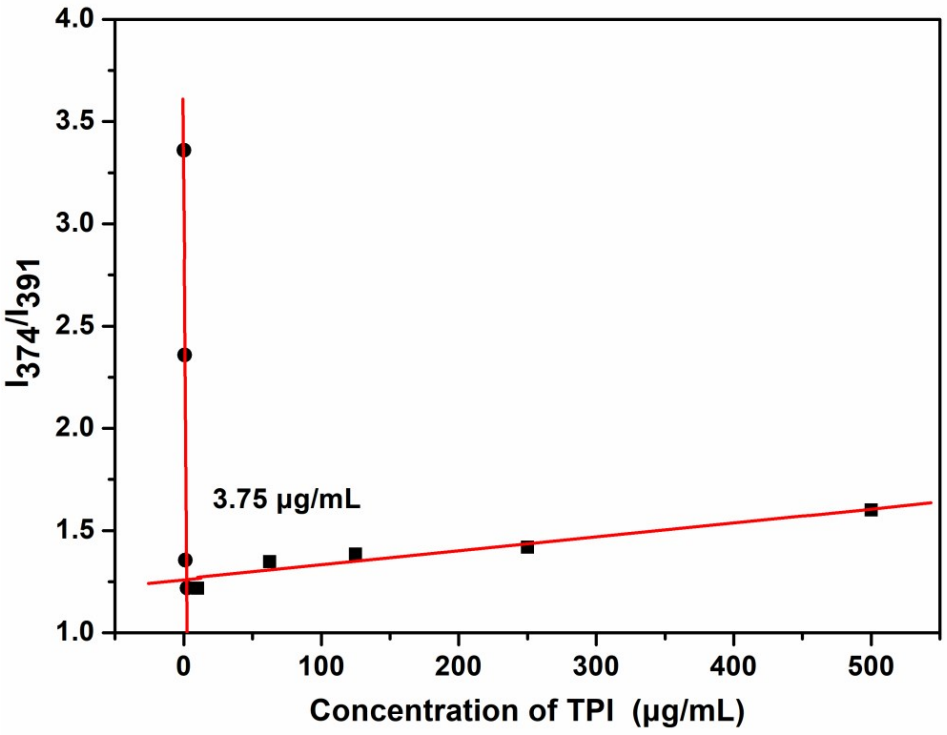

Figure S3. I_{374}/I_{391} intensity ratio versus the concentration of TPI

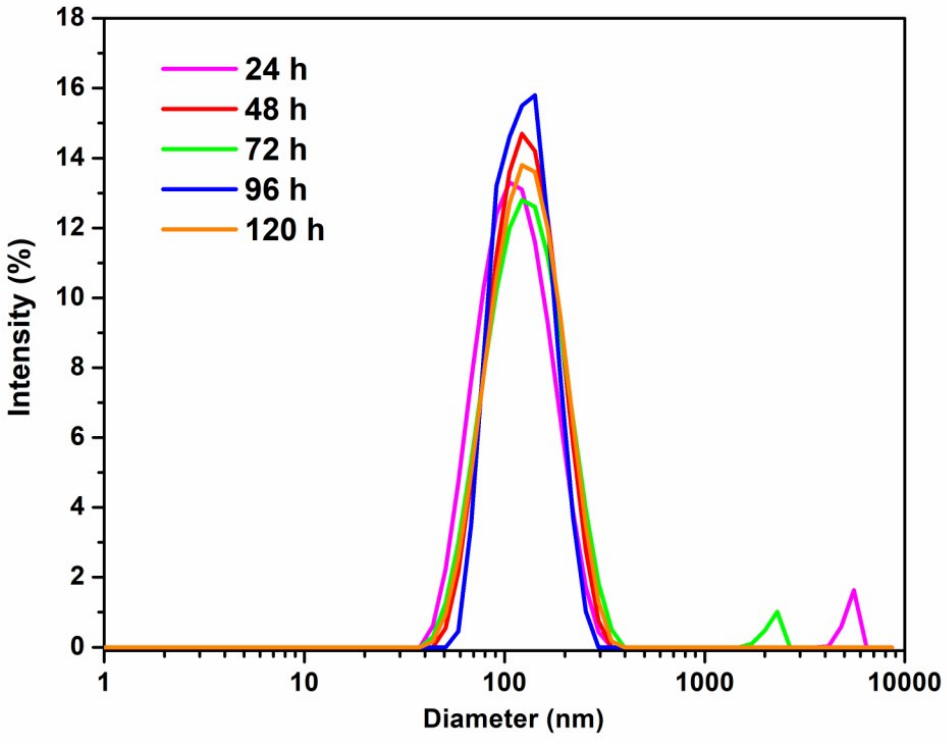

Figure S4. Hydrodynamic diameter of TPI incubated in plasma during 120 h.

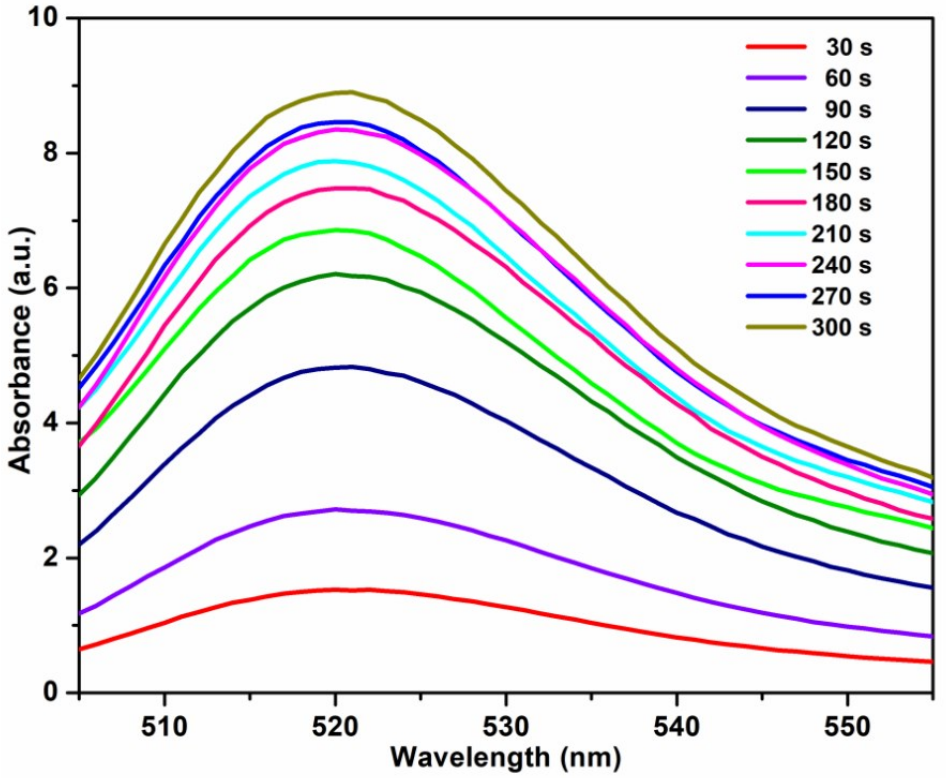

Figure S5. SOSG fluorescence spectra of ICG in TPI at different US irradiation time.

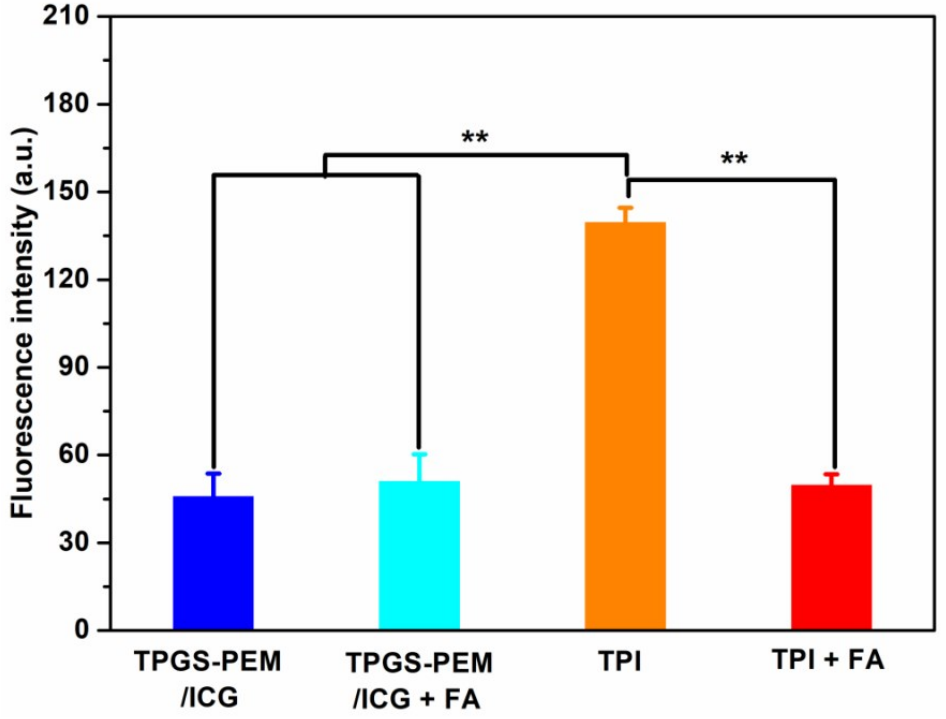

Figure S6. Cellular uptake of TPGS-PEM/ICG and TPI with/without FA.

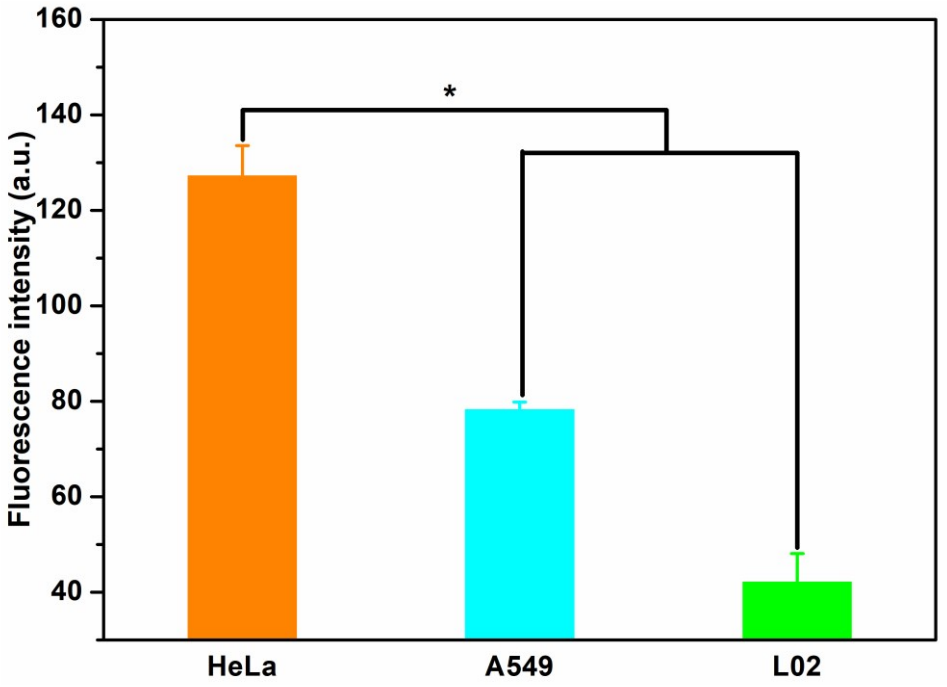

Figure S7. Cellular uptake of TPI in HeLa, A549 and L02 cells.

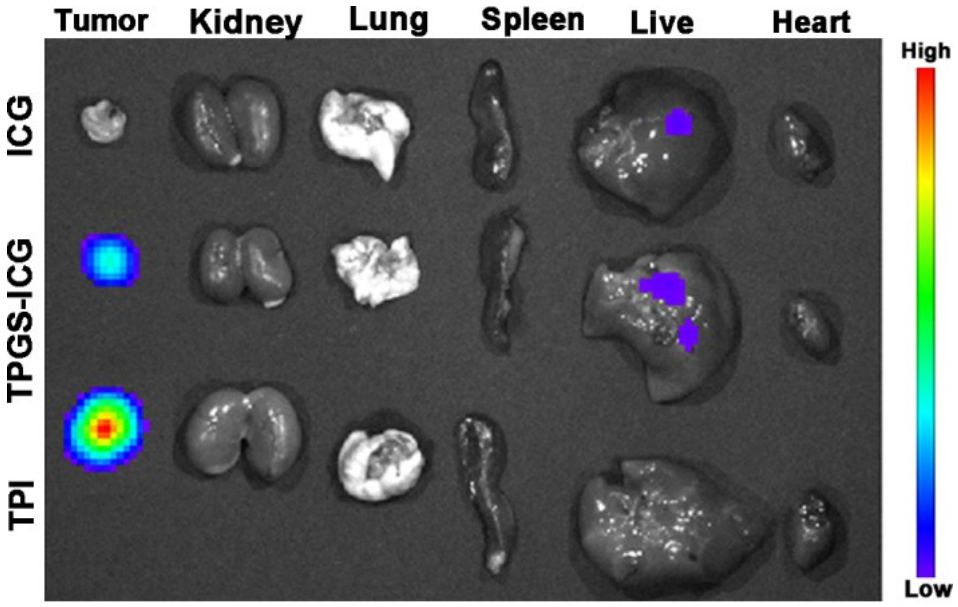

Figure S8. *Ex vivo* fluorescence imaging of tumors and major organs at 24 h post-injection of free ICG, TPGS-ICG and TPI.

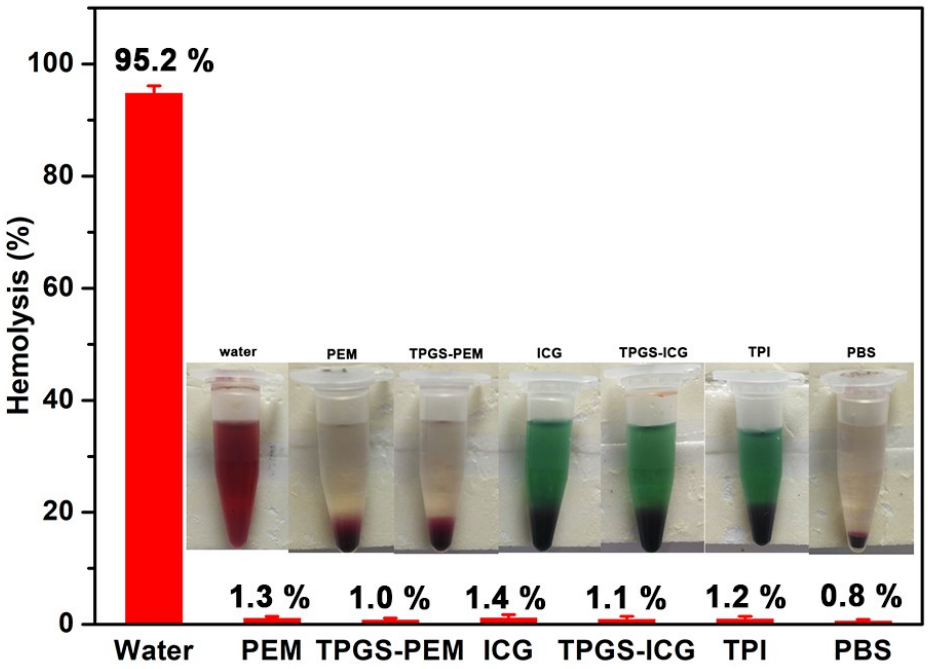

Figure S9. Hemolysis test of PEM, TPGS-PEM, ICG, TPGS-ICG, TPI in red blood cells. Error bars represent mean ± SD (n = 3).