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Supporting information

Raman/Fluorescence Dual-Modal Imaging Guided Synergistic Photothermal and Photodynamic Therapy Nanoplatform for Precision Cancer Theranostics

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1. General experimental section

1.1 Instruments

The SERS spectra were recorded by using a DXR Raman microscope (Thermo Scientific, USA). Transmission electron microscopy (TEM) images were acquired on a JEM-1400 transmission electron microscope (JEOL, Japan). Mice fluorescence imaging was performed on Perkinelmer IVIS Lumina XRMS Series III In Vivo Imaging System. Mice IR thermal imaging was performed by Infrared Thermal Camera (TESTO 865). Fluorescence spectra were obtained by a HORIBA Scientific Fluoromax-4 spectro fluorometer with a Xenon lamp and 1.0-cm quartz cells. Absorption spectra were measured on Thermo Scientific NanoDrop 2000/2000C spectrophotometer. Mass spectra were taken on LCQ Fleet LC-MS System (Thermo Fisher Scientific). ^{1}H NMR, ^{13}C NMR spectra were recorded on a Bruker AVANCE III TM 500 spectrometer. CCK-8 kit was carried out by a microplate reader (Tecan, Austria). The fluorescence images of cells were taken using a confocal laser scanning microscope (Japan Olympus Co., Ltd) with an objective lens (×40). Flow cytometry and intracellular fluorescence detection was carried out on flow cytometry (Aria, BD) with excitation at 633 nm and emission at 750-810 nm. The mean particle size was determined by DLS Zetasizer Nano ZS90 (Malvern Instruments Ltd, UK) and by TEM (JEOL, model JEM-1230, Japan).

1.2 Materials.

The stock solutions of C-S-NPs, Cy-C-S-NPs were solute in ultrapure water and maintained in refrigerator at 4 °C. All solvents were purchased from Sinopharm Chemical Reagent Co., Ltd. Other chemicals were purchased from Sigma-Aldrich unless otherwise stated and straightforward used without further purification, unless otherwise stated. The purity of Cy-DM was separated on a Shimadzu LC-20AT HPLC system equipped with fluorescence and UV-vis absorption detectors. HEPES was obtained from Aladdin. All reactions were performed under argon protection and dark, monitored by TLC (Hailang, Yantai). Flash chromatography was carried out using silica gel (300-400 mesh). Human hepatocellular carcinoma cell lines (HepG2 cells) were obtained from the Committee on Type Culture Collection of the Chinese Academy of Sciences. The antibody of Caspase 9, Hsp70, Cytochrome c, Bax and β-Actin were obtained from Cell Signaling Technology (Beverly, MA. USA). Water used in all experiments was doubly distilled and purified by a Milli-Q system (Millipore, Bedford, MA, USA).

1.3 Absorption and Fluorescence Analysis.

UV-visible spectra were obtained from a 1.0-cm glass dish. Add Cy-C-S-NPs (ultra-pure water, 60 μg/mL) and C-S-NPs (ultra-pure water, 60 μg/ml) into 10.0-mL colorimetric tube, and with HEPES buffer (10 mM, pH 7.4). Then, the Cy-C-S-NPs solution was continuously bubbed with N_2 for 10 minutes to remove the dissolved oxygen in the solution and establish an anoxic environment. In this process, the temperature is always at 37 ℃. Fluorescence spectra were obtained with a Xenon lamp and 1.0-cm quartz cells. Cy-C-S-NPs (ultrapure water, 60 μg/mL) were added to a 5.0-mL color comparison tube, after with HEPES buffer (10 mM, pH 7.4). Then, as above, hypoxia treatment was carried out for 10 minutes. In this process, the temperature is always at 37 ℃. Then the fluorescence emission spectra of Cy-C-S-NPs were integrated from 720 to 900 nm with excitation at 700 nm.

1.4 Quantitative analysis of Cy-DM on the surface of C-S-NPs

UV-vis titration was performed at pH 7.4. Then, 60 μg/mL Cy-C-S-NPs solution was treated with DTT (20 mM) and NaCl (4 M) for 30 min. The strong binding ability of DTT and C-S-NPs separated Cy-DM from C-S-NPs. The mixture is then centrifuged. The supernatant was collected and its absorbance at 780 nm was determined by UV-vis-NIR spectroscopy. The concentration of Cy-DM in supernatant was determined by standard calibration method. UV-vis-NIR spectra showed that about 6 μM Cy-DM was equivalent to 60 μg/mL Cy-C-S-NPs.

1.5 Photothermal effect and photothermal conversion efficiency.

The solutions of Cy-C-S-NPs at the concentrations of 60 μg/mL in 0.5 mL glass vials were irradiated by 808 nm laser for 6 min. Meanwhile, the temperatures of solutions were recorded using a thermometer and infrared thermal camera at an interval of 30 s. To assess the photothermal conversion efficiency, the solutions of Cy-C-S-NPs, free Cy-DM, free C-S-NPs and Cy-C-S-NPs + hypoxia in quartz cuvettes were irradiated at 808 nm. When the temperature reached a plateau, the irradiation was removed for cooling down to room temperature. The temperature of the solutions was recorded at an interval of 30 s, and then their photothermal conversion efficiencies were calculated.

1.6 Cell line and culture.

The human hepatoma cell lines (HepG2) used in this study were purchased from Chinese Academy of Sciences Model Culture Committee. HepG2 cells were maintained in normoxic or hypoxic conditions at 37 ℃ in a recommended medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. Normoxic conditions are grown in a humidified incubator of 5% CO_2 at 37 ℃ (Thermo Science 3111, USA), and anoxic conditions are supplemented by a modular incubator (Billups-Rothenberg) with a mixture of humidified O_2 (1%), CO_2 (5%) and balanced N_2.

1.7 Cell Imaging

Fluorescence images were captured on an Olympus FV1000 confocal laser scanning microscope with an objective (×40). The cells were inoculated on (Φ= 20 mm) petri dish and adhered to the wall for 24 hours before imaging. 60 μg/mL Cy-C-S-NPs was added to the petri dish. After incubating with the cells for 3 hours, the cells were put into a modular incubator for hypoxia treatment. Imaging was performed by laser scanning confocal microscopy. Fluorescence changes were quantified by flow cytometry.

1.8 Single Oxygen Detection in Lving Cells.

The green singlet oxygen sensor (SOSG, Invitgen Co., USA) is used to evaluate singlet oxygen production of Cy-C-S-NPs in living cells. HepG2 cells were cultured in 2.0×10^5 cells/well with 6-well plates and incubated for 24 h. After cell adherence, 60 μg/mL Cy-C-S-NPs was added and incubated at 37 ℃ for 3 h. Cy-C-S-NPs was not added as control group. Then, we placed one group of Cy-C-S-NPs added cells in a modular incubator (Billup-Rothenberg) and cultured them without oxygen for half an hour, while the other group was cultured normally. Then SOSG (1 μM) was added to water containing 2% methanol and the cells were irradiated at 808 nm and 1.5W/cm^2 for 6min. After incubation at 37 ℃ for 20 min, the cells were washed, resuspended by PBS, and analyzed by flow cytometry. The fluorescence images of HepG2 cells were rapidly detected by laser scanning confocal microscopy. Emission at 500-600 nm was generated under excitation of a light source with a wavelength of 494 nm. Singlet oxygen production was quantified by flow cytometry.

1.9 CCK-8 assay was used to detect the cytotoxicity.

HepG2 cells were inoculated in 96-well cell culture plates with a final density of 5×10^3 cells/well. Then, different concentrations of Cy-DM samples were added to the well, with concentrations of 0 μM, 2 μM, 4 μM, 6 μM, 8 μM and 10 μM, respectively. The concentrations of C-S-NPs and Cy-C-S-NPs were 0 μg/mL, 20 μg/mL, 40 μg/mL, 60 μg/mL, 80 μg/mL, 100 μg/mL. Then the cells were incubated at 37 ℃ and 5% CO_2 for 24h. Then the cells were divided into four groups by different treatments: ① Normoxia + Laser off; ②Hypoxia + Laser off; ③Normoxia + Laser on; ④ Hypoxia + Laser on. Add CCK-8 solution (final concentration 100 μL/mL) into each well. The laser power was adjusted to 1.5W /cm^2 and the irradiation time was 6 min. The cell survival rate was calculated by the absorbance (OD) at 450nm using a microplate reader (Tecan, Austria) after 1h treatment at 37 ℃ and 5% CO_2. Calculate the IC_{50} value according to Huber and Koella. The result is the average standard deviation of six independent measurements.

1.10 Western blot analysis.

1×10^6 HepG2 cells were seeded in 6-well plate and incubated overnight. The cells were divided into two groups, one that was hypoxic and one that was cultured under normal conditions. They were treated with 60

μg/mL Cy-C-S-NPs for 24 h incubation. Then, cells were irradiated with NIR laser light (808 nm, 1.5 W/cm^2) for 6 min respectively. In order to investigate the effect of PDT or PTT, the cells were maintained below 4 °C by ice treatment to scavenge photothermal conversion or treated with 10 mM N-acetylcysteine (NAC, ROS scavenger) to scavenge ROS, during laser irradiation. The Cy-C-S-NPs dark incubation group (without laser irradiation) was used as positive control. After 6 min laser irradiation, all cells were washed with PBS, protein was extracted with 200 μL RIPA lysis buffer containing 2μL PMSF (Solarbio, China). Then the extracts were quantified with BCA protein assay kit (Biogot, China). After denatured, the equal amounts of protein were electrophoresed on 10–12% SDS-polyacrylamide gels (Bio-Rad, USA) and transferred to PVDF membranes. Then the membranes were blocked with 5% milk and incubated with primary antibodies overnight at 4 ℃. Appropriate secondary antibody conjugated to horseradish peroxidase (HRP) (Cell Signaling Technology, USA) was used to quantify protein and an enhance chemiluminescence (ECL) detection system was used to detect the signals. The results were analyzed with ImageJ software and Graphpad Prism software.

1.11 In Vitro Synergetic PDT, PTT Effect of Cy-C-S-NPs.

HepG2 cells were used to evaluate the synergistic effect of PTT and PDT on Cy-C-S-NPs. First, 5×10^3 cells were inoculated into 6-well plates and cultured overnight, then Cy-C-S-NPs was cultured with 60 μg/mL Cy-C-S-NPs for 3 hours under normoxic and hypoxic conditions, respectively. In the control group, cells were directly incubated with the same concentration of Cy-C-S-NPs for 3 h without laser irradiation. Afterward, the cells of experimental groups (single group of PDT, PTT; synergistic PDT/PTT group) were irradiated by an NIR (808 nm) laser with energy density of 1.5 W/cm^2 for 6 min. Incubation 3 h later, cells were stained with 2 μM Calcein AM and with 2 μM PI for visualization of live cells or visualization of dead/late apoptotic cells respectively. Cellular apoptosis and necrosis were evaluated by Annexin V-FITC Apoptosis Detection Kit and observed by flow cytometry. Lastly, the fluorescent images of cells in all groups were acquired with a biological inverted microscope after being rinsed with PBS. The synergistic anticancer effect of Cy-C-S-NPs on HepG2 cells was further intuitively verified by Calcein AM and PI co-staining. The green color of Calcine AM and the red color of PI show living and dead cells respectively. Both Calcein AM and PI co-staining and flow cytometry proved the synergistic anti-tumor effect of PTT/PDT of Cy-C-S-NPs. The same method as Western blot was used to study the effect of PDT or PTT alone.

1.12 Establishment of the HepG2 Transplanted Tumor Nude Mice

5-week-old specific pathogen free nude mice were housed in individual ventilated cages and fed a SPF laboratory diet and water ad libitum. 2×10^6 cells were suspended in media and implanted subcutaneously into nude mice. HepG2 xenografts were established in nude mice until the tumor volumes typically reached to about 80-100 mm^3. All experiments were performed in accordance with the guidelines established by the Committee of Animal Research Policy of Binzhou Medical University.

1.13 Histological Experiments.

Tumors from nude mice were excised. The issues were fixed in 10% formaldehyde and embedded in paraffin. Then the paraffin masses were cut and dewaxed. The sections were dehydrated using graded ethanol series and washed by distilled water. The tumor sections were stained with hematoxylin and eosion to observe the tumor tissue structure.

1.14 Imaging Mice in vivo.

A Bruker In-vivo Imaging System was employed to image tumor bearing nude mice. The excitation and emission wavelengths were chosen as described in paper. The mice were anesthetized prior to injection and during S4 imaging. After in vivo imaging, the organs (lung, heart, liver, kidney and spleen) and tumors were excised to perform ex vivo imaging.

1.15 H&E staining.

Heart, liver, spleen, lung and kidney of tumor-bearing mice in each group, tumor tissue of tumor-bearing

mice in each group were all excised and fixed in 10% formaldehyde and embedded in paraffin and stained with hematoxylin and eosin (H&E) to confirm histology.

2. Synthetic Procedures and Characterization Details of Cy-C-S-NPs.

2.1 Synthesis of C-S-NPs

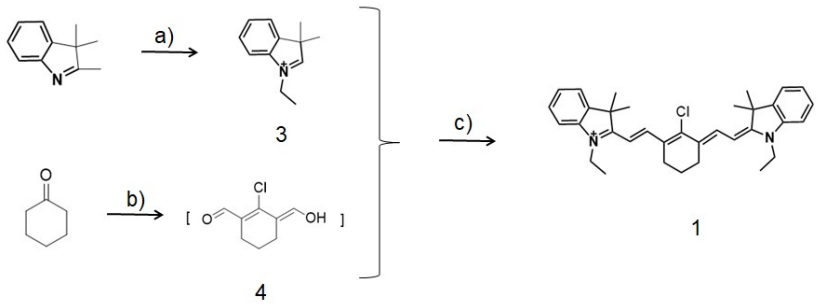
Synthesis of Au NSs: Au NSs that served as core were synthesized according to a reported method with some minor modifications. Briefly, the seed solution was prepared by reducing $HAuCl_4$ (0.5 mM, 5 mL) in CTAB (0.2 M, 5 mL) with freshly prepared ice-cold $NaBH_4$ (10 mM, 0.6 mL) at 27 °C. After 3 h, 50 μL of the synthetic seed solution was mixed with CTAC (0.2 M, 2 mL), AA (0.1 M, 1.5 mL), followed by the addition of $HAuCl_4$ solution (0.5 mM, 2 mL). The reaction was allowed to continue at 27 °C for 15 min. The product was collected by centrifugation twice at 14 000 rpm for 40 min and then concentrated to 1 mL. The diameter of the product was 10 nm. Finally, CTAC (0.1 M, 40 mL), AA (10 mM, 2.6 mL), and the 10 nm seeds (100 μL) were mixed, followed by the addition of $HAuCl_4$ solution (0.5 mM, 40 mL) using a syringe pump at an injection rate of 40 mL/h. The reaction was allowed to proceed at 27°C for 10 min after the injection had beenfinished.

Synthesis of Liposomes: Liposomes were prepared by a conventionalfilm hydration method. Briefly, the phospholipid was dissolved in ethanol (5 mg/mL), followed by the removal of the solvent using a rotary evaporator at 35 °C. A dried lipidfilm was obtained by further placing it under vacuum for 2 h at 40 °C. The driedfilm was fully hydrated in 5 mL of water to form multilamellar vesicles, which were then broken into small unilamellar vesicles by using probe sonication (150 W, 30 min), indicated by the solution turning from turbid to clear.

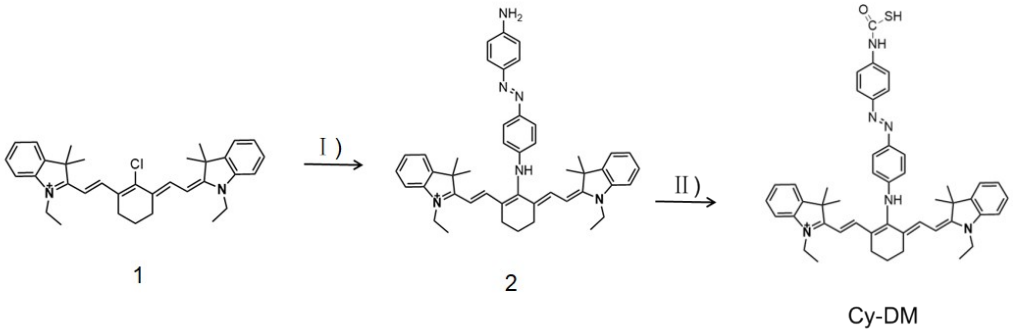
Synthesis of Lipid Bilayer-Coated Au NSs: A volume of 1 mL of Au NS solution was centrifuged at 3000 rpm for 10 min, and the supernatant was discarded. The NS pellet was resuspended in 1 mL of liposome solution (5 mg/mL) and incubated at room temperature overnight. Then, the mixture was centrifuged at 3000 rpm for 10 min, and the pellet was dispersed in 50 μL of water, followed by a dropwise addition of the dispersion into 1 mL of the liposome solution and stirring for 4 h at 50 °C. The lipid bilayer-coated Au NSs were finally obtained by centrifuging the mixture at 4000 rpm for 10 min and dispersing in 1 mL of water.

Synthesis of C−S NPs: A volume of 1 mL of the bilayer-coated Au NSs (OD_{540nm}= 1.0) was mixed with AA (0.1 M, 0.1 mL), followed by the addition of 0.1 mL of $HAuCl_4$ with concentrations (2.5 mM) under vigorous stirring at room temperature[1].

2.2 Synthetic routes for Cy-DM



Scheme S1. Synthetic Approaches of Fluorophore. a) Iodoethane, acetonitrile, refluxed for 12 h, 90%; b) DMF, CH_2Cl_2, $POCl_3$, 45 °C, 3 h, 85%; c) n-Butyl alcohol : benzene = 7:3 (v/v), refluxed, 3 h, 70%



Scheme S2. The general synthetic routes for the probe Cy-DM: i) 4-4 '- diaminoazobenzene Et_3N, NaH, DMF, 40 ºC, 24 h. ii) TGA, NHS, EDC·HCl, DMF, 20 ℃, 2 h

Synthesis of Compound 3: 2,3,3-Trimethylindolenine (24 g, 150 mmol) and iodoethane (23 g, 150 mmol) were mixed in 80 mL anhydrous acetonitrile in 250 mL round flask, then the mixture was refluxed at 130 ℃ for 12 h, then stopped heating and cooled down. The precipitate was filtered through a buchner funnel, and the solid product was washed by diethyl ether and dried in vacuum to afford pink product (21.8 g, yield: 90%).

Synthesis of Compound 4: A solution of 40 mL of anhydrous N, N-dimethylformamide (DMF) and 40 mL of anhydrous CH_2Cl_2 was placed in a 250 mL round-bottom flask, chilling the solution to -10 °C and then stirring for 20 min. Phosphorus oxychloride (37 mL), with 35 mL of anhydrous CH_2Cl_2 was dropwise added into above solution through a constant pressure drop of liquid funnel. Cyclohexanone (10 g, 101.9 mmol) was added into the mixture in batches, the solution changed from colorless into yellow immediately. Then the solution was slowly heated to 45 ℃ for 3 h, then cooled down, poured into a lot of ice, and allowed to stand overnight. The yellow solid was collected through a buchner funnel and dried in vacuum (12.8 g, yield: 85%).

Synthesis of Compound 1: Compound 3 (0.376 g, 2 mmol) and 4 (0.17 g, 1 mmol) were resolved in 100 mL mixed solution of n-butyl alcohol and benzene (7:3, v/v) in 250 mL round flask, refluxed at 145℃ for 3 h, dried in vacuum, to obtain green solid. The crude product was purified by silica gel chromatography using EtOAc/CH_3OH (7:1, v/v) as eluent to afford compound 1 as green solid (0.681 g, yield:70%).

Synthesis of Compound 2: 4-4 '- diaminoazobenzene (2.12 g, 10 mmol), NaH (0.12 g, 5 mmol), TEA(1.01 g,1.4 mL,10 mmol) dissolved in 30 mL anhydrous DMF, mixing 0.5 h at room temperature, and then chase with constant pressure drop funnel drop join Compound1(0.511 g, 1mmol), stir solution under 40 ℃ for 24 h, the reaction is always under the Ar atmosphere[2]. After the reaction, the insoluble organic components were filtered out by a Brucellosis funnel, and then vacuum-dried to obtain a black mixture. The crude product was purified by silica gel chromatography and eluted with EtOAc /CH_3OH (5:1, V/V) to obtain a green solid compound 1 (0.148g, yield: 22%).

Synthesis of Compound Cy-DM : N-hydroxysuccinimide (NHS, 0.46 g, 4 mmol) , n-(3-dimethylaminopropyl) -n'-ethylcarbodiimide hydrochloride and Mercaptoacetic acid (EDC•HCl, 0.766 g, 4 mmol), Mercaptoacetic acid (TGA, 0.184 g, 2 mmol) were activated at room temperature for 30 minutes, then the product compound 2(0.687 g, 1 mmol) dissolved by DMF was added drop by drop in a constant pressure drop funnel, and the reaction was performed at 25 ℃ for 2 hours. After the reaction, the product was extracted with CH_2Cl_2, washed with ultrapure water for 5 times, and vacuum dried to obtain green product compound 3.

2.3 Synthesis of Cy-C-S-NPs

Compound 3 was added drop by drop into C-S-NPs solution through a constant pressure drop funnel, and the reaction lasted for 24 h. After the reaction, centrifuge at 12000 rpm for 15 minutes, repeat three times to remove excess compound 2 and obtain the final product Cy-C-S-NPs. Cy-C-S-NPs was suspended in ultra-pure water and stored at 4 °C[3].

3. SERS spectra of Cy-C−S NPs solutions.

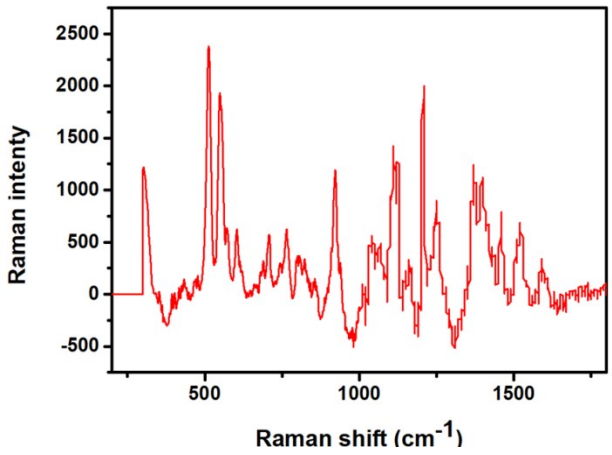


Figure S1. SERS spectra of Cy-C−S NPs solutions.

4. Effect of pH Values on Cy-C-S NPs.

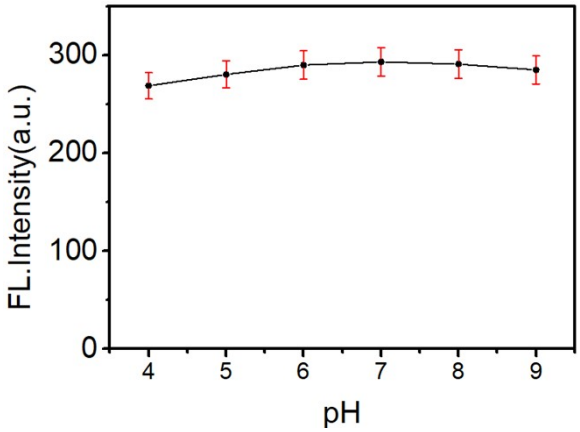


Figure S2. Effect of PH on Cy-C-S NPs under hypoxia. PH ranges from 4.0 to 9.0.

5. The Bright Field of Cells in Fig 2a.

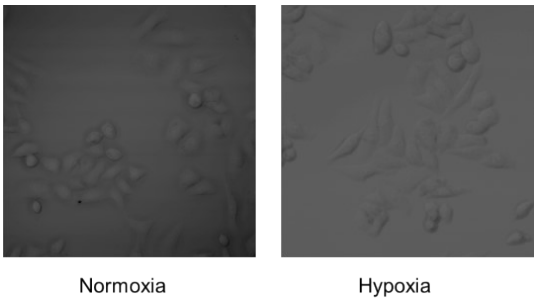


Figure S3. The bright field of cells in Fig 2a. Scale bar = 20 μm.

6. The Mean Fluorescence Intensity of Fig 2a.

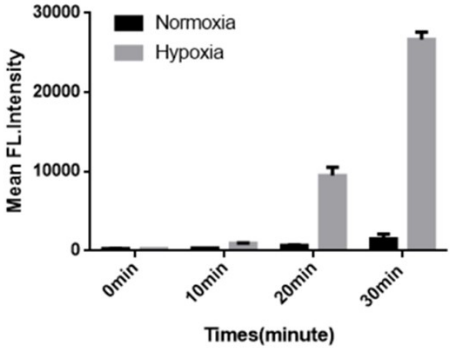


Figure S4. The mean fluorescence intensity of Fig 2a.

7. The Bright Field of Cells in Fig 3a.

Figure S5.The bright field of cells in Fig 3a. Scale bar = 20 μm.

8. The mean fluorescence intensity of Fig 3a

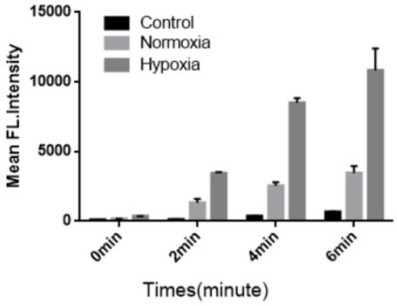


Figure S6.The mean fluorescence intensity of Fig 3a.

9. Statistical analysis of apoptosis rate detected by flow cytometry.

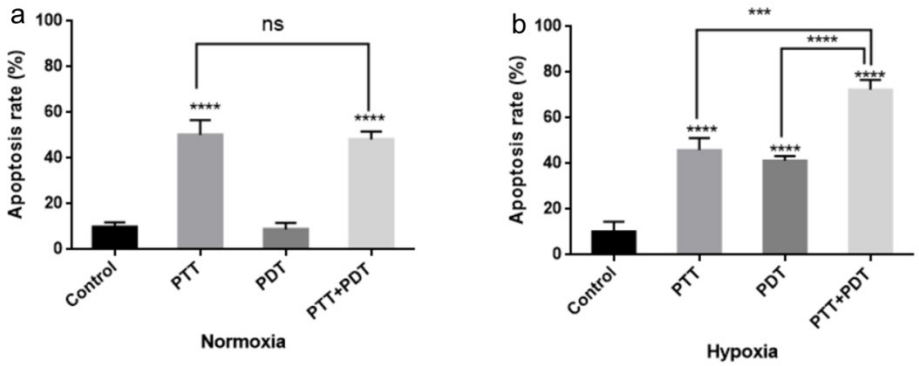
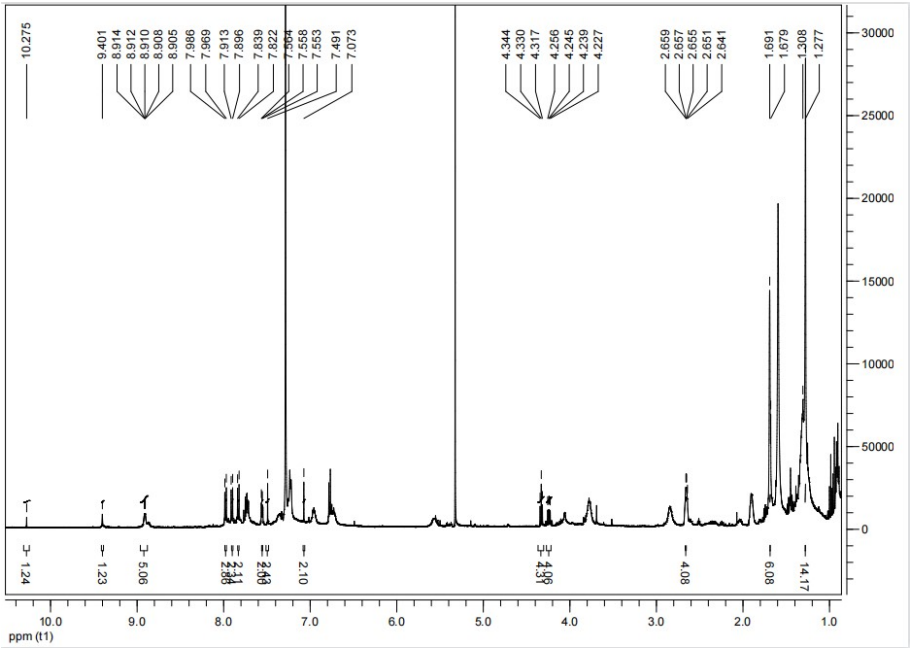


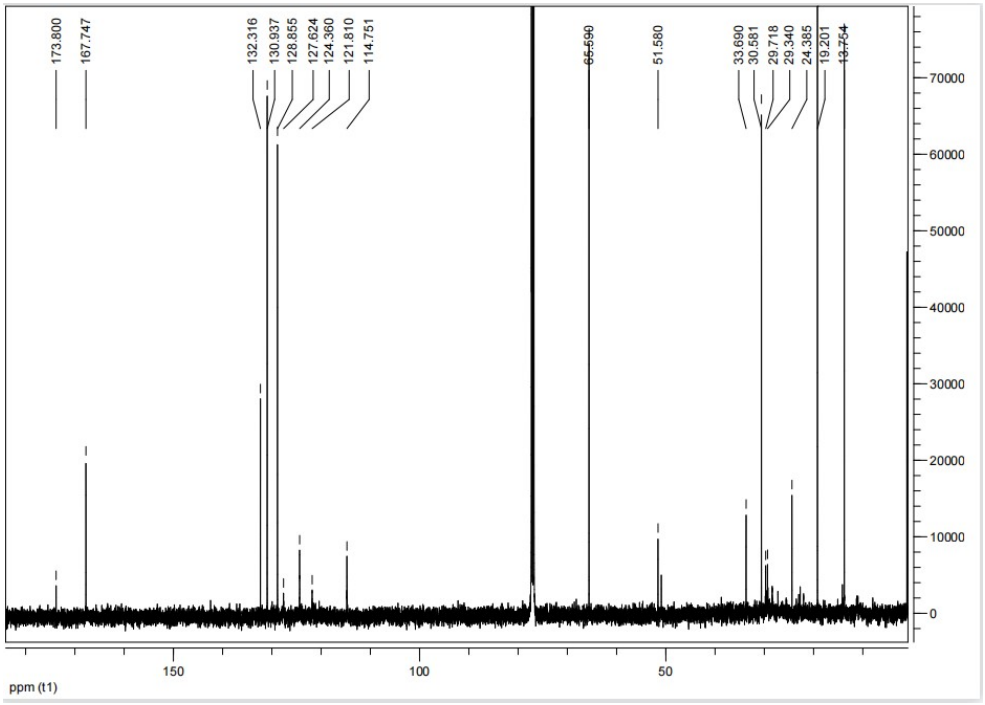
Figure S7. a) Statistical analysis of Annexin V-PE under blank, synergetic PDT and PTT, single PTT or single PDT in normoxia condition using flow cytometry. b) Statistical analysis of Annexin V-PE under blank, synergetic PDT and PTT, single PTT or single PDT in hypoxia condition using flow cytometry. The symbol "ns" indicates no significant difference (P>0.05). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

10. ^{1}H NMR, ^{13}C NMR and LC-MS of Compound 2, Cy-DM

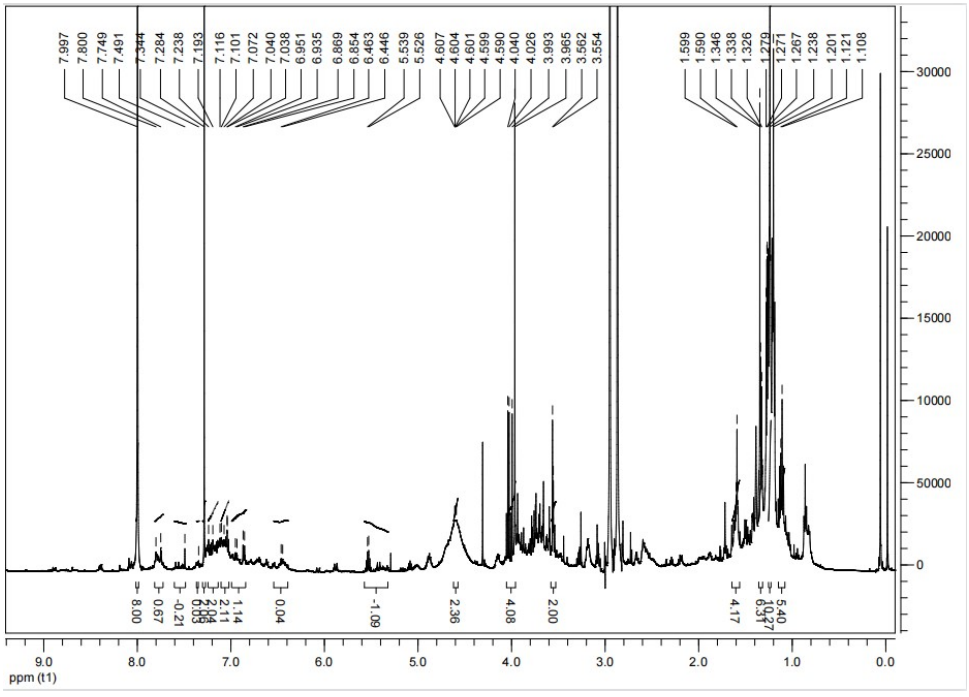
^{1}H NMR of Compound 2



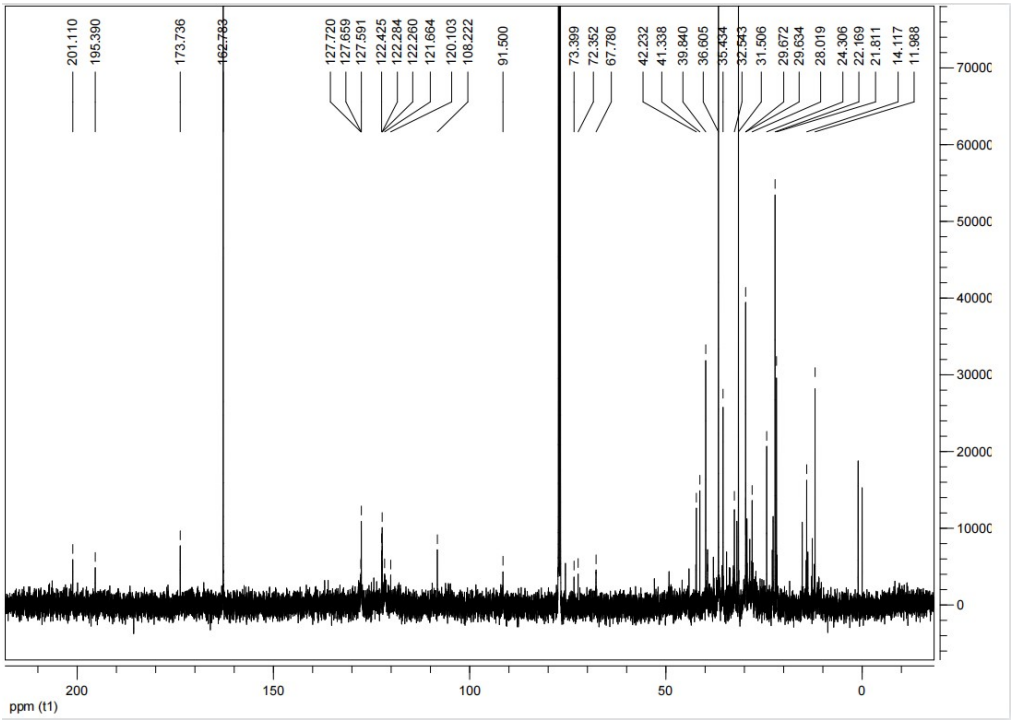
^{13}C NMR of Compound 2



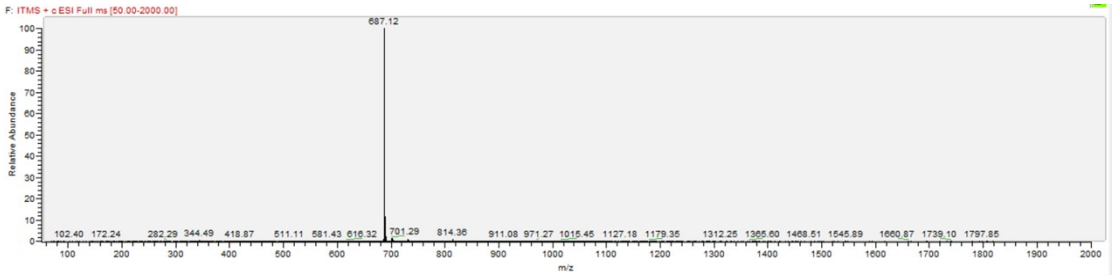
^{1}H NMR of Cy-DM



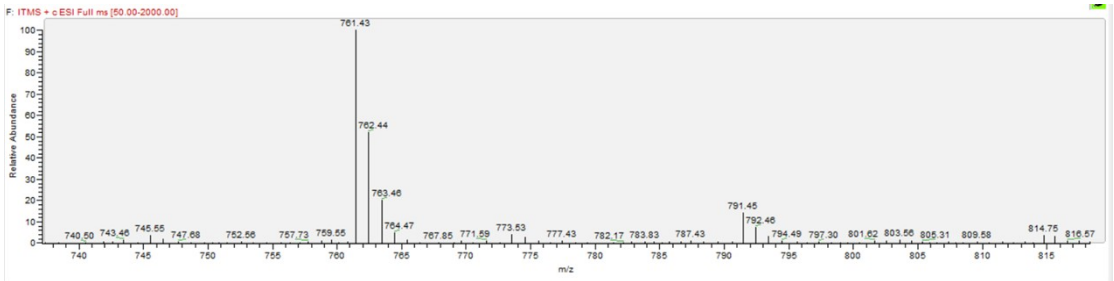
^{13}C NMR of Cy-DM



LC-MS of Compound 2



LC-MS of Cy-DM



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