Pillar[5]arene-based Supramolecular Photosensitizer for Enhanced Hypoxic-Tumor Therapeutic Effectiveness

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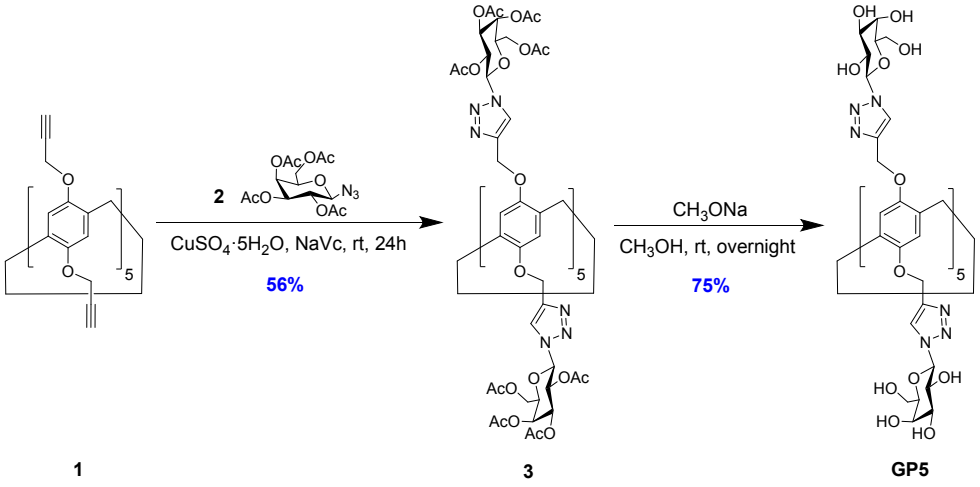
Supporting Information

1. General information

All reagents were purchased from commercial suppliers and used without further purification unless specified. Water used in this work was triple distilled. Doxorubicin hydrochloride was purchased from Sangon Biotech. ^{1}H NMR spectra were recorded on a Bruker 500 MHz Spectrometer, with working frequencies of 500 MHz for ^{1}H and 125 MHz for ^{13}C nuclei, respectively. SEM image was obtained using a Nano SEM-450 (FEI, U.S.A.) with an accelerating voltage of 10.0 kV. TEM image was obtained by TECNAI G2 SPIRIT BIO (FEI, U.S.A.). DLS measurements were performed on a DelsaTM Nano system (Beckman Coulter, U.S.A.). K_a was measured from Nano-ITC SV (TA-Waters LLC, U.S.A.). UV-vis spectra were recorded with Shimadzu 1750 UV-visible spectrophotometer (Japan) at 298 K. Water surface tension was recorded with BZY-3B surface tension measurer (China). Cell culture was carried out in an incubator with a humidified atmosphere of 5% CO_2 at 37 °C. Dihydrorhodamine 123 (DHR123), Dihydroethidium (DHE), hydroxyphenyl fluorescein (HPF), singlet oxygen sensor green (SOSG) were purchased from MKbio (China).

2. Synthesis and characterizations

Synthesis of compound GP5



Scheme S1: Synthesis of the host molecule GP5. [S1-S2]

As shown in Scheme S1, GP5 was synthesized and purified according to our previously reported procedures. [S1] The ^{1}H NMR spectrum of 1 was shown in Figure S1. ^{1}H NMR (500 MHz, $CDCl_3$, 298 K) δ (ppm): 6.83 (s, 10H), 4.54 (d, J = 2.16 Hz, 20H), 3.81 (s, 10H), 2.29 (s, 10H). The ^{1}H NMR spectrum of 2 was shown in Figure S2. ^{1}H NMR (500 MHz, $CDCl_3$, 298 K) δ (ppm): 5.41 (d, J = 2.7

Hz,1H), 5.14 (t, *J* = 9.3 Hz, 1H), 5.02 (dd, *J* = 10.2, 3.2 Hz, 1H), 4.59 (d, *J* = 8.9 Hz, 1H), 4.18-4.11 (m, 2H), 4.00 (t, *J* = 6.4 Hz,1H), 2.15 (s, 3H), 2.08 (s, 3H), 2.05 (s, 3H), 1.97 (s, 3H). The ^{1}H NMR spectrum of 3 was shown in Figure S3. ^{1}H NMR (500 MHz, $CDCl_3$, 298 K) δ (ppm): 8.18 (s, 10H), 6.77 (s, 10H), 5.92 (d, *J* = 9.3 Hz, 10H), 5.64 (t, *J* = 9.8 Hz, 10H), 5.55 (d, *J* = 3.0 Hz, 10H), 5.35 (dd, *J* = 10.2, 3.3 Hz, 10H), 4.84 (dd, *J* = 33.9, 11.9 Hz, 20H), 4.37 (t, *J* = 6.6 Hz, 10H), 4.31-4.28 (m, 10H), 4.19-4.16 (m, 10H), 3.75 (s, 10H),2.23 (s, 30H), 2.00 (s, 60H),1.87 (s, 30H). The ^{1}H NMR spectrum of GP5 was shown in Figure S4. ^{1}H NMR (500 MHz, DMSO-d_6, 298 K) δ (ppm): 8.46 (d, *J* = 10.3 Hz, 10H), 7.06 (s, 10H), 5.53-5.43 (m, 10H), 5.41-5.32 (m, 10H), 5.17 (s, 10H), 4.99-4.71 (m, 30H), 3.77 (s, 10H), 3.62 (s, 10H), 3.57-3.46 (m, 20H).

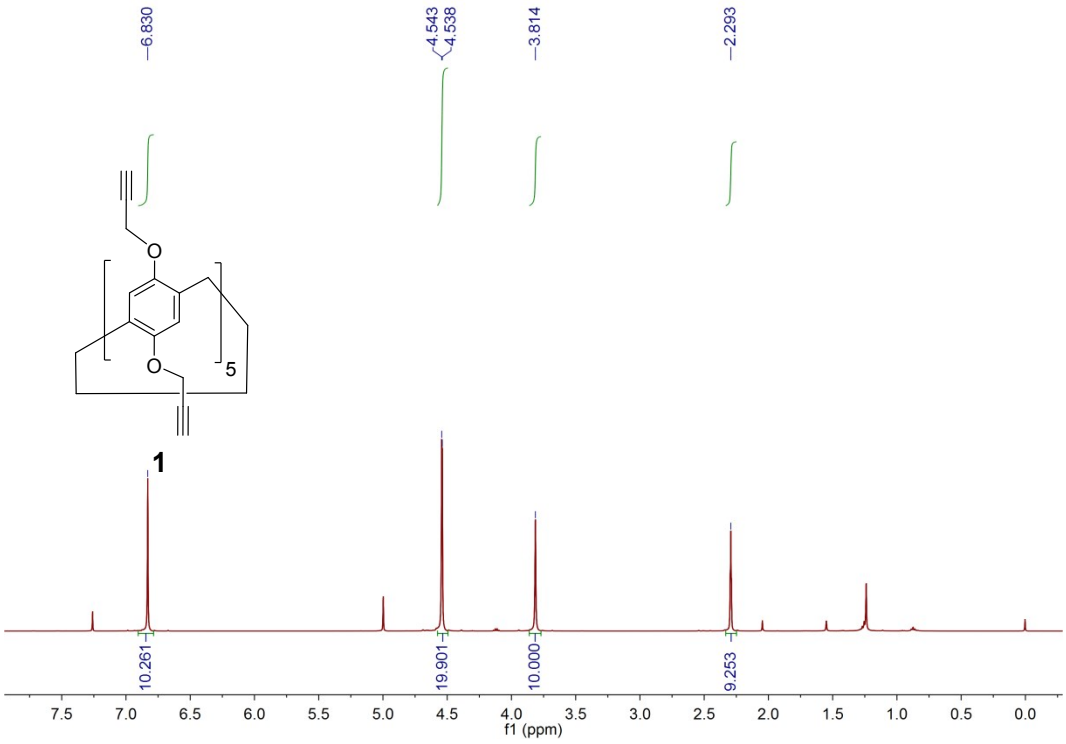


Figure S1: ^{1}H NMR (500 MHz, $CDCl_3$, 298 K) spectrum of Compound 1.

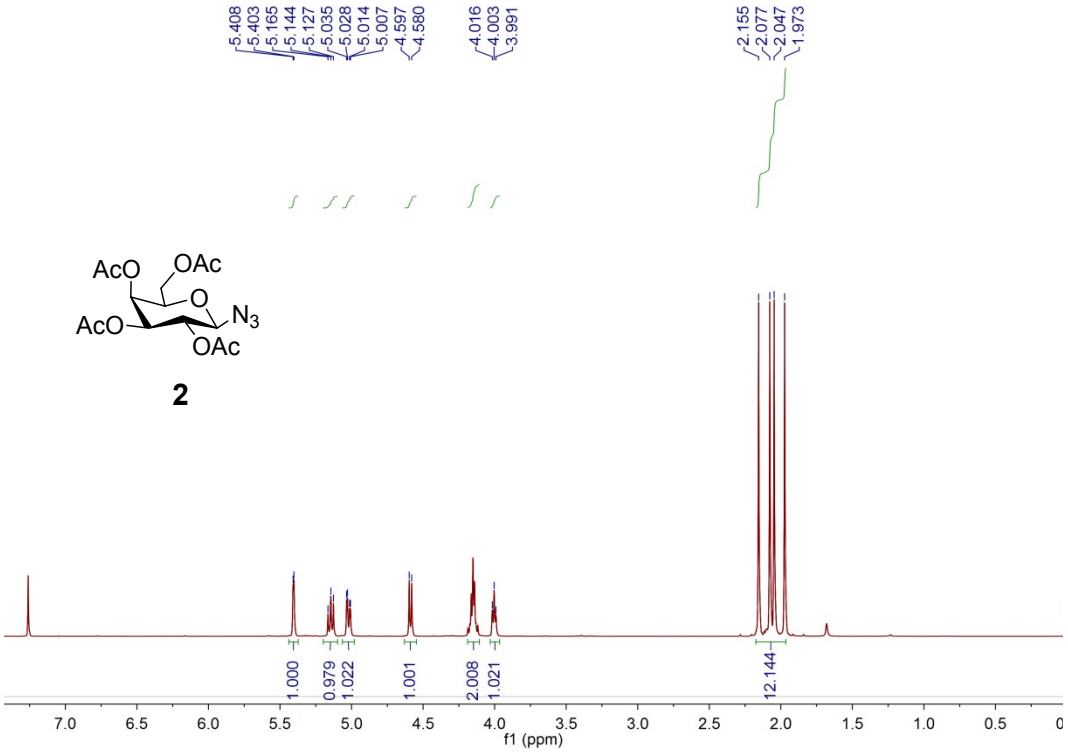


Figure S2: ^{1}H NMR (500 MHz, $CDCl_3$, 298 K) spectrum of Compound 2.

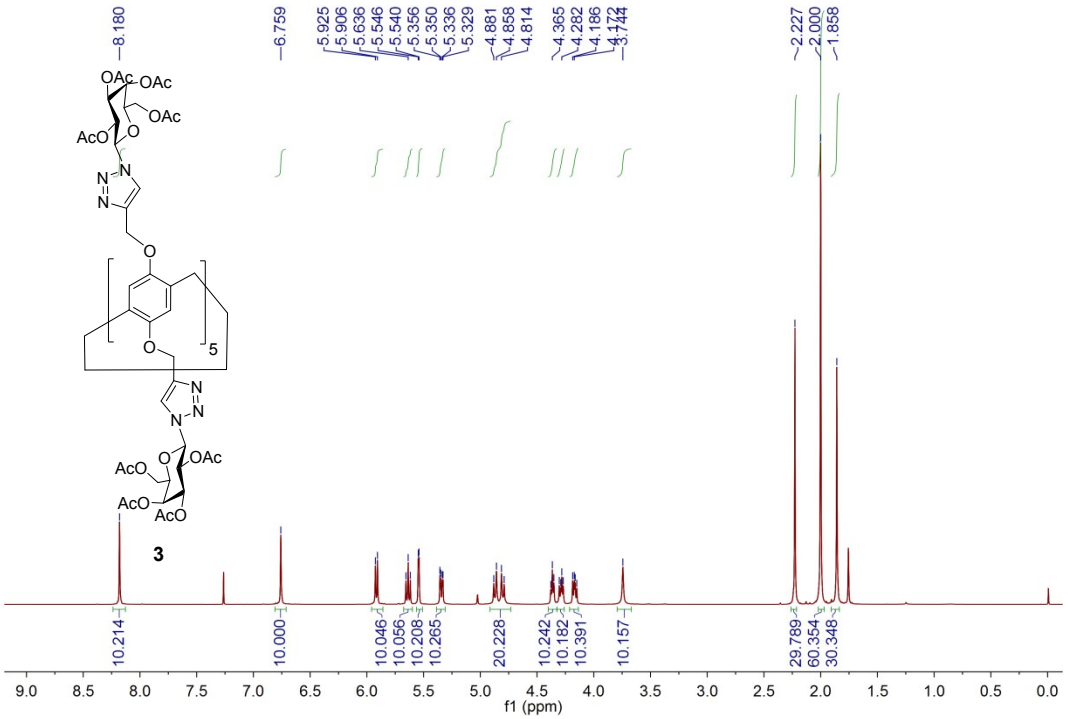


Figure S3: ^{1}H NMR (500 MHz, $CDCl_3$, 298 K) spectrum of Compound 3.

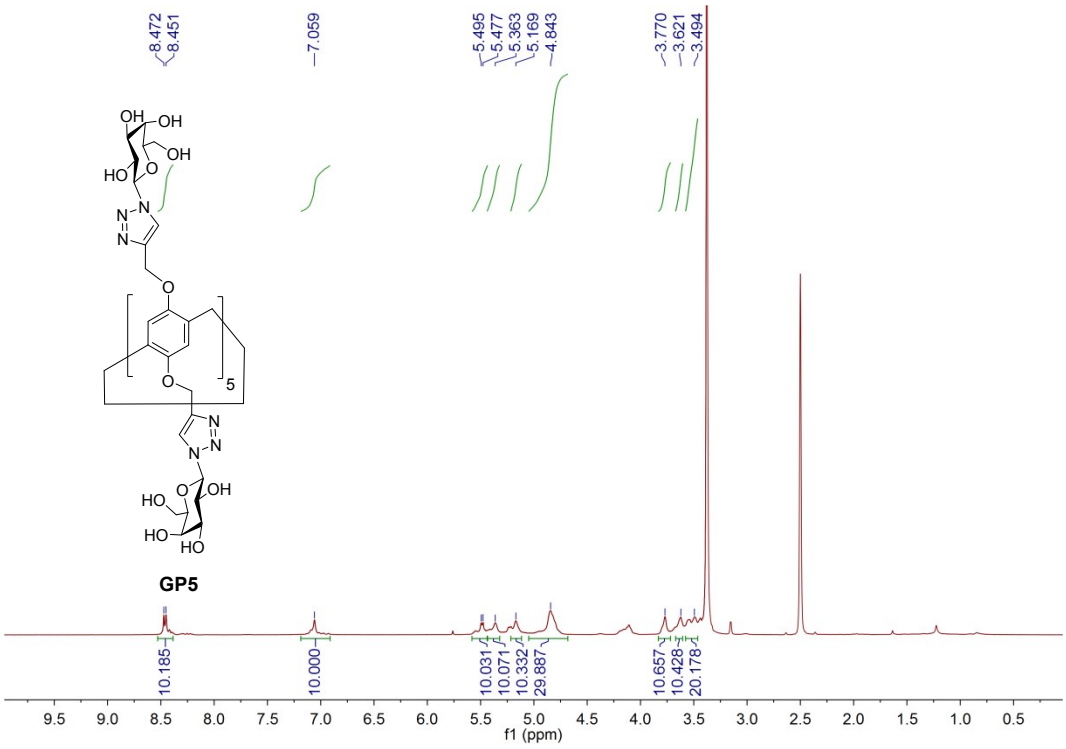
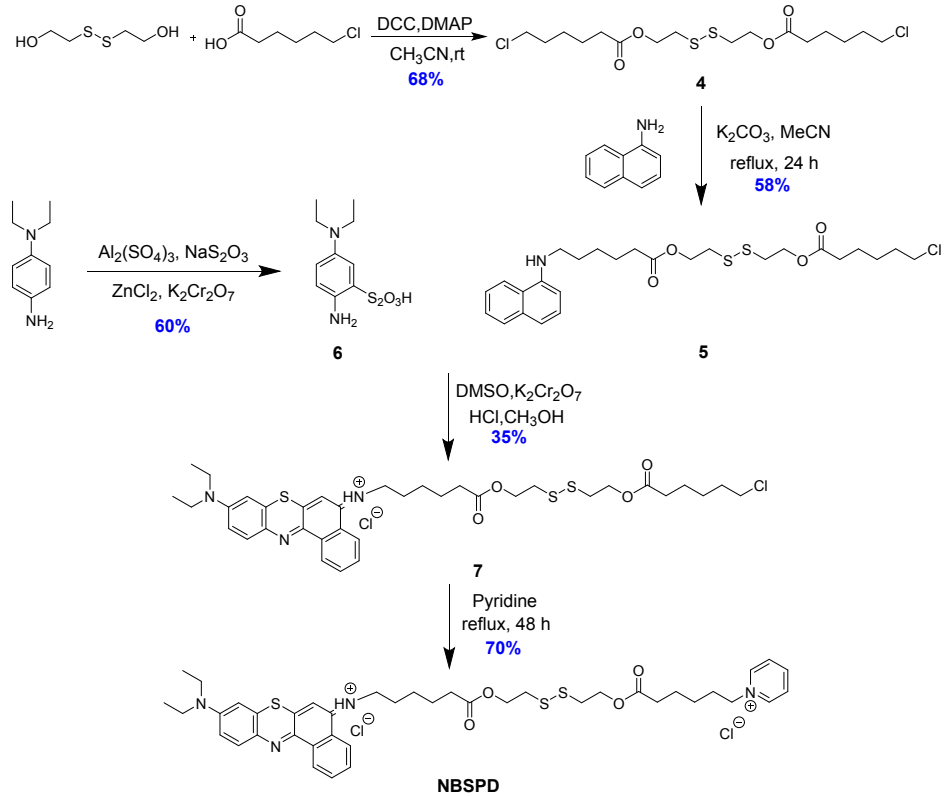


Figure S4: ^{1}H NMR (500 MHz, DMSO-d_6, 298 K) spectrum of Compound GP5.

Synthesis of compound NBSPD



Scheme S2: Synthesis of the host molecule NBSPD. [S3-S5]

Synthesis of compound 4

A magnetically stirred solution of bis(2-hydroxyethyl) disulfide (1.00 g, 6.48 mmol) and 6-chlorocaproic acid (2.93 g, 19.44 mmol) in MeCN (30 mL) was treated with DCC (4.01 g, 19.44 mmol) and DMAP (0.08 g, 0.648 mmol), and the ensuing solution was stirred for 72 h at room temperature before being filtrated. The resulting filtrate was concentrated by rotary evaporator to obtain the crude product residue. This residue was dissolved in DCM and then washed with H_2O and sat. NaCl (3 × 10 mL), successively. The combined organic phases were dried (Na_2SO_4) and concentrated under reduced pressure to give the crude product. The crude product was purified by column chromatography, using petroleum ether/ethyl acetate (PE/EA = 10:1, *v/v*) as eluent to afford a white solid (1.85 g, 4.41 mmol, 68 %). The ^{1}H NMR spectrum of 4 was shown in Figure S5. ^{1}H NMR (500 MHz, $CDCl_3$, 298 K) δ (ppm): 4.33 (t, J = 6.55 Hz, 4H), 3.40 (t, J = 7.75 Hz, 4H), 2.92 (t, J = 6.55 Hz, 4H), 2.34 (t, J = 7.45 Hz, 4H), 1.90-1.84 (m, 4H), 1.69-1.62 (m, 4H), 1.51-1.44 (m, 4H). The ^{13}C NMR spectrum of 4 was shown in Figure S6. ^{13}C NMR (125 MHz, $CDCl_3$, 298 K) δ (ppm): 173.3, 62.2, 37.4, 34.0, 33.6, 32.5, 27.7, 24.1.

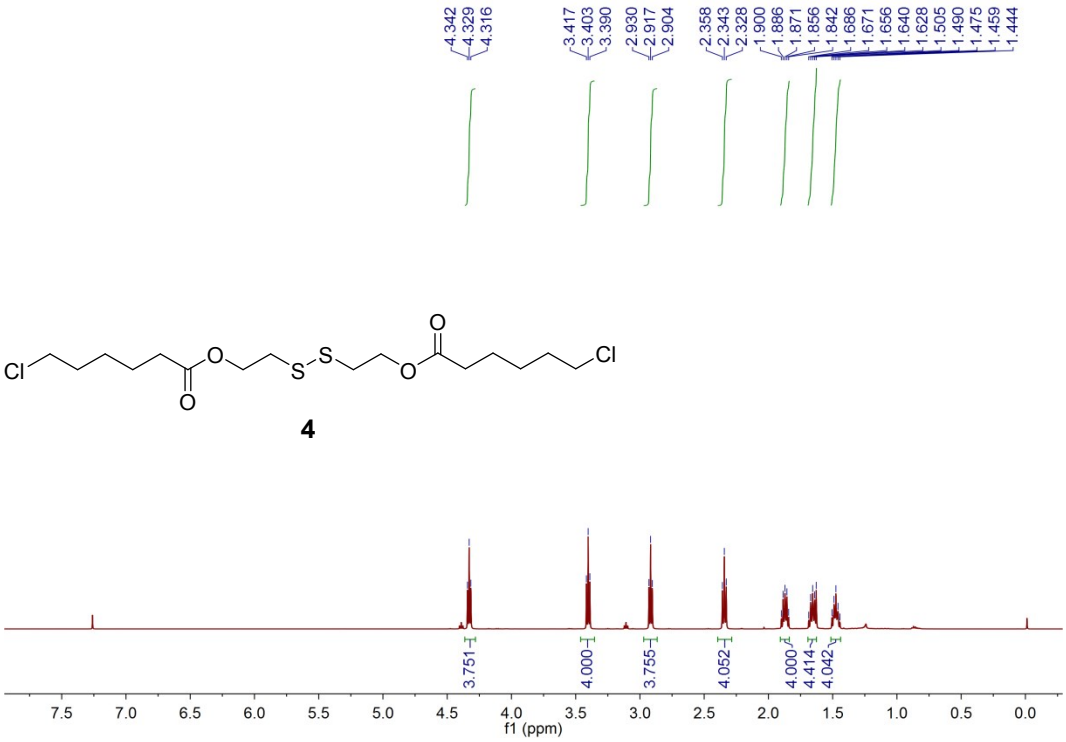


Figure S5: ^{1}H NMR (500 MHz, $CDCl_3$, 298 K) spectrum of Compound 4.

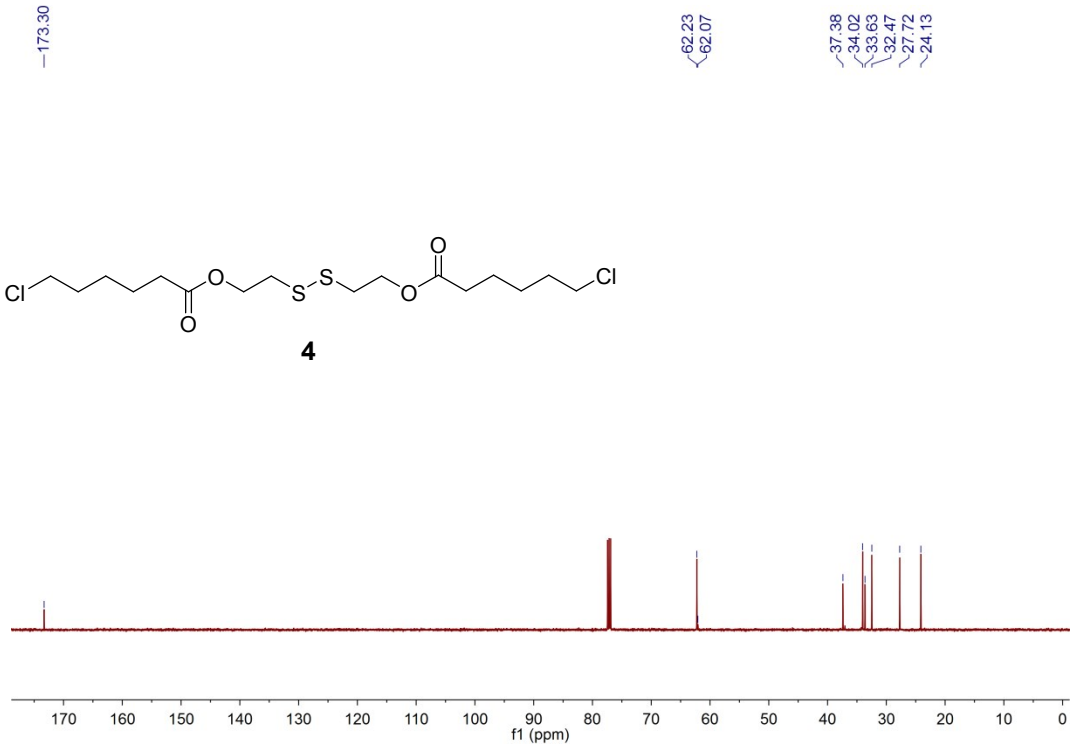


Figure S6: ^{13}C NMR (500 MHz, $CDCl_3$, 298 K) spectrum of Compound 4.

Synthesis of compound 5

A magnetically stirred solution of 1-Naphthylamine (0.143 g, 1.0 mmol) and compound 4 (0.419 g, 1.0 mmol) in MeCN (30 mL) was treated with potassium carbonate (0.205 g, 1.5 mmol) and NaI (0.030 g, 0.20 mmol), and the ensuing solution was stirred for 12 h under reflux before being filtrated. The resulting filtrate was concentrated by rotary evaporator to obtain the crude product residue. This residue was dissolved in DCM and then washed with H_2O and sat. NaCl (3×10 mL), successively. The combined organic phases were dried (Na_2SO_4) and concentrated under reduced pressure to give the crude product. The crude product was purified by column chromatography, using petroleum ether/ethyl acetate (PE/EA = 8:1, *v/v*) as eluent to afford a pale pink solid (0.305 g, 0.58 mmol, 58 %). The ^1H NMR spectrum of 5 was shown in Figure S7. ^1H NMR (500 MHz, $CDCl_3$, 298 K) δ (ppm): 7.81-7.77 (m, 2H), 7.45-7.40 (m, 2H), 7.34 (t, *J* = 15.7 Hz, 1H), 7.21 (d, *J* = 8.2 Hz, 1H), 6.59 (d, *J* = 7.5 Hz, 1H), 4.32 (q, *J* = 6.4 Hz, 4H), 3.38 (t, *J* = 6.8 Hz, 2H), 3.27 (t, *J* = 7.1 Hz, 2H), 2.90 (td, *J* = 6.6, 3.0 Hz, 4H), 2.37 (t, *J* = 7.4 Hz, 2H), 2.32 (t, *J* = 7.4 Hz, 2H), 1.85-1.82 (m, 2H), 1.79-1.76 (m, 2H), 1.74-1.71 (m, 2H), 1.66-1.60 (m, 2H), 1.55-1.49 (m, 2H), 1.47-1.43 (m, 2H). The ^{13}C NMR spectrum of 5 was shown in Figure S8. ^{13}C NMR (125 MHz,

$CDCl_3$, 298 K) δ (ppm): 173.4, 173.2, 143.5, 134.3, 128.7, 126.7, 125.7, 124.6, 123.3, 119.9, 117.1, 104.2, 62.1, 44.0, 37.3, 37.2, 34.1, 33.9, 33.6, 32.4, 29.0, 27.6, 26.8, 24.7, 24.0.

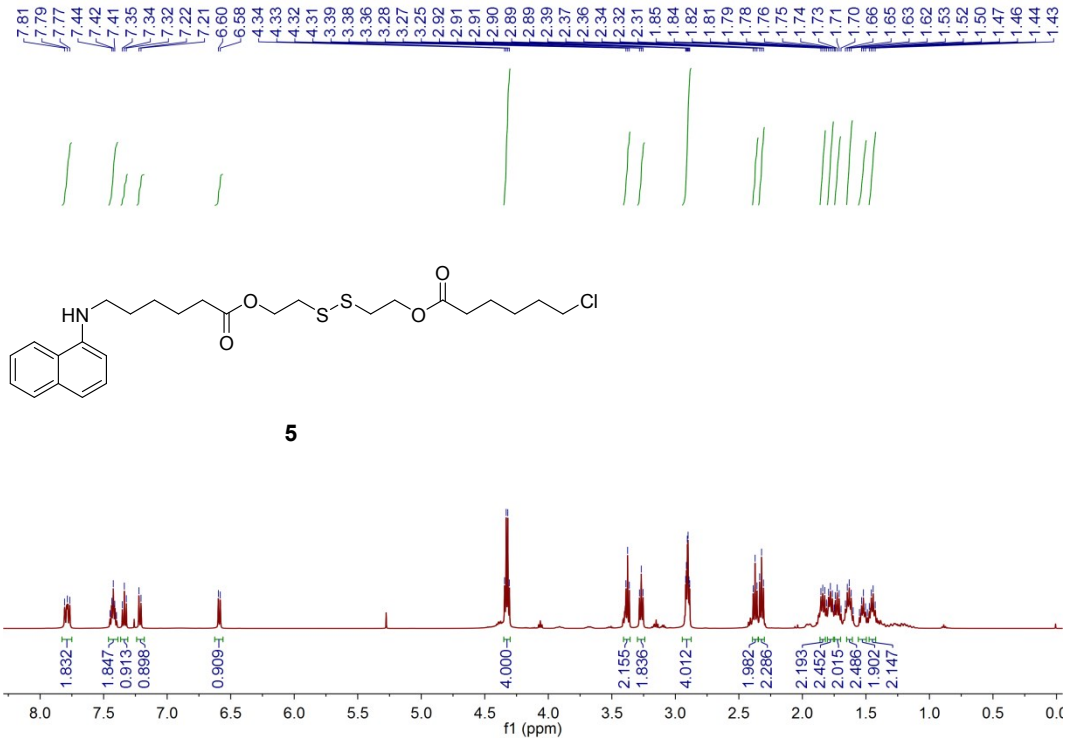


Figure S7: ^{1}H NMR (500 MHz, $CDCl_3$, 298 K) spectrum of Compound 5.

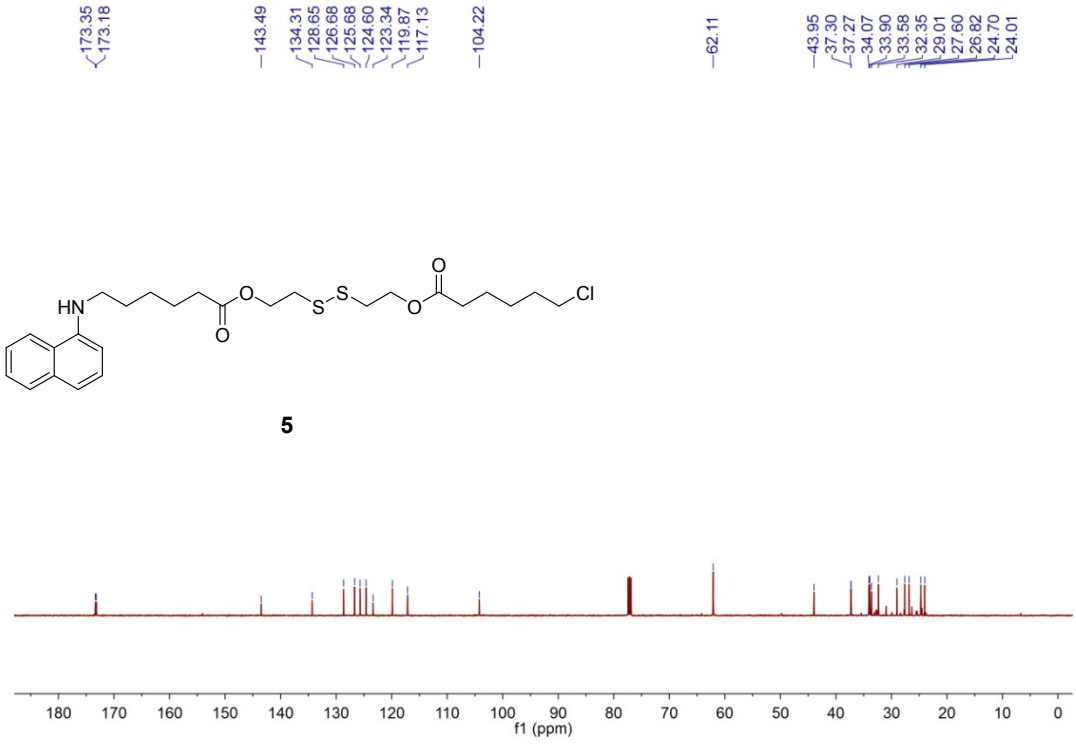


Figure S8: ^{13}C NMR (500 MHz, $CDCl_3$, 298 K) spectrum of Compound 5.

Synthesis of compound 7

Compound 6 was prepared according to previously reported procedures.[S5]

Compound 5 (2.74 g, 5.20 mmol) and compound 6 (1.05 g, 3.80 mmol) were dissolved in DMSO (20 mL), then potassium dichromate (1.2 g, 4.05 mmol) was added. After the mixture was stirred at room temperature for 20 min, 200 mL methanol and 20 mL hydrochloric acid (2 mol/L) were added to above mixture. Then, the mixture was stirred for another 40 min at r.t., and the reaction progress was monitored by TLC. When the reaction was complete, methanol and water were removed under reduced pressure, and the remaining solution was slowly poured into 100 mL saturated sodium chloride. The blue precipitate was filtrated, collected and dried, and crude product was purified by silica gel column chromatography (DCM/MeOH = 6:1, v/v) to give a dark blue solid (1.38 g, 1.82 mmol, 35 %). The ^{1}H NMR spectrum of 7 was shown in Figure S9. ^{1}H NMR (500 MHz, $CDCl_3$, 298 K) δ (ppm): 11.22 (s, 1H), 9.12 (d, *J* = 7.6 Hz, 1H), 8.73-8.71 (m, 1H), 7.74 (d, *J* = 9.4 Hz, 1H), 7.63-7.59 (m, 2H), 7.01 (dd, *J* = 9.4, 2.5 Hz, 1H), 6.8 (s, 1H), 6.75 (d, *J* = 2.5 Hz,1H), 4.29-4.26 (m, 4H), 3.72 (t, *J* = 7.3 Hz, 2H), 3.58 (q, *J* = 7.1 Hz, 4H), 3.49 (t, *J* = 6.6 Hz, 2H), 2.87 (t, *J* = 6.5 Hz, 4H), 2.34-2.28 (m, 4H), 1.90-1.84 (m, 2H), 1.76-1.57 (m, 6H), 1.53-1.47 (m, 2H), 1.45-1.39 (m, 2H), 1.30 (t, *J* = 7.1 Hz, 6H). The ^{13}C NMR spectrum of 7 was shown in Figure S10. ^{13}C NMR (125 MHz, $CDCl_3$, 298 K) δ (ppm): 173.4, 173.2, 154.0, 150.4, 139.7, 136.8, 135.3, 133.3, 131.9, 131.1, 130.1, 125.8, 125.2, 124.8, 116.0, 104.6, 102.7, 62.2, 62.1, 45.8, 44.9, 44.4, 37.3, 37.2, 33.96, 33.2, 28.9, 26.6, 26.4, 24.6, 24.2, 12.9. HRMS (ESI) (Figure S11): m/z calcd. for $C_{36}H_{47}ClN_3O_4S_3^+$ = 716.2412, found m/z = 716.2398.

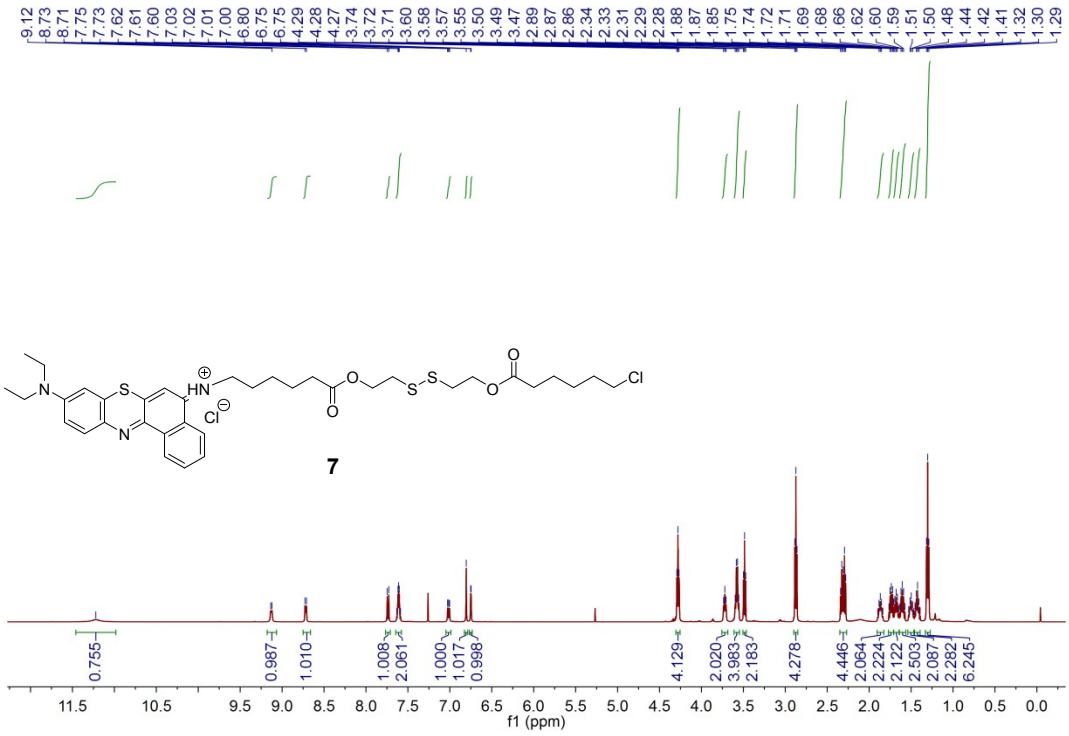


Figure S9: ^{1}H NMR (500 MHz, $CDCl_3$, 298 K) spectrum of Compound 7.

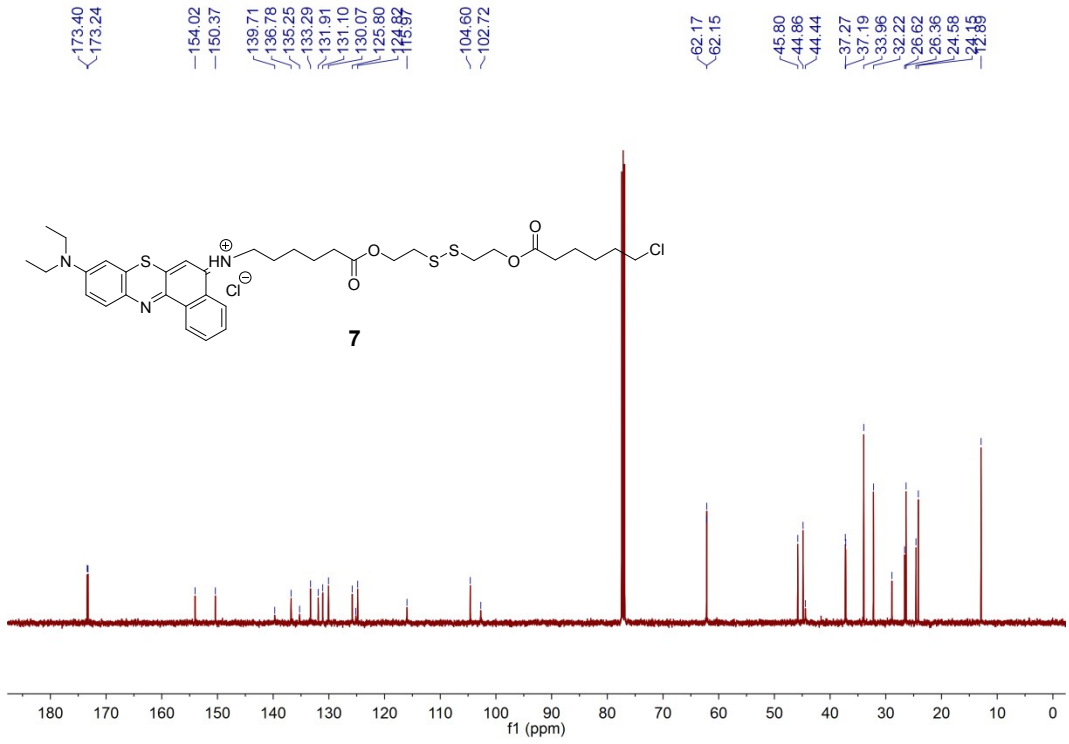


Figure S10: ^{13}C NMR (500 MHz, $CDCl_3$, 298 K) spectrum of Compound 7.

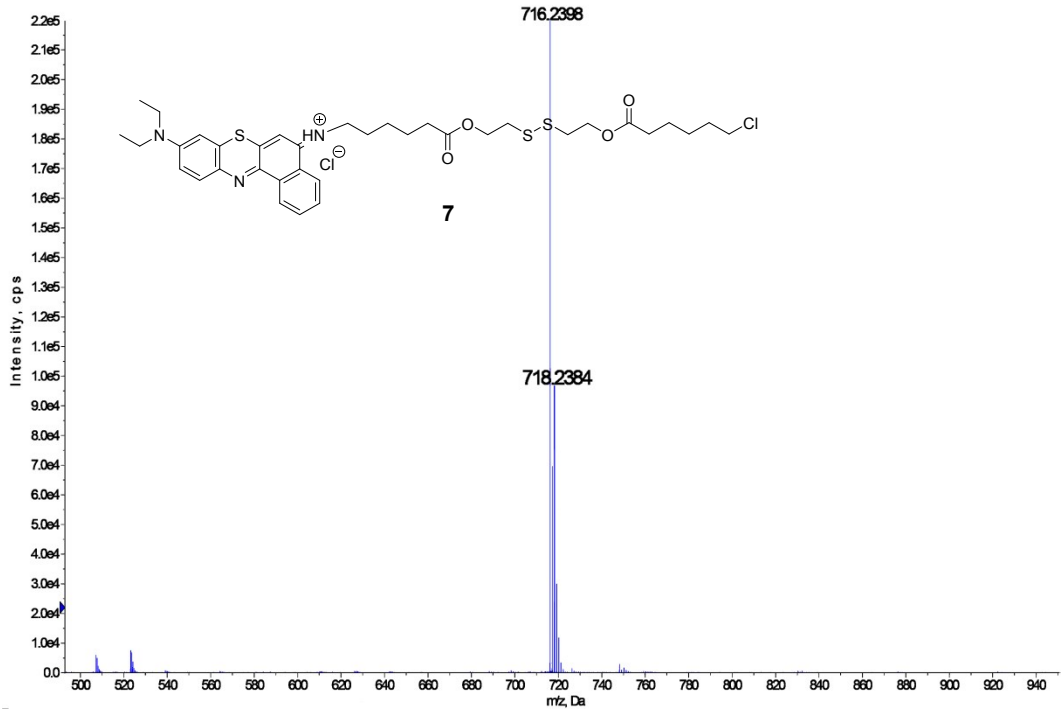


Figure S11: HRMS spectrum of compound 7.

Synthesis of compound NBSPD

Compound 7 (1.50 g, 2.00 mmol) was dissolved in pyridine, the mixture was reflux under nitrogen atmosphere for 24 h before being cooled to room temperature. After removal of the solvent under reduced pressure, the crude product was purified by silica gel chromatography using DCM/MeOH (5:1, *v/v*) as the eluent to afford compound NBSPD as a dark blue solid (1.16 g, 1.40 mmol, 70 %). The ^{1}H NMR spectrum of NBSPD was shown in Figure S12. ^{1}H NMR (500 MHz, DMSO-d_6, 298 K) δ (ppm): 10.85 (s, 1H), 9.22 (m, 2H), 8.96 (d, *J* = 7.2 Hz, 1H), 8.88 (d, *J* = 7.4 Hz, 1H), 8.61 (t, *J* = 7.1Hz, 1H), 8.17 (s, 2H), 7.87 (s, 2H), 7.77 (t, *J* = 7.4 Hz, 1H), 7.51(s, 1H), 7.32 (s, 1H), 4.66 (t, *J* = 7.2 Hz, 2H), 4.26-4.17 (m, 4H), 3.7 (s, 2H), 3.66-3.59 (m, 4H), 2.95-2.91 (m, 4H), 2.35-2.27 (m, 4H), 1.95-1.87 (m, 2H), 1.80-1.73 (m, 2H),1.65-1.49 (m, 4H), 1.47-1.39 (m, 2H),1.30-1.18 (m, 8H). The ^{13}C NMR spectrum of NBSPD was shown in Figure S13. ^{13}C NMR (125 MHz, DMSO-d_6, 298 K) δ (ppm): 172.7, 172.5, 153.1, 150.6, 145.5, 144.8, 136.6, 133.8, 133.1, 131.7, 131.1, 129.4, 128.0, 124.5, 124.4, 120.0, 116.9, 105.3, 103.4, 99.5, 61.7, 61.6, 60.3, 59.4, 45.2, 45.0, 43.7, 41.0, 36.6, 36.4, 33.3, 33.0, 30.4, 28.1, 25.8, 24.7, 24.1, 23.7, 12.6. HRMS (ESI) (Figure S14): m/z calcd. for $C_{41}H_{52}N_4O_4S_3^{2+}$ = 380.1570, found m/z = 380.1603.

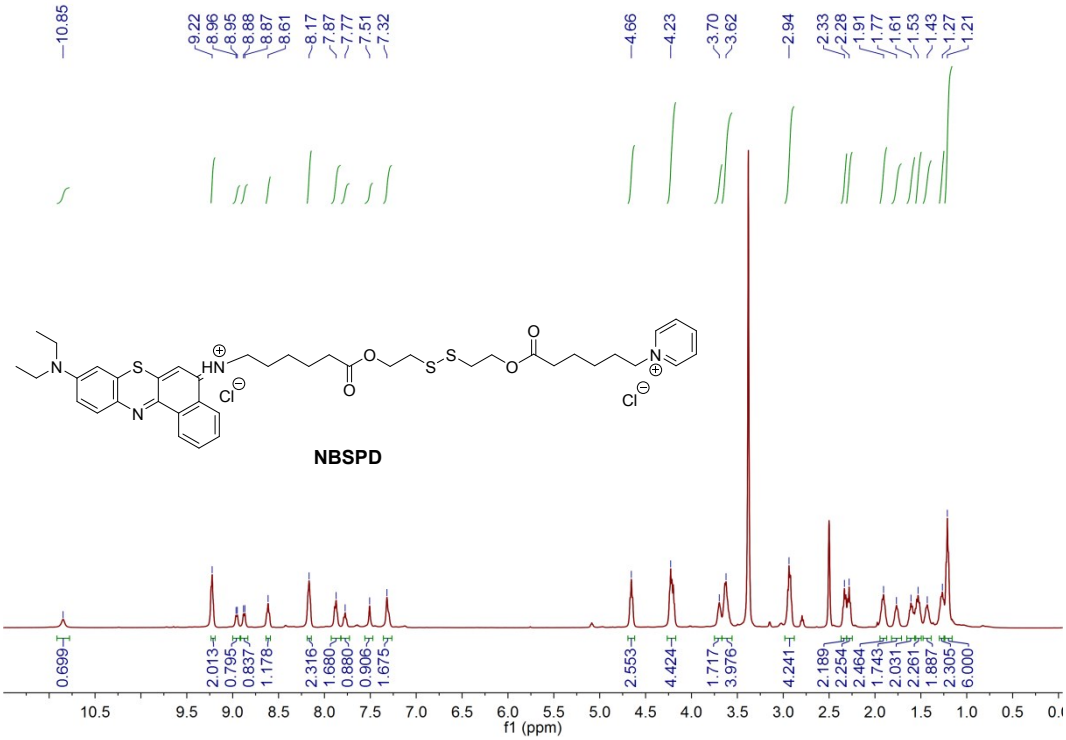


Figure S12: ^{1}H NMR (500 MHz, DMSO-d_6, 298 K) spectrum of Compound NBSPD.

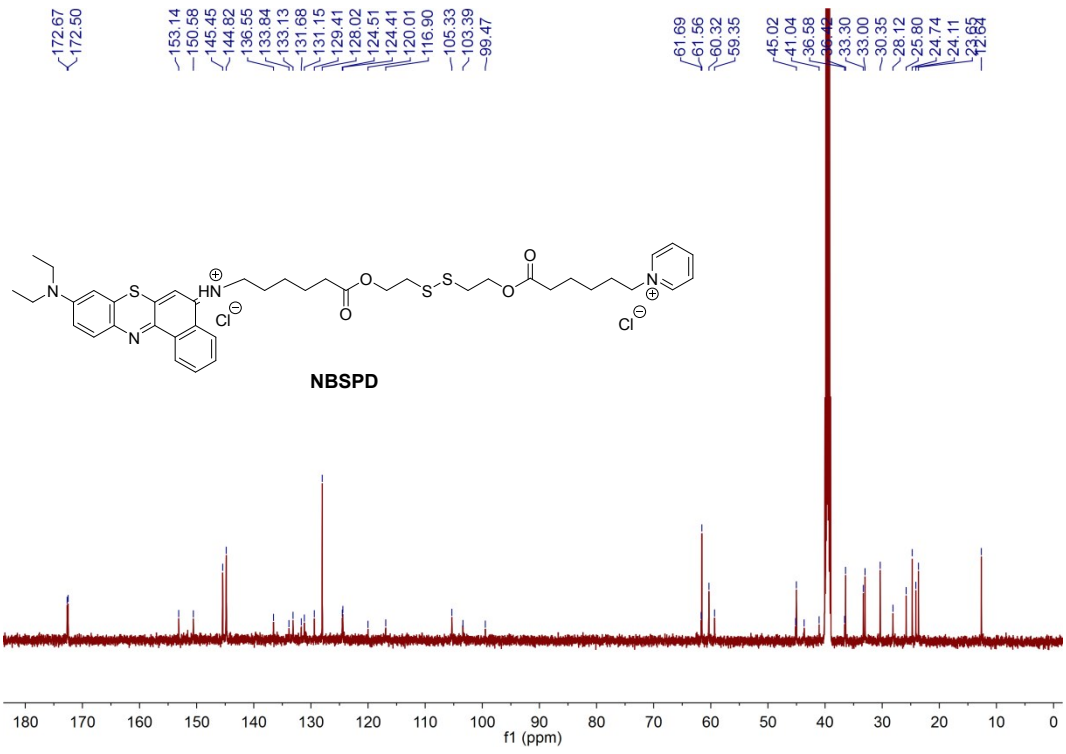


Figure S13: ^{13}C NMR (500 MHz, DMSO-d_6, 298 K) spectrum of Compound NBSPD.

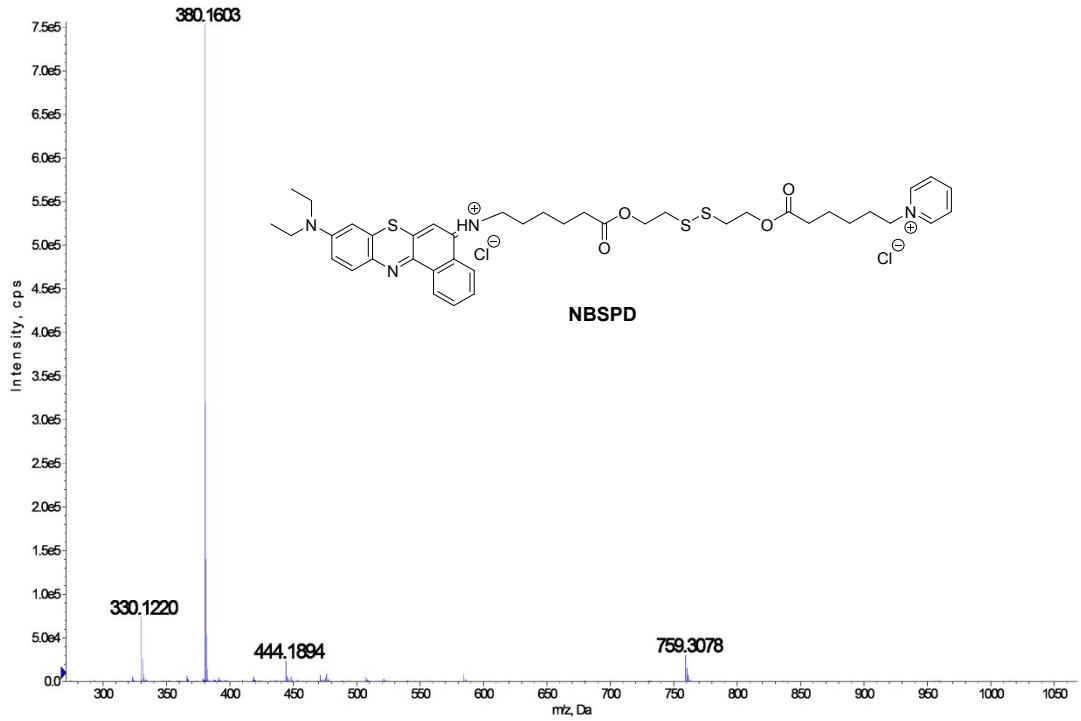


Figure S14: HR-MS spectrum of compound NBSPD.

3. Host-guest complexation of GP5 and NBSPD

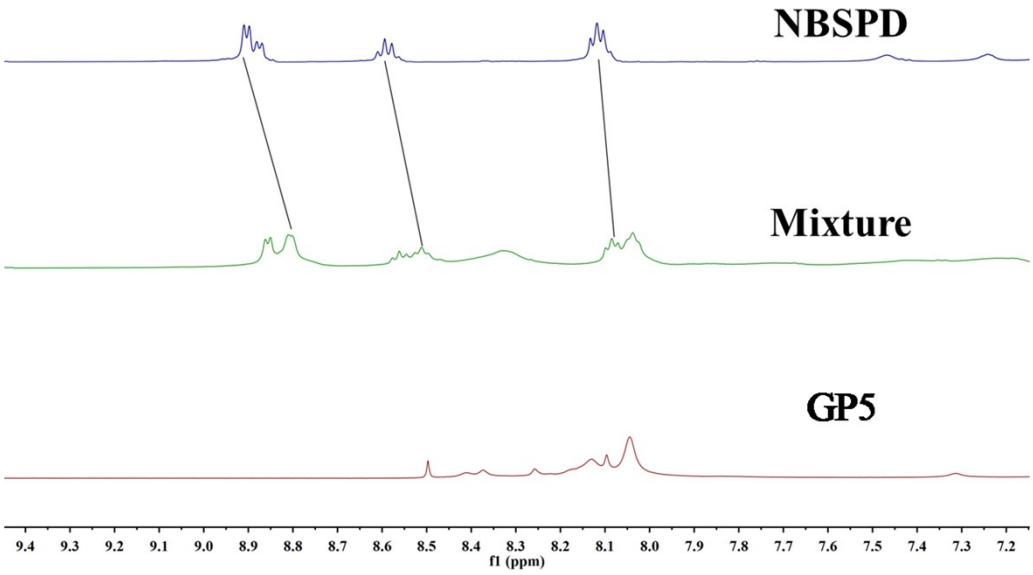


Fig. S15: ^{1}H NMR spectra (500 MHz, D_2O = 1:1, 298 K) of NBSPD (10.00 mM), GP5 + NBSPD (GP5 : NBSPD = 1:1) and GP5 (10.00 mM).

4. Job's Plot for GP5⊃NBSPD

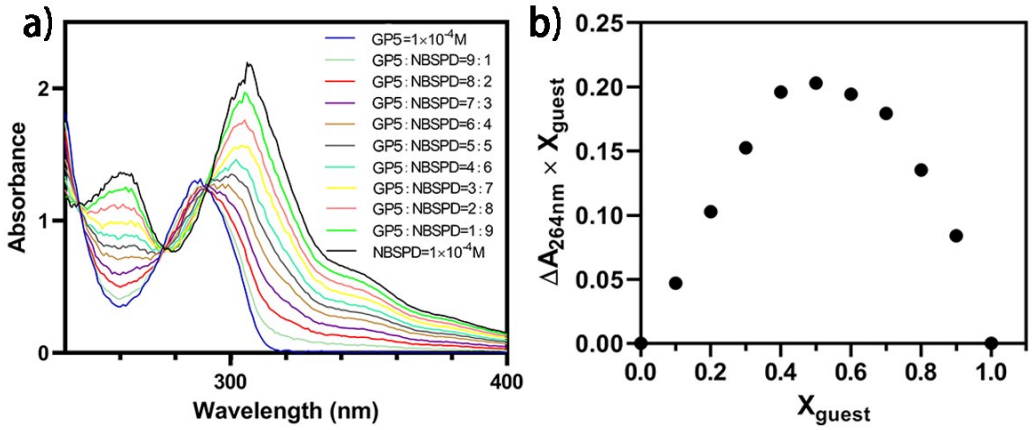


Figure S16: (a) Absorption intensity of the mixture of GP5 and NBSPD in water at different molar ratio while [GP5] + [G] = 1 ×10^{-4} M. (b) Job's Plot showing 1:1 stoichiometry of the complex between GP5 and NBSPD by plotting the difference fluorescence intensity at 264 nm.

5. Raw ITC data of GP5 with NBSPD in water

The binding constant between GP5 and NBSPD was performed using a thermostated and fully computer operated Nano-ITC SV calorimeter purchased from TA-Waters LLC. The microcalorimetric titrations were performed in D.I. water at atmospheric pressure. Each solution was degassed and thermostated before titration. [S6]

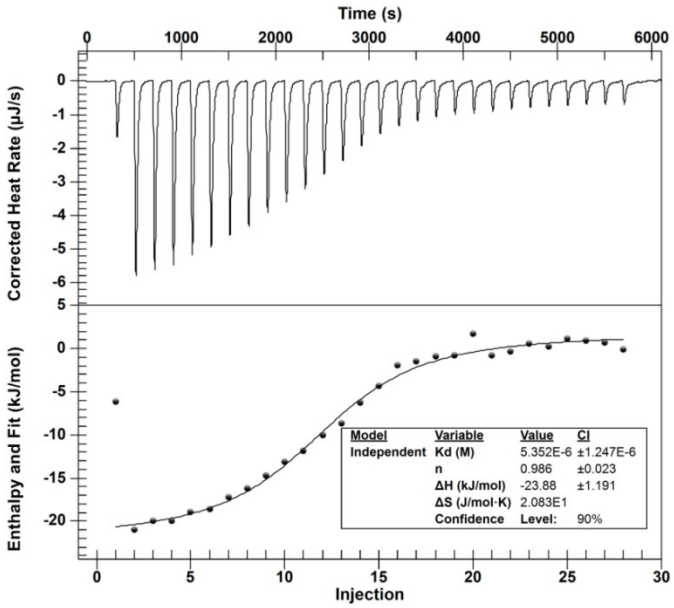


Figure S17: Microcalorimetric titration of GP5 and NBSPD in D.I. water at 298.15K. (TOP) Raw ITC data for 28 sequential injections (3.54 μL per injection) of a GP5 solution (2.00 mM) into a NBSPD solution (0.20 mM). (Bottom) Net reaction heat obtained from the integration of the calorimetric traces.

6. Tyndall effect and TEM Image of GP5⊃NBSPD NPs

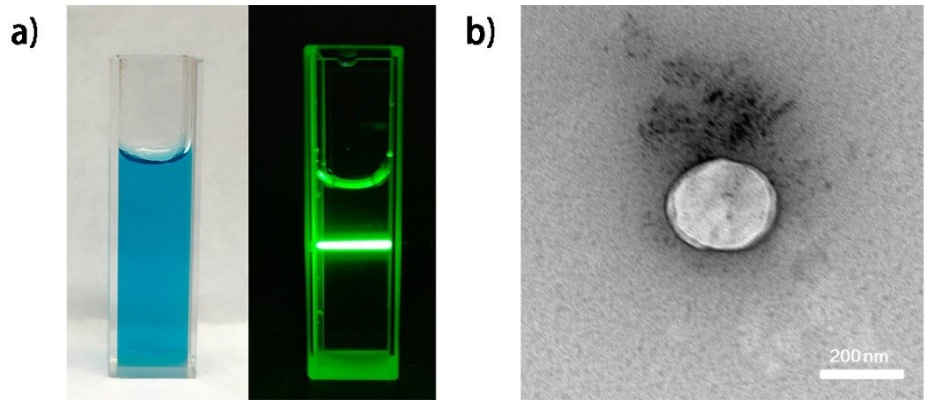


Figure S18: The obvious Tyndall effect and TEM image of GP5⊃NBSPD NPs

7. The Fluorescence Curves of ROS Detection

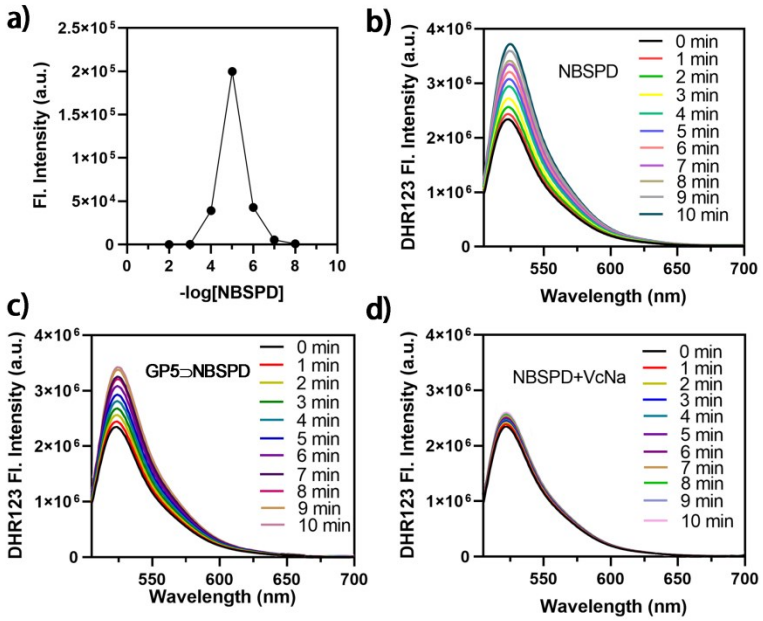


Figure S19: a) The relationship curves between fluorescence intensity and concentration of NBSPD; b-d) $O_2^{-\bullet}$ production and characterization using DHR123 as fluorescence probe: Fluorescence spectra of DHR123 (10 μM) induced by b) NBSPD (10 μM), c) GP5⊃NBSPD (10 μM) and d) NBSPD (10 μM) + VcNa (50 μM) after 630 nm light irradiation (20 mW/cm^2). [S5]

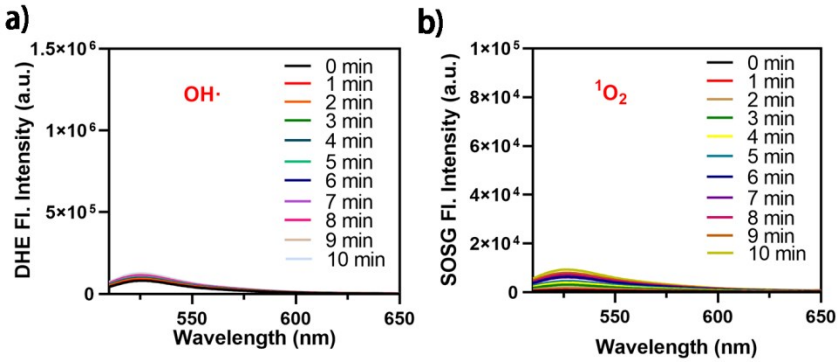


Figure S20: Fluorescence curves of a) HPF (10 μM) for OH· characterization and b) SOSG (10 μM) for $^1O_2$ detection induced by NBSPD (10 μM). [S5]

8. Confocal laser scanning microscopy (CLSM)

Generally, HepG2 or HL7702 cells were plated onto 35 mm confocal dishes and incubated at 37 ℃ under 5% CO_2 for 24 h. Liquid paraffin covering method was used to simulate the tumor hypoxic environment. Liquid paraffin was added on the surface of cell culture medium and incubated at 37 ℃ under 5% CO_2 for 24 h. Dihydroethidium (DHE) (λ_{ex} = 535 nm, λ_{em} = 610 nm), hydroxyphenyl fluorescein (HPF) (λ_{ex} = 490 nm, λ_{em} = 515 nm) and singlet oxygen sensor green (SOSG) (λ_{ex} = 504 nm, λ_{em} = 525 nm) as the specific indicators for $O_2^{-\bullet}$, OH· and $^1O_2$ in intracellular. The anaerobic indicator ROS-ID was used to prove intracellular hypoxia. In addition, HepG2 or HL7702 cells e incubated with 1 μM GP5⊃NBSPD NPs for 0.5 h followed by incubation with 10 μM SOSG for 30 min, 10 μM HPF for 60 min or 1 μM SOSG for 30 min. After that, cells were washed with PBS and then irradiated with 630 nm red LED light for 12.5 min at a power density of 20 mW/cm^2. The fluorescence was immediately observed using CLSM.

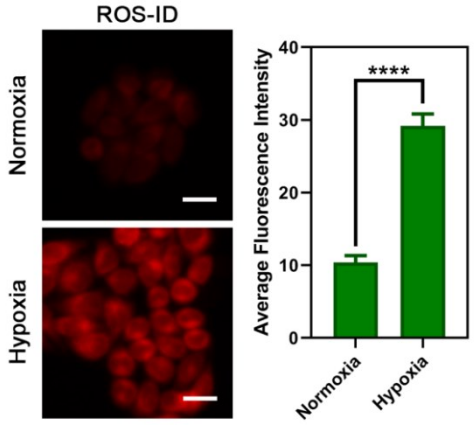


Figure S21: Intracellular hypoxia imaging using ROS-ID as anaerobic indicator. The scale bar is 20 μm.

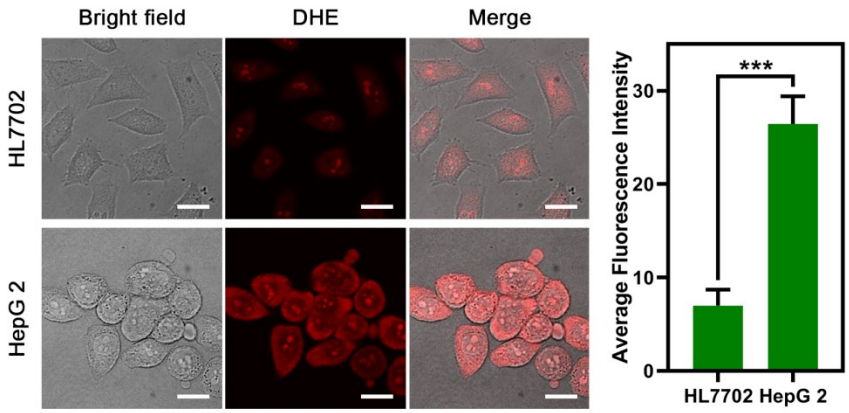


Figure S22: Comparison of intracellular $O_2^{-\bullet}$ generation in HL7702 and HepG2 cells. The scale bar is 20 μm.

9. Preparation of GP5⊃NBSPD and Loading DOX

Firstly, GP5 (0.01 mmol) and NBSPD (0.01 mmol) were dissolved in 10 mL distilled water, and the resultant suspension was sonicated for 30 min at room temperature. Subsequently, the mixture was stirred for 12 h at room temperature to obtain GP5⊃NBSPD NPs. In addition, Doxorubicin hydrochloride (DOX) was loaded into GP5⊃NBSPD NPs. A standard curve of DOX has been drawn. Both DOX, GP5 and NBSPD were dissolved in distilled water with the ratio of 1:1:1 to prepare DOX loaded GP5⊃NBSPD NPs (DOX@GP5⊃NBSPD). The resultant mixture was sonicated for 30 min at room temperature. Subsequently, the mixture was stirred for 12 h at room temperature under dark circumstance. After that, the unloaded DOX was removed by dialysis (molecular weight cut-off (MWCO) = 2,000 Da) against distilled water for 24 h until water outside the dialysis tube exhibited negligible DOX fluorescence. The DOX loading capability was calculated using the following equation:

$$\text{Loading Capability (\%)} = (m_{\text{DOX loaded}}/m_{\text{DOX@GP5⊃NBSPD}}) \times 100\%$$

$m_{\text{DOX loaded}}$ and $m_{\text{DOX@GP5⊃NBSPD}}$ refer to the mass of DOX encapsulated in GP5⊃NBSPD and the mass of the DOX@GP5⊃NBSPD, respectively.

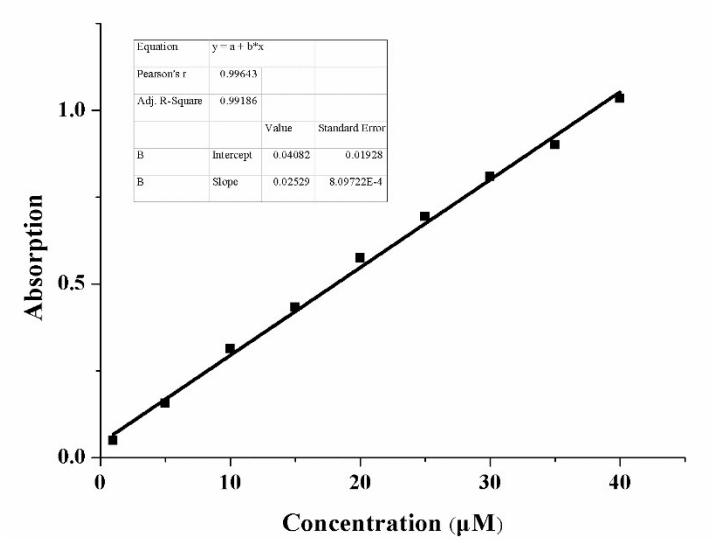


Figure S23: The standard curve of DOX (λ abs = 494 nm).

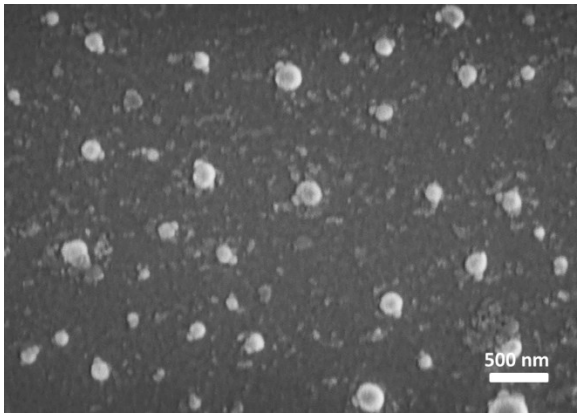


Figure S24: SEM image of DOX@GP5⊃NBSPD NPs.

10. Stimuli-Responsive Behaviour of the GP5⊃NBSPD NPs

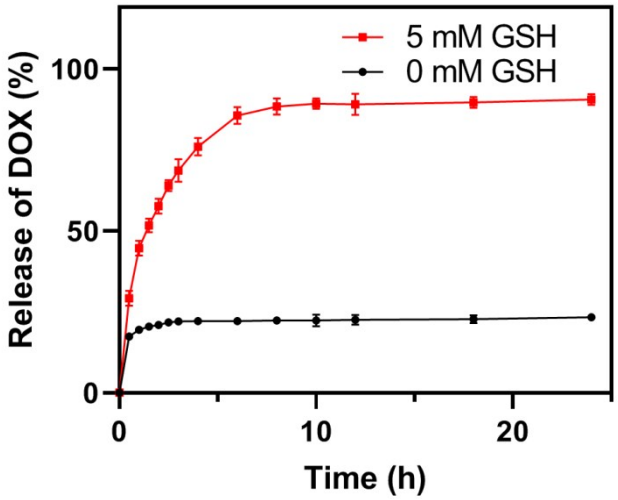


Figure S25: DOX release profiles from the GP5⊃NBSPD NPs in PBS of 0 mM and 5mM GSH (pH=7.4).

11. Flow Cytometry

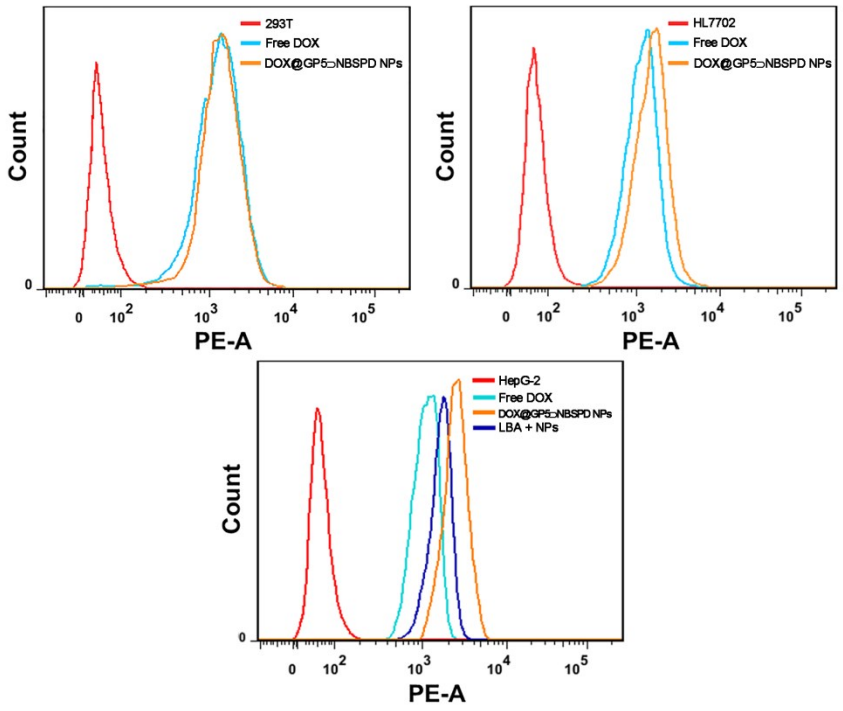


Figure S26: Flow cytometry analyses of 293T, HL7702 and HepG2 cells.

12. Cytotoxicity Evaluation

HepG2 and HL7702 cells were cultured in RPMI 1640 medium containing 10% FBS, 1% penicillin/streptomycin (complete RPMI 1640 medium) in 5% CO_2 at 37 °C. Liquid paraffin covering method was used to simulate the tumor hypoxic environment. The relative cytotoxicity of free DOX, GP5⊃NBSPD NPs and DOX@GP5⊃NBSPD NPs were evaluated *in vitro* by MTT assay, respectively. The cells were seeded in 96-well plates. Liquid paraffin was added on the surface of cell culture medium and incubated at 37 ℃ under 5% CO_2 for 24 h to obtain hypoxic state cells. The dark toxicity of GP5⊃NBSPD NPs was demonstrated on HepG2 and HL7702 cells for 24 h. The phototoxicity of free DOX, GP5⊃NBSPD NPs and DOX@GP5⊃NBSPD NPs were demonstrated on HepG2 cells presence of light irradiation (15 J/cm^2) under normoxia and hypoxia conditions for 24 h. Subsequently, cells were washed and the fresh medium containing MTT (0.5 mg/mL) was added into each plate. The cells were incubated for another 4 h. After that, the medium containing MTT was removed and dimethyl sulfoxide (100 μL) was

added to each well to dissolve the formazan crystals. Finally, the plate was gently shaken for 10 min and the absorbance at 490 nm was recorded with a microplate reader.

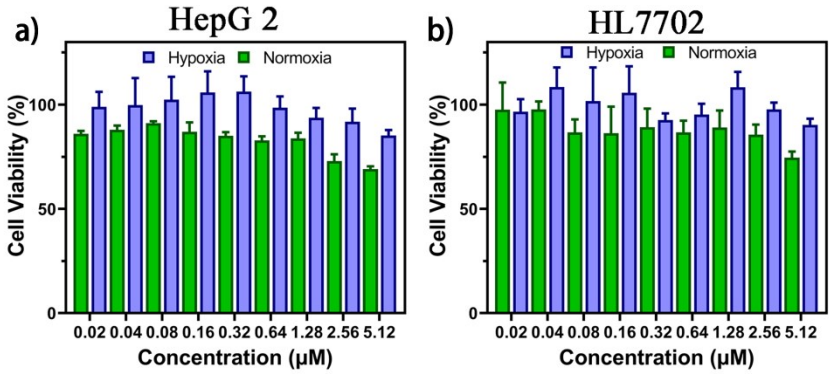


Figure S27: The dark toxicity of GP5⊃NBSPD NPs on HepG2 (a) and HL7702 (b) cells presence of light irradiation under normoxia (a) and hypoxia (b) conditions for 24 h.

13. References

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