Therapeutic nanosystem consisting of singlet-oxygen-responsive prodrug and photosensitizer excited by two-photon light

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Supporting Information

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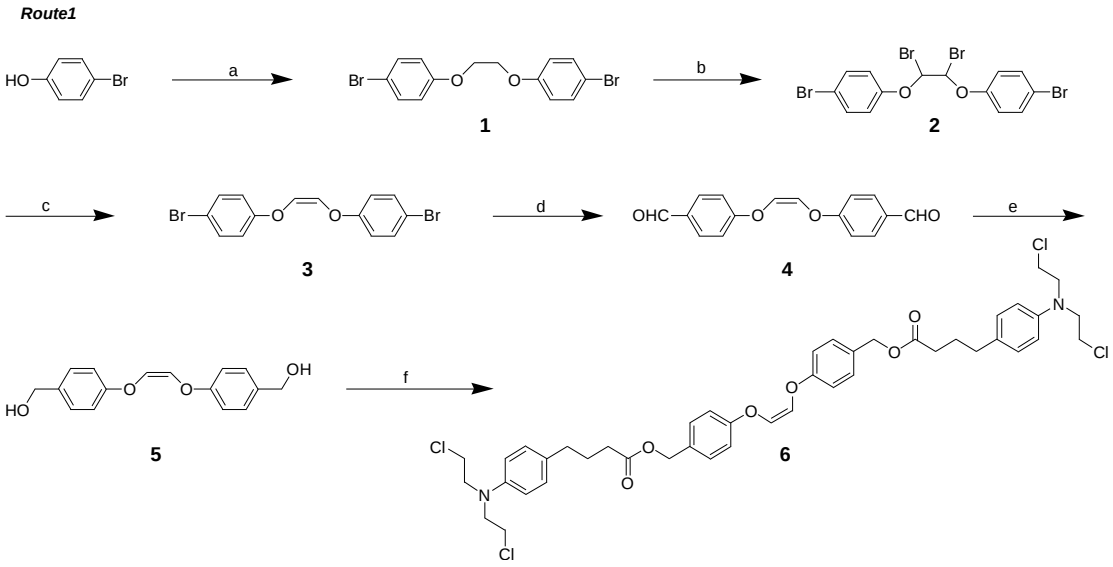
1. Experimental Materials and Synthesis

1.1 Materials and Reagents:

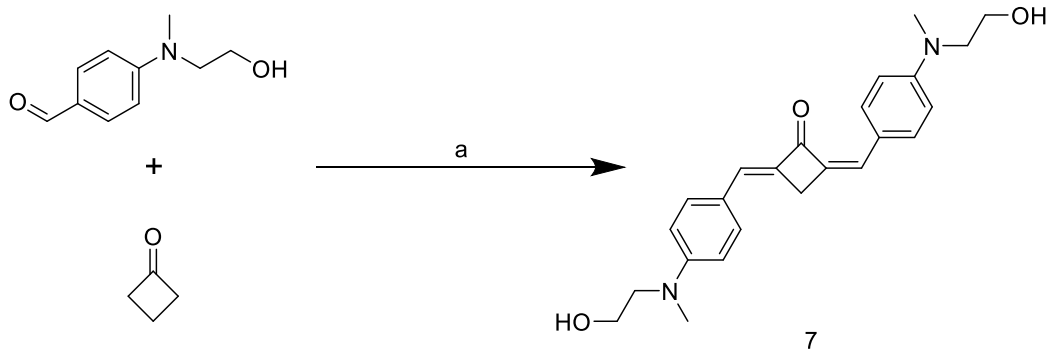
4-Bromophbenol, 1,2-dibromoethane, N-bromosuccinimide (NBS), benzoyl peroxide, odium iodine, n-butyllithium (n-BuLi), sodium borohydride, carbodiimide hydrochloricde (EDC), 4-dimethylaminopyridine (DMAP) and N-methyl-N-(2-hydroxyethyl)-4-aminobenzaldehyde were purchased from Sigma-Aldrich Reagents without further purification. The solvents including carbon tetrachloride, acetone, dichloromethane (DCM) and N,N-dimethyl-formamide (DMF) was dried with 5A molecular sieve and distilled under nitrogen atmosphere. Other solvents used in this study were analytical grade reagents and used without further purification.

1.2 Synthesis



Scheme S1. Synthetic route for the prodrug (compound **6)**. *Reagents, conditions and yeilds:* a) NaOH, H_2O, 65-100℃, 6h, yield 54%; b) NBS, BPO, CCl_4, reflux, 4h, yield 21%; c) NaI, acetone, r.t.,2 h, yield 87%; d) t-BuLi, DMF,, THF, -78℃ to r.t., 3 h, yield 53%; e) $NaBH_4$, MeOH, r.t., 14 h, yield 95%; f) DMAP, EDC, CHB, r.t., 6h, yield 58%.

Route2



Scheme S2. Synthetic route for the photosensitizer (the PS, compound **7)**. *Reagents and Conditions:* a) NaOH, EtOH, r.t., 12h, yield 61%.

Compounds **1-5** were synthesized according to previously reported methods.[1]

Compound 1 (white solid, 54%). ^{1}H NMR (600 MHz, Chloroform-d) δ 7.39 (d, J = 9.0 Hz, 4H), 6.82 (d, J = 9.0 Hz, 4H), 4.27 (s, 4H).

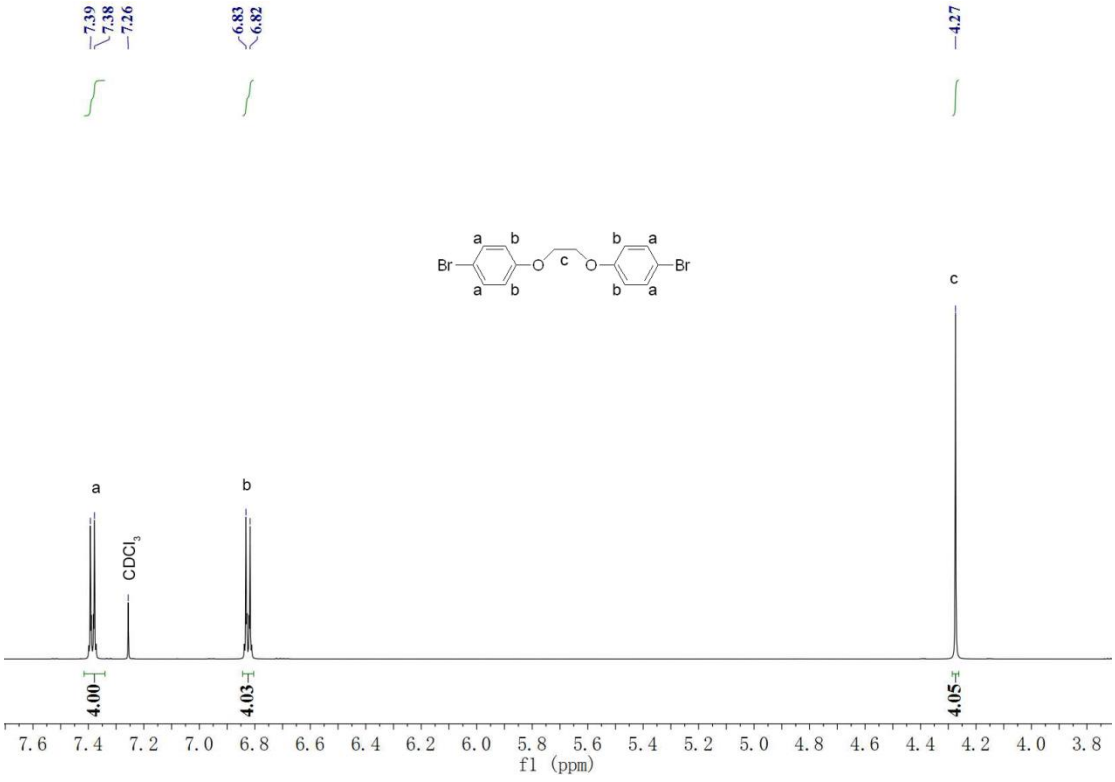


Figure S1. ^{1}H NMR spectrum of **1** (in $CDCl_3$).

Compound 2 (white solid, 20%). ^{1}H NMR (600 MHz, Chloroform-d) δ 7.53 (d, J = 8.8 Hz, 4H), 7.13 (d, J = 8.8 Hz, 4H), 6.47 (s, 2H).

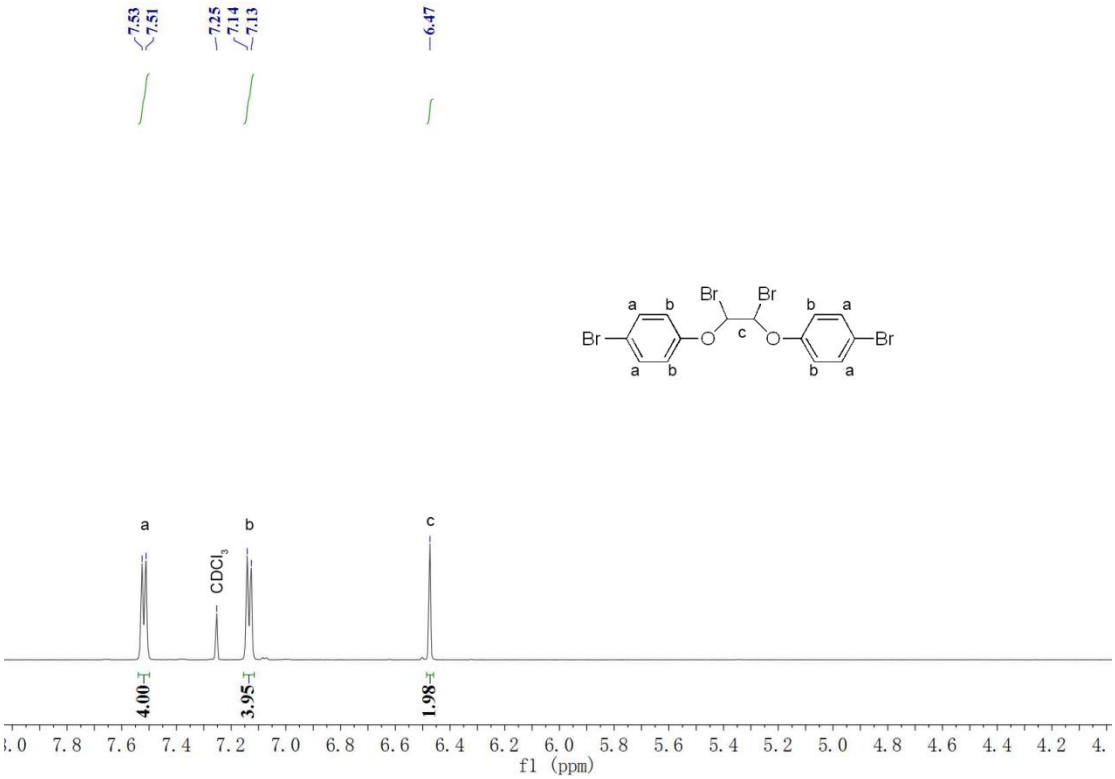


Figure S2. ^{1}H NMR spectrum of **2** (in $CDCl_3$).

Compound 3 (white solid, 90%). ^{1}H NMR (600 MHz, Chloroform-d) δ 7.42 (d, J = 9.0 Hz, 4H), 6.97 (d, J = 9.0 Hz, 4H), 6.12 (s, 2H).

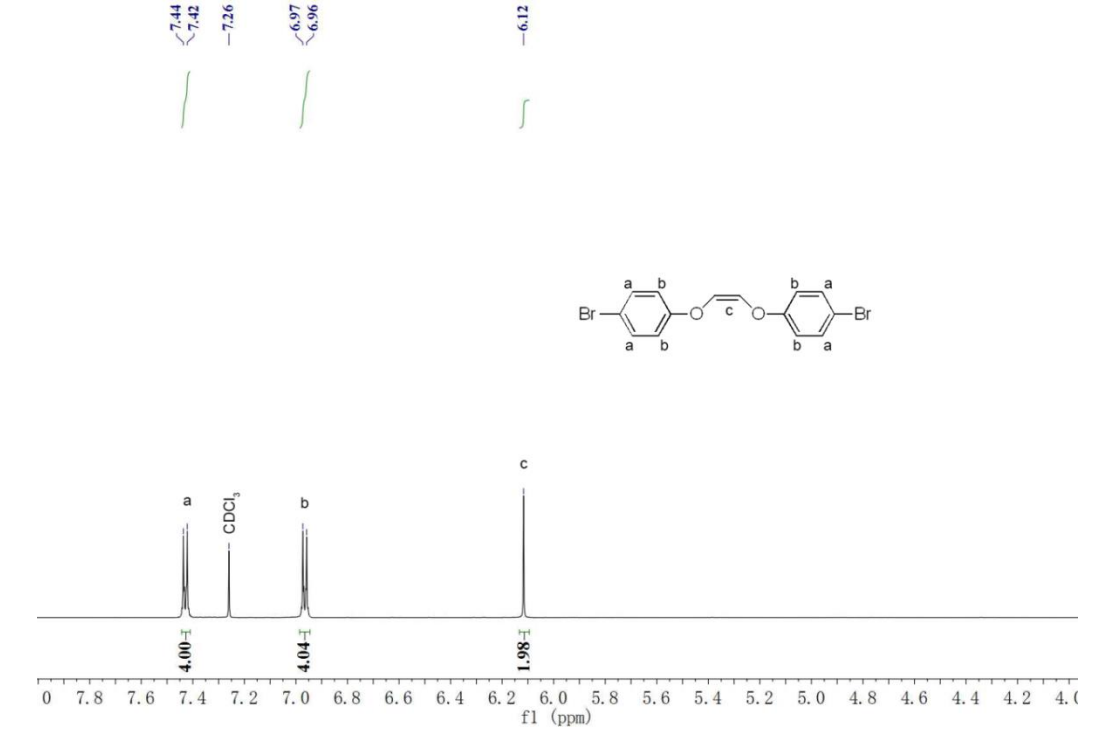


Figure S3. ^{1}H NMR spectrum of **3** (in $CDCl_3$).

Compound 4 (white solid, 50%) . ^{1}H NMR (600 MHz, Chloroform-*d*) δ 9.95 (s, 2H), 7.90 (d, *J* = 8.7 Hz, 4H), 7.18 (d, *J* = 8.7 Hz, 4H), 7.04 (s, 2H).

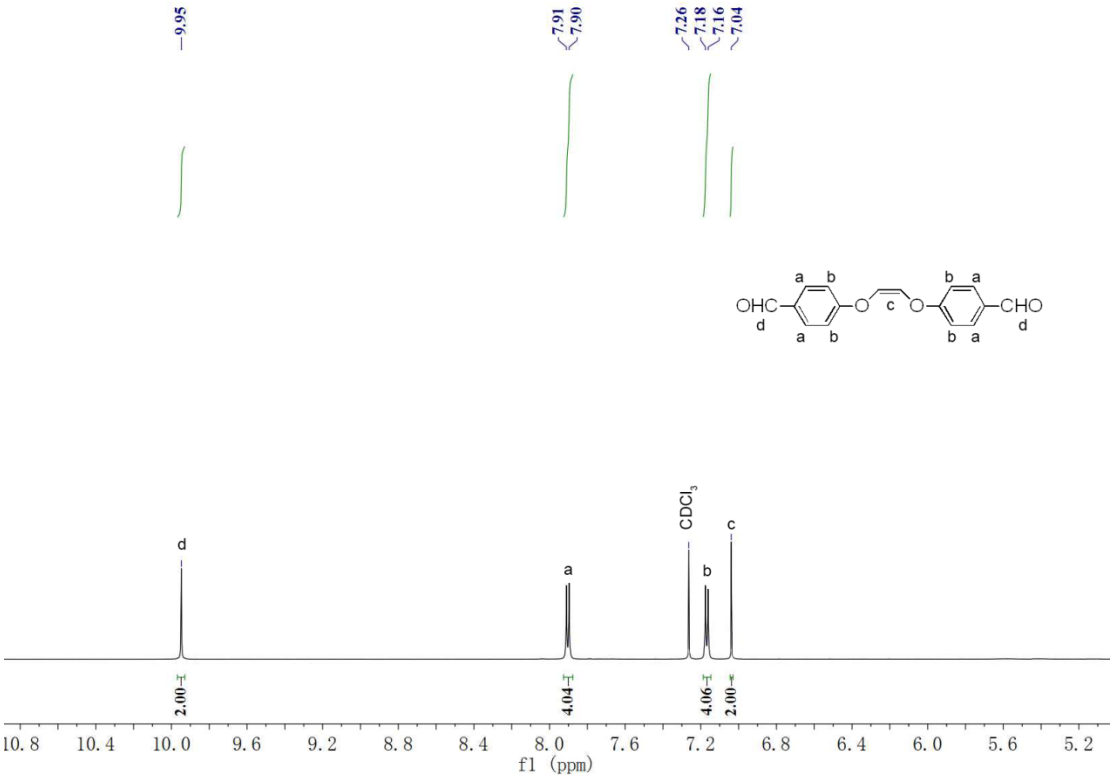


Figure S4. ^{1}H NMR spectrum of **4** (in $CDCl_3$).

Compound 5 (white solid, 95%). ^{1}H NMR (600 MHz, Chloroform-*d*) δ 7.33 (d, *J* = 6.9 Hz, 4H), 7.08 (d, *J* = 8.6 Hz, 4H), 6.15 (s, 2H), 4.65 (s, 4H).

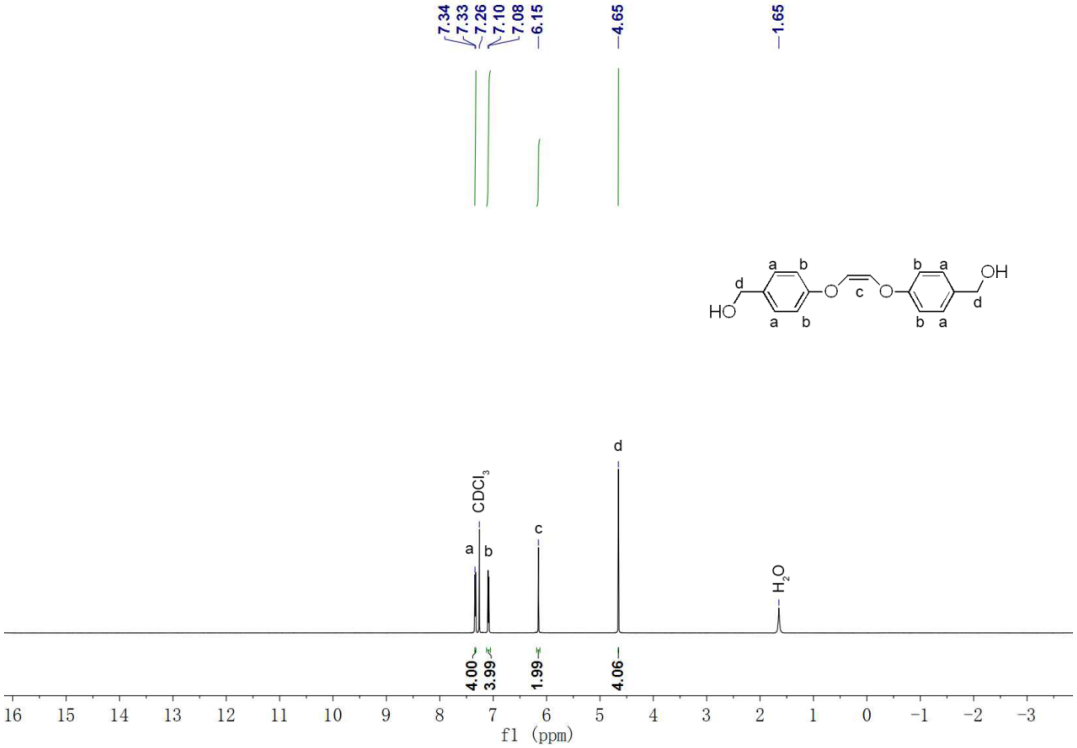


Figure S5. ^{1}H NMR spectrum of **5** (in $CDCl_3$).

1.2.1 Synthesis of ((ethene-1,2-diylbis(oxy))bis(4,1-phenylene))bis(methylene) bis(4-(4-(bis(chloromethyl)amino)phenyl)butanoate) (6, the prodrug):

111.76 mg (0.367 mmol) of chlorambucil, 50 mg (0.184 mmol) of **5**, 8.18 mg (0.067 mmol) of DMAP and 191.7 mg (1 mmol) of EDC were added into 10 mL of DCM. After stirring for 6 h at room temperature, the solvent was evaporated and the crude product was purified by column chromatography (30:1 methanol/dichloromethane) to yield **6** (mike white oil, 58%). HPLC retention time: 1.83 min; purity, 97.52%. ^{1}H NMR (600 MHz, Chloroform-*d*) δ 7.33 (d, *J* = 8.6 Hz, 4H), 7.08 (d, *J* = 8.6 Hz, 4H), 7.05 (d, *J* = 8.4 Hz, 4H), 6.63 (d, *J* = 8.5 Hz, 4H), 6.16 (s, 2H), 5.06 (s, 4H), 3.69 (t, J = 7.0 Hz, 8H), 3.61 (t, J = 6.9 Hz, 8H), 2.54 (t, J = 7.6 Hz, 4H), 2.35 (t, J = 7.5 Hz, 4H), 1.91 (m, 4H). HR-MS (ESI): calculated for $C_{44}H_{51}Cl_4N_2O_6^+$ ([M+H]$^+$) 843.2490, found: 843.2498.

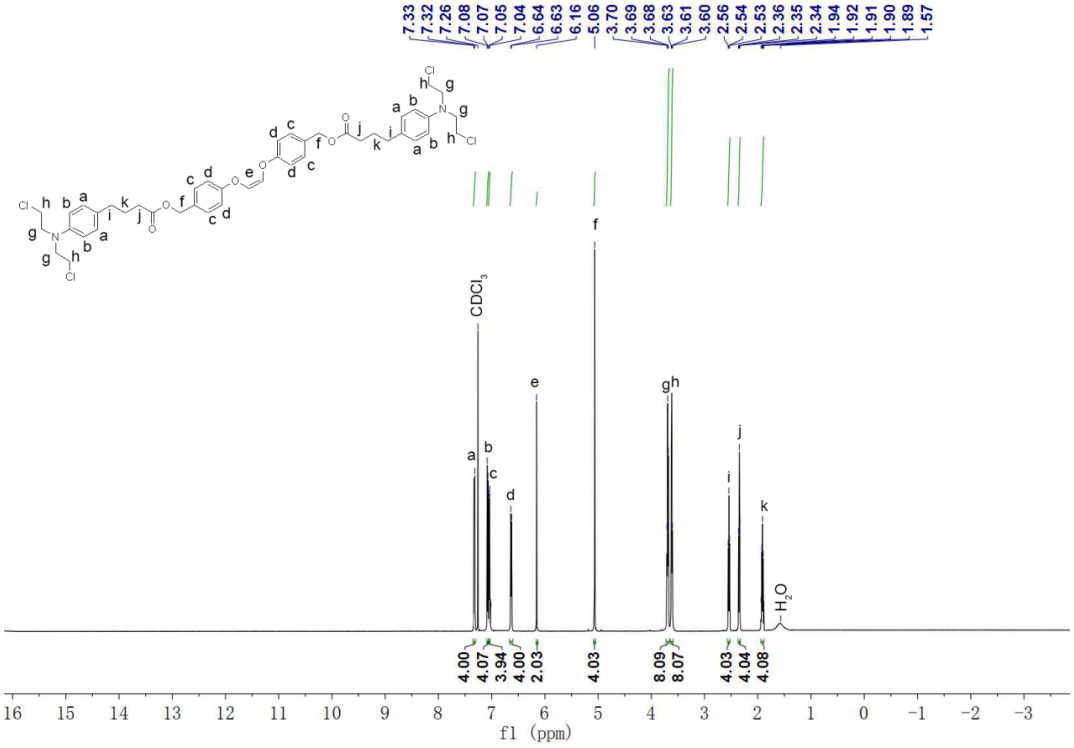


Figure S6. ^{1}H NMR spectrum of **6** (in $CDCl_3$).

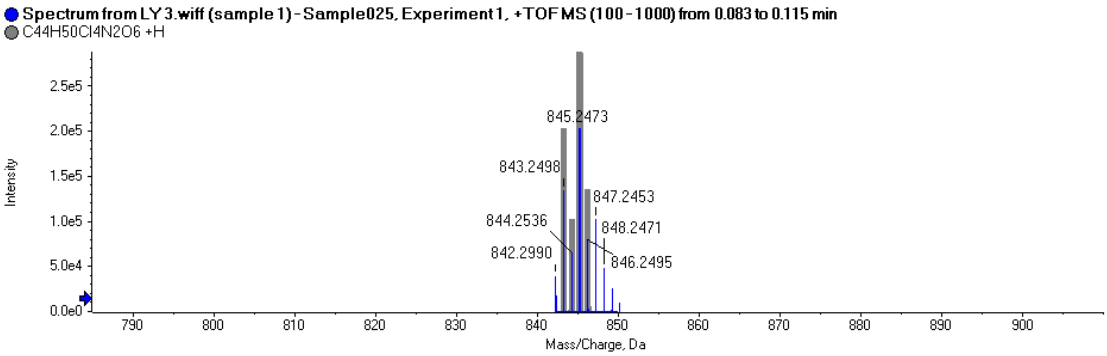


Figure S7. HR-MS (ESI) spectrum of **6**. m/z ([M+H]$^+$) 843.2498.

1.2.2 Synthesis of 2,4-bis(4-((2-hydroxyethyl)(methyl)amino)benzylidene) cyclobutanone (7, the photosensitizer):

350.45 mg (5 mmol) of cyclobutanone and 1.969 g (11 mmol) of N-methyl-N-(2-hydroxyethyl)-4-aminobenzaldehyde were dissolved in 50 mL of ethanol containing 0.2 g of NaOH as catalyst. The mixture was stirred at room temperature for 12 h, and then the solvent was evaporated. The residue was purified by silica gel column chromatography (50:1 ethyl acetate/methanol) as the eluent to yield deep-red solid **7** (61%). HPLC retention time: 2.06 min; purity, 98.76%. ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.48 (d, J = 8.9 Hz, 4H), 7.00 (s, 2H), 6.76 (d, J = 8.9 Hz, 4H), 4.75 (t, J = 5.4 Hz, 2H), 3.70 (s, 2H), 3.57 (m, 4H), 3.48 (m, 4H), 3.35 (s, 6H). HR-MS (ESI): calculated for $C_{24}H_{29}N_2O_3^+$ ([M+H]$^+$) 393.2173, found: 393.2169.

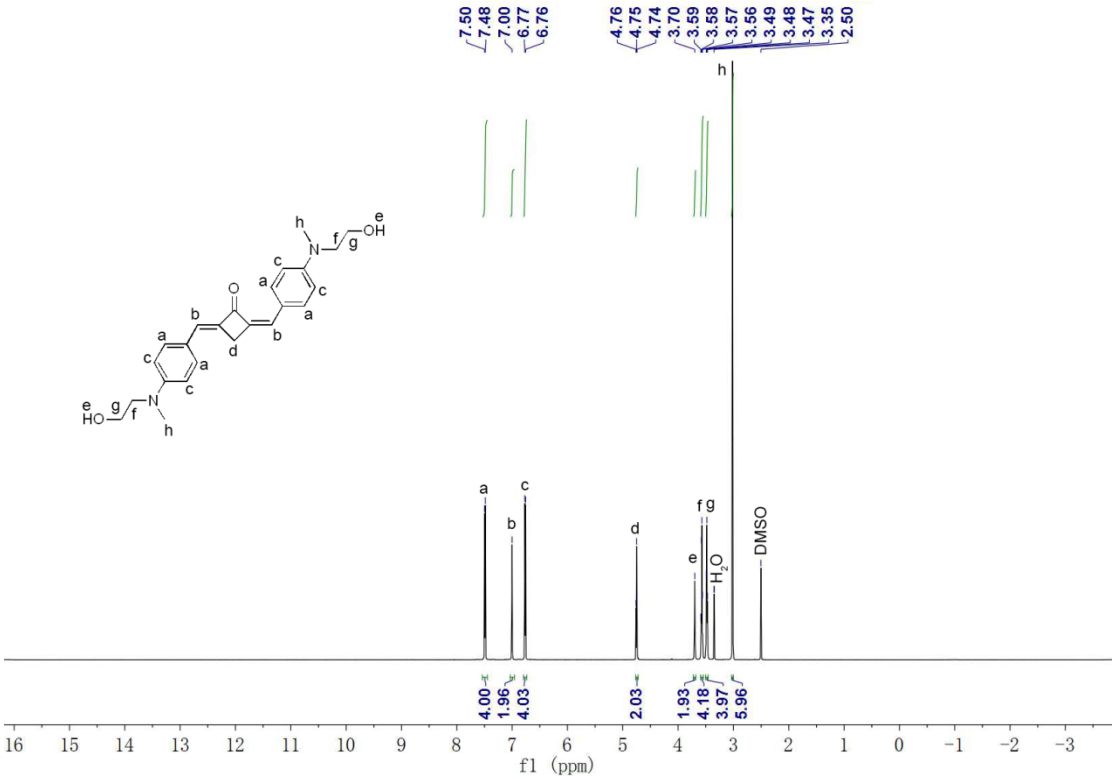


Figure S8. ^{1}H NMR spectrum of **7** (in DMSO-d6).

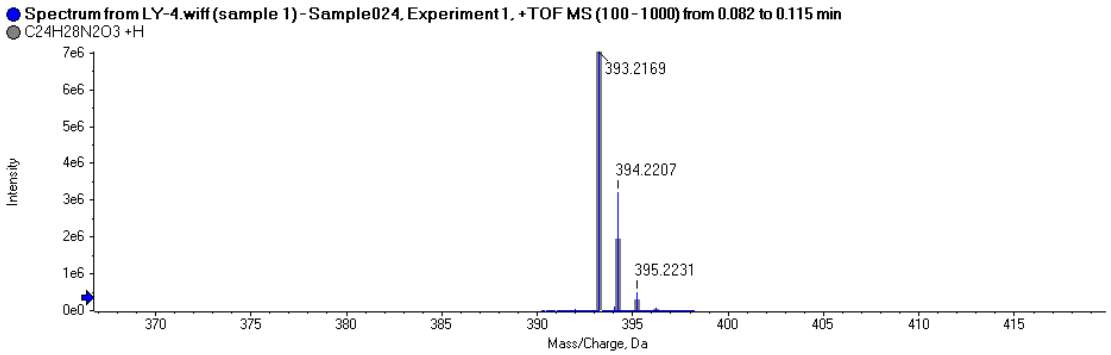


Figure S9. HR-MS (ESI) spectrum of **7**. m/z ([M+H]$^+$) 393.2169.

1.3 Characterization and spectra

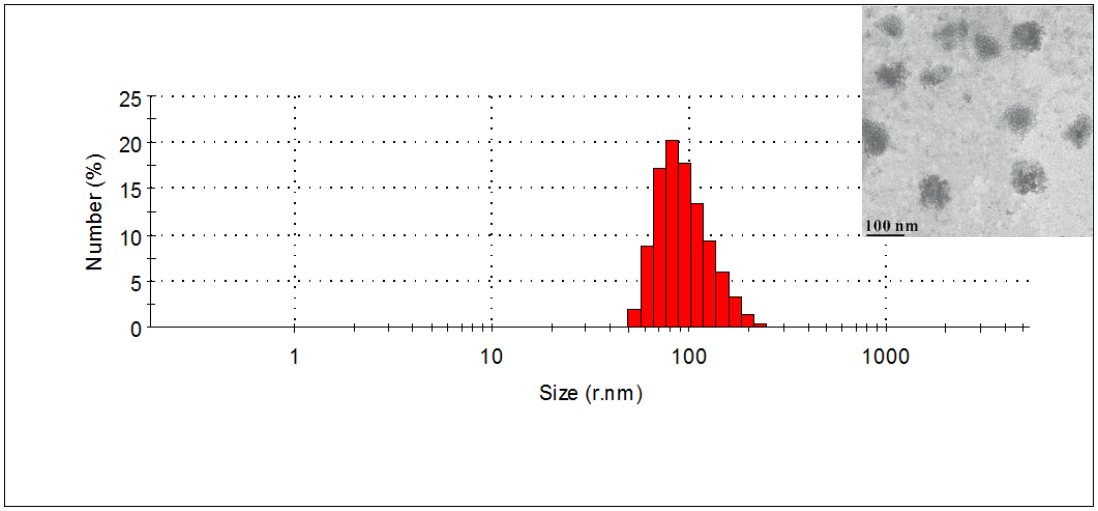


Figure S10. Particle size distribution obtained from dynamic light scattering (DLS) and images from transmission electron microscopy (TEM) for LIP-Pro-Stm.

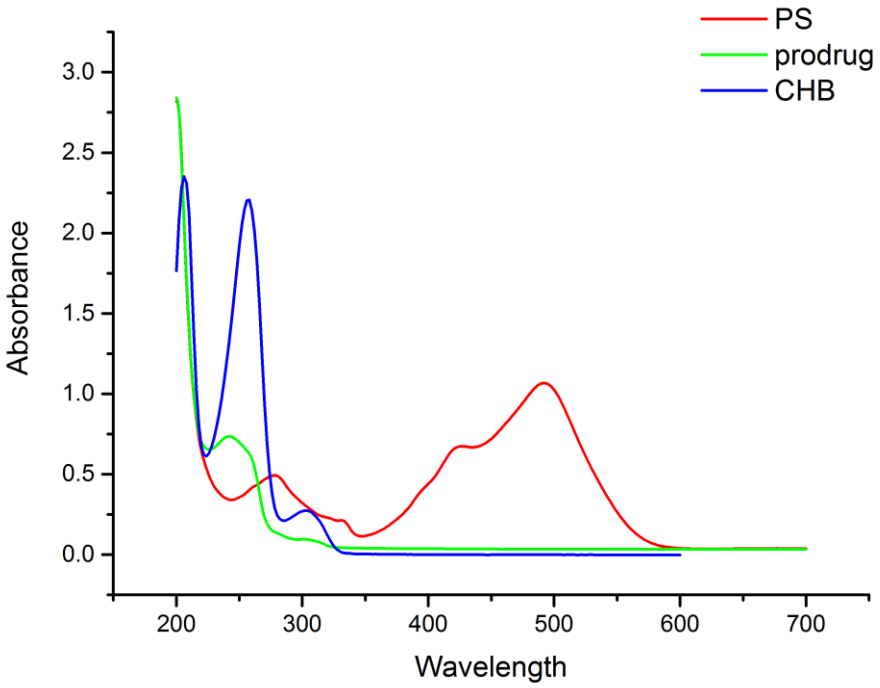


Figure S11. Absoption spectra of the PS (photosensitizer), the prodrug and CHB.

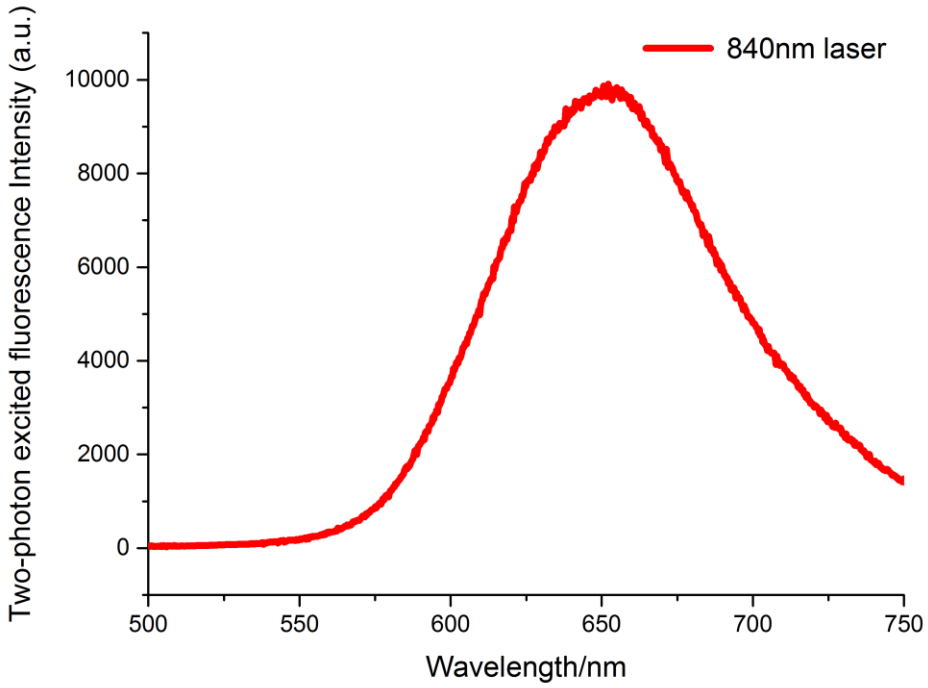


Figure S12. Typical two-photon excited fluorescence spectrum (the PS (5 mM) excitaed by 840 nm laser).

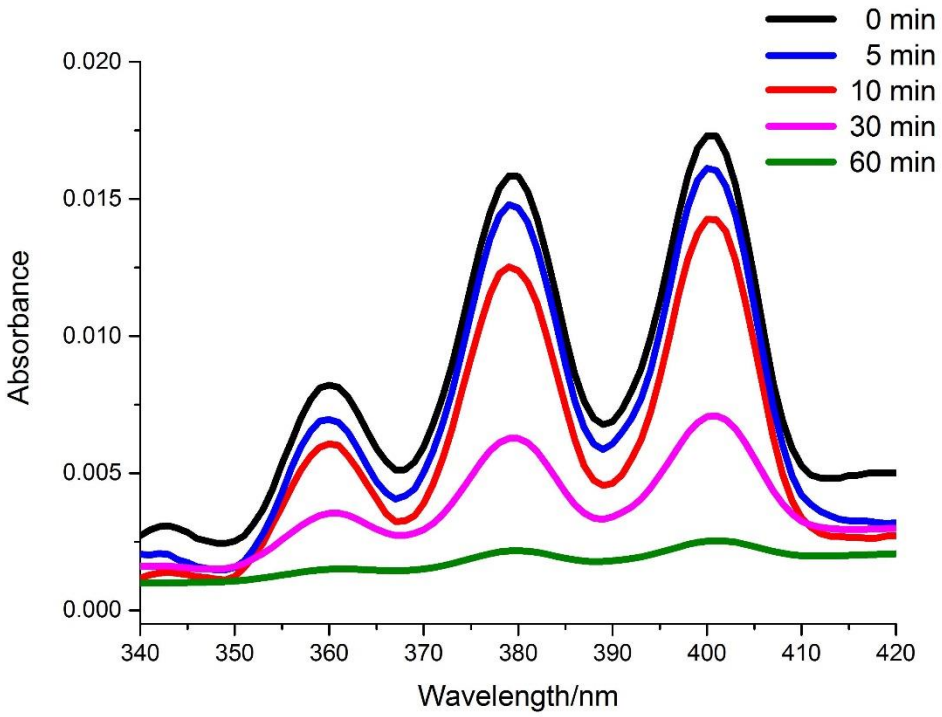


Figure S13. Photobleaching of ABDA (10 μM) by singlet oxygen generated by PS (5 μM) solution over different periods of time under light irradiation (500 nm, 30mW·cm^{-2}).

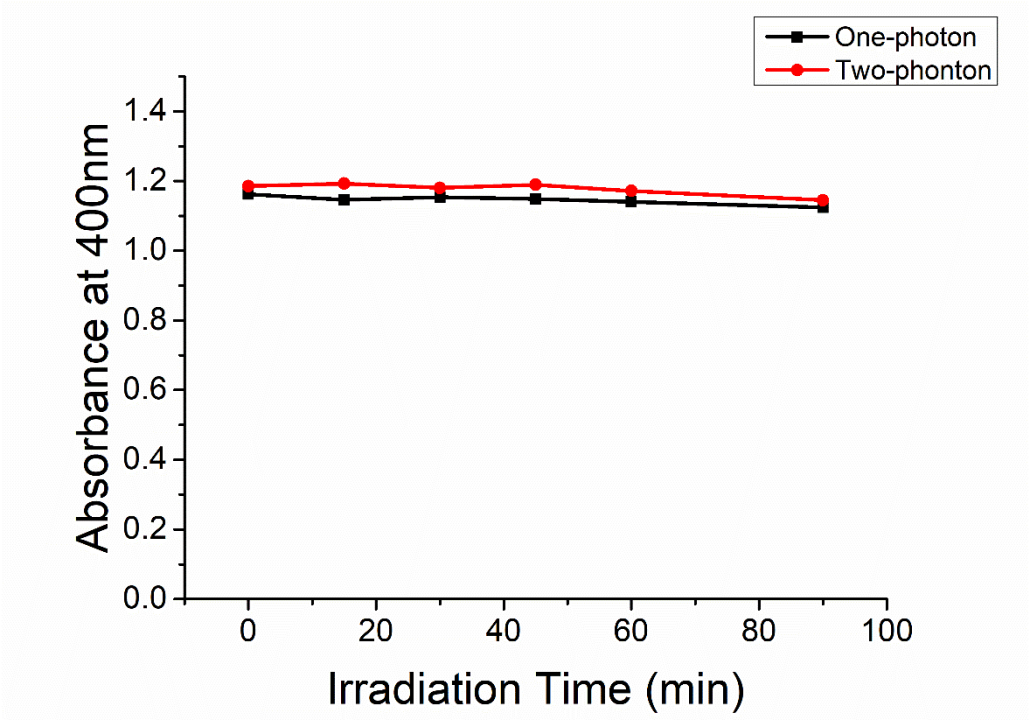


Figure S14. Photostability of ABDA (10 mM) over different periods upon one-photon (500 nm, 30mW·cm^{-2}) and two-phonton (840 nm 55 mW·cm^{-2} femtosecond laser) light irradition (under nitrogen atmosphere).

2. Methods

2.1 Cell culture:

HeLa cells and A549 cells were maintained according to the supplier's protocols. The two cell lines were incubated with 10% FBS (fetal bovine serum, GIBCO)-containing Dulbecco's modified eagle medium (DMEM) with 1% penicillin and streptomycin (GIBCO) at 37 ℃ in a humidified atmosphere containing 5% of CO_2. When the cell density reached 70-80% of confluence, a subculture was considered complete. The medium was changed approximately every 1 to 2 days.

2.2 Cell imaging:

HeLa cells and A549 cells were incubated in DMEM supplemented with 10% FBS for 2 hours incubation. Then cells were seeded in a 6-well plate. Cells were allowed to 50–70% confluence and washed with DMEM, and the cells were incubated with the prodrug system or Lip-Pro-Stms in a humidified incubator (37 ℃, 5% CO_2) for 2 hours. Afterwards, the 6-well plate was washed with PBS for three times. Fluorescence images were obtained using an Olympus IX71 inverted fluorescence microscope equipped with a DP72 color CCD.

2.3 Cell viability assay:

Cells (5000 cells/well) were seeded in 96-well plates and incubated with the prodrug and the PS of varied concentrations from 0 to 14 μM under 60 minutes of light radiation or in the dark at 37 ℃. The cells without the treatment were used as control. After incubation for 48 h, the wells were washed three times with PBS buffer and treated with DMEM medium containing 0.5 mg/mL MTT for another 4 h. The resulting formazan crystal was dissolved in 150 μL of DMSO after carefully removing the medium and the absorbance was recorded at 570 nm. The cell viability assays were performed using a Thermo MK3 ELISA plate reader. The independent experiments performed in six replicates were used to obtain the statistical mean and standard deviation.

2.4 Apoptosis Analysis by Annexin V-FITC and Propidium Iodide (PI) Double Staining:

HeLa and A549 cells were seeded in 6-well plate at the density of 1.0×10^6 cells/well for 12 h at 37 ℃. When the cell density reached 80-90% of confluence, the medium was replaced with fresh medium, or the fresh medium containing the PS, or that containing CHB, or that containing both the prodrug and the PS, or that containing the

Lip-Pro-Stm , with or without light radiation for 15 to 60 min, then cells were further incubated for 2 hours. The cells were then washed with PBS and harvested after trypsinization and underwent centrifugation at 2000 rpm for 10 min. Subsequently, the cells were collected and resuspended in 1 mL PBS and then mixed with Annexin V Binding buffer (400 μL) containing 5 μL Annexin V-FITC and 10 μL PI. Finally, the flow cytometry analyses were performed using BD Accuri C6 flow cytometer and the data were analyzed using the BD Biosciences software.

2.5 Measurements:

^{1}H NMR spectra were recorded on a Bruker Avance 600 MHz NMR spectrometer. All chemical shifts are reported in ppm value using the peak of residual proton signals of TMS as an internal reference. Mass spectra were obtained on a Bruker Esquire HCT Plus mass spectrometer. High resolution mass spectra were obtained on AB Sciex Triple TOF 5600+ mass spectrometer. UV-vis spectra were recorded on a Hitachi U-3010 UV-vis spectrophotometer. Fluorescence spectra were recorded on a Hitachi F-4600 fluorescence spectrophotometer. Fluorescence images were obtained using an Olympus IX 71 with a DP72 color CCD.

2.6 Determination of fluorescence quantum yield of the PS:

The fluorescence quantum yield (Φ) of the PS was calculated by comparing its integrated fluorescence intensity (excitation at 500 nm) and absorbance value at 500 nm with those of rhodamine 6g (Rho-6G).

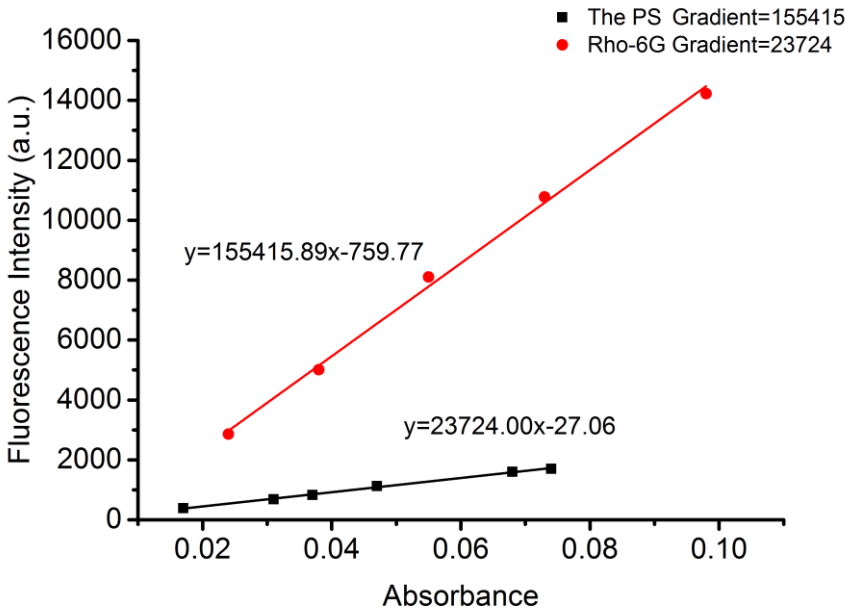


Figure S15. Linear plots of fluorescence intensity against absorbance for the PS and Rho-6G.

Rho-6G (φ_r = 0.95)[2] was dissolved in ethanol (refractive index: 1.3628) and the PS was dissolved in ethanol (refractive index: 1.3628). Quantum yield can be calculated according to the following equation:

$$\phi_s = \phi_r \times \left[\frac{Grad_s}{Grad_r}\right] \times \frac{n_s^2}{n_r^2}$$

where φ is the fluorescence quantum yield of fluorophore, Grad is the slope of the plot of integrated fluorescence intensity vs absorbance (as shown in Figure S13); n is the refractive index at 25 °C of the solvent. Subscript 'r' stands for reference, and 's' stands for samples. In order to minimize the re-absorption effects, absorbance values in the 10 mm fluorescence cuvettes was maintained under 0.1 at the excitation wavelength. Excitation and emission slit widths were set at 5.0 nm when recording their fluorescence spectra.

As a result, The φ_s of the PS was therefore calculated to be 0.15.

2.7 Determination of two-photon absorption cross-section:

In this study, two-photon excited fluorescence (TPEF) intensity was obtained and used to determine the two-photon absorption cross-section of the PS. Rho-6G was selected as the reference (5×10^{-5} M in ethanol) and its two-photon absorption cross-section was acquired according to a literature reported[3]. The PS of the same concentration as that of Rho-6G in ethanol was used as the sample for cross-section determination. Since the input beam parameters are the same for all experiments, we can calculate the two-photon absorption cross-section (δs) of the PS using the known two-photon absorption cross-section of Rho-6G (the reference) according to the following equation:

$$\delta_s = \frac{S_s \Phi_r \phi_r C_r}{S_r \Phi_s \phi_s C_s} \delta_r$$

where S is the integrated fluorescence intensities measured at the same power of the excitation beam; Φ is the fluorescence quantum yields; C is the concentrations, which is same between the PS and Rho-6G. ϕ is the collection efficiencies for the sample and the reference, respectively. Fluorescence quantum yields Φs = 0.15 for the PS was measured according to the Figure S15. Rho-6G (Φr = 0.95) was dissolved in ethanol as the standard.

As for the collection efficiencies, it is noteworthy that, the experimental condition and setup were kept completely identical when measuring each fluorescence spectra for both the reference and samples. In this case, the collection efficiency of the experimental setup ϕ is the same for both the reference and samples. Thus, the ratio of ϕ(r) / ϕ(s) is equal to 1, and the actual value of ϕ is not essential to be measured or calculated in this case.

And the output intensity of two-photon excited fluorescence was linearly dependent on the square of the input laser power (Slope = 1.94, Figure S16), which is very close to 2; and this confirms an obvious two-photon excitation process.

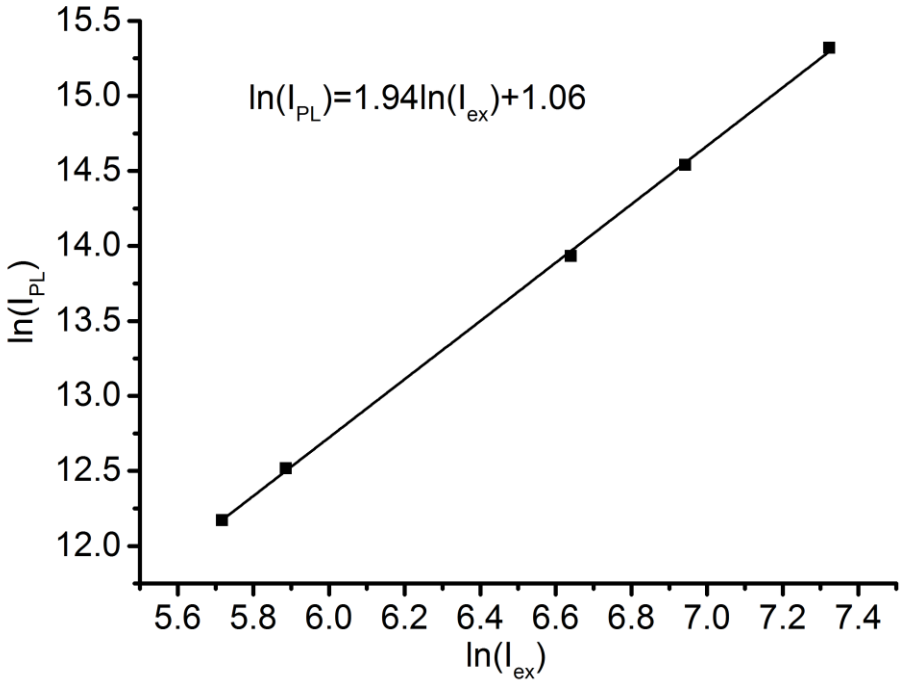


Figure S16. The plot (ln-ln) of emission intensity against incident power at 840 nm of the PS.

2.8 Fabrication of the Liposomal Prodrug system (Lip-Pro-Stm) :

0.4 mg of the PS and 1.4 mg of the prodrug were dissolved with THF (1 mL), followed by the addition of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC, 10 mg) in 1 mL THF. The mixture was then added dropwise into 20 mL water under sonication for 20 minutes at 4 ℃. The mixture was then stirred at room temperature for 24 hours. Afterwards, THF was removed from the mixture by evaporation under reduced pressure at 37 °C. The resultant solution was dialyzed against water for 24 hours at room temperature. The resultant suspension was centrifuged at 12,000 rpm for 15 min at 4 ℃ to collect the liposomal particles (Lip-Pro-Stm). After being washed for three times with water, the particles were resuspended in the water of designated volume (10 mL).

2.9 Determination of drug loading capacity

The loading efficiency and content of the PS and the prodrug in the LIP-Pro-Stm were determined using a UV-vis spectrophotometer at 500 nm for the PS and 240 nm for the prodrug. LIP-Pro-Stm was dissolved in DMSO, and the calibration curves of 500

nm for the PS and 240 nm for the prodrug were obtained using DMSO solutions with different concentrations of the PS and the prodrug.
The entrapment efficiency and the drug loading efficiency of the PS and the prodrug were calculated using the following formulas:

$$Entrapment\ efficiency\ (\%) = \frac{weight\ of\ loaded\ drug}{weight\ of\ drug\ in\ feed} \times 100\%$$

$$Drug\ Loading\ Efficiency\ (\%) = \frac{weight\ of\ loaded\ drug}{weight\ of\ liposome} \times 100\%$$

The entrapment efficiency and the drug loading efficiency was found to be 92.8% and 3.2 wt% of the PS and 89.3% and 10.7 wt% of the prodrug into the LIP-Pro-Stm respectively.

2.10 General Procedure for Linearization of Plasmid pBR322 by EcoR I

Supercoiled pBR322 DNA (30 μL, 30 μg) was incubated with EcoR I (20 μL, 300 u), EcoR1 buffer (10 ×, 20 μL) and 146 μL of sterile H_2O at a humidified incubator (37 ℃, 5% CO_2). After 3 h, NaOAc (40 μL, 3 M) and ethanol (1400 μL) were added into the solution of the catalyzed DNA and incubated at -20 ℃ overnight. The mixture was centrifuged (12000 rpm) for 15 min at 4 ℃, followed by the removal of supernatant. The residue was freeze-dried to obtain a white solid. The obtained linearized DNA was suspended in 100 μL sterile H_2O.

2.11 Interstrand DNA cross-linking reactions

The linear DNA was linearized from supercoiled pBR322 by EcoR I. Linearized Plasmid pBR322 (1 μg/sample) was mixed with 50 μg/mL of LIP-Pro-Stm respectively in 10 μL phosphate buffer (10 mM, pH 7.4) at 37 ℃ under 500 nm light radiation for 15 to 60 min. The reaction was allowed to proceed for 2 h, and the resultant solution was diluted to the final volume of 20 μL for agarose gel electrophoresis

2.12 Purity determination:

HPLC analyses for compounds **6** and **7**. HPLC analysis was performed under reversed phase conditions on a C18 (150 x 4, 6 mm I.D.) column, using a binary mixture (A/B) of methanol/water (80/20, v/v) as mobile phase with a flow-rate of 0.1 mL/min. The HPLC analyses were performed on Agilent 1260 high performance liquid chromatography (with DAD).

2.13 Drug release from the prodrug molecule

HPLC analysis was performed under reversed phase conditions on a C18 (150 x 4, 6 mm I.D.) column, using a binary mixture (A/B) of methanol/water (80/20, v/v) as mobile phase with a flow-rate of 0.1 mL / min. The HPLC analyses were performed on an Agilent 1260 high performance liquid chromatography (with DAD).

The quantification of drug release from the prodrug molecule is obtained as follows:

First, a calibration curve was obtained by using a series of CHB calibration solutions with varied concentrations. The calibration curve is the plot of the peak area of CHB versus the concentration of the drug CHB.

Then, as for a prodrug sample (the concentration of the prodrug molecules is predetermined) irradiated for a certain time, the peak area corresponding to the CHB released from the prodrug molecule was measured; based on the calibration curve, the concentration of released CHB was determined. Then, by dividing the concentration of released CHB by the concentration of prodrug, the drug release for this irradiation time point was obtained.

For the prodrug samples undergoing irradiation for different time periods, the same procedures were employed for determining drug release.

3. Drug release, DNA crosslink and cell cytotoxicity

3.1 Drug release

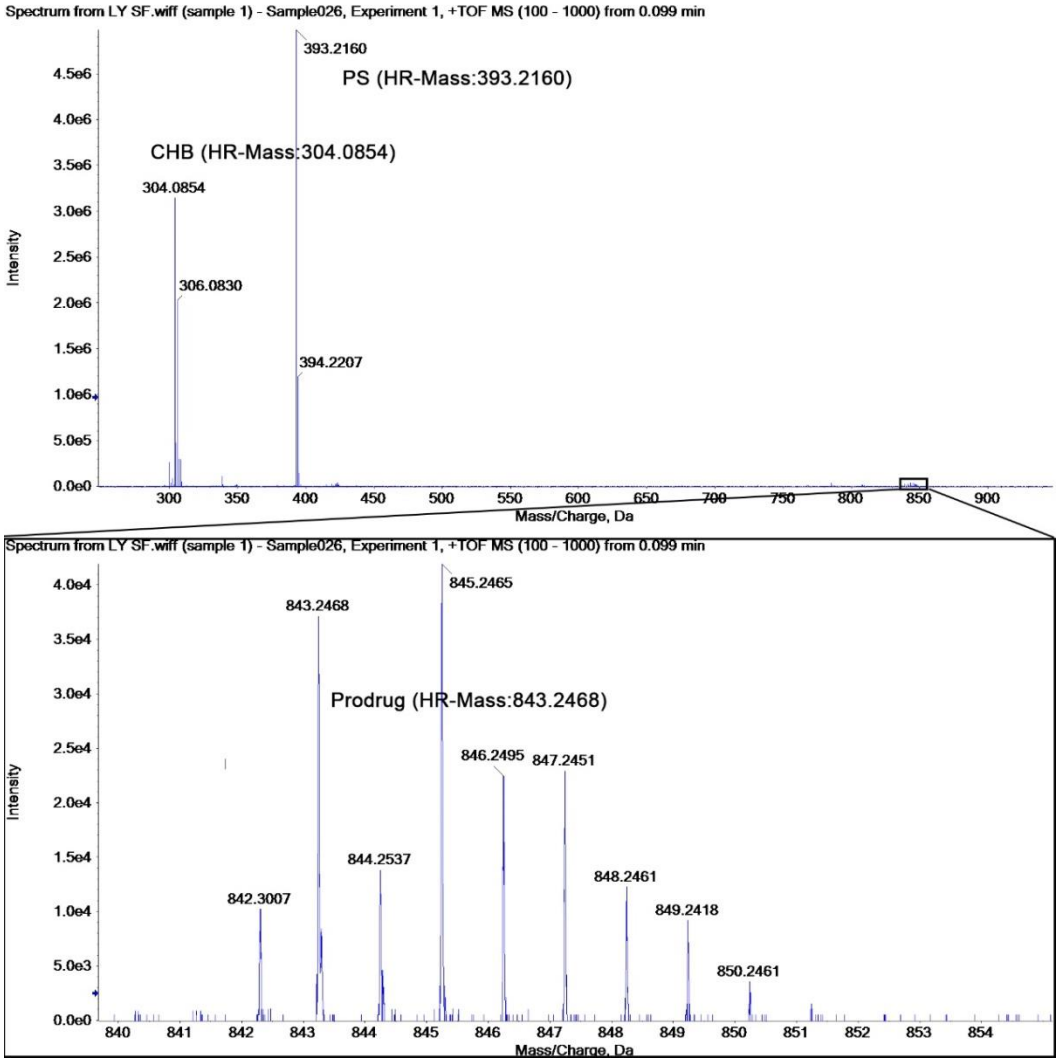
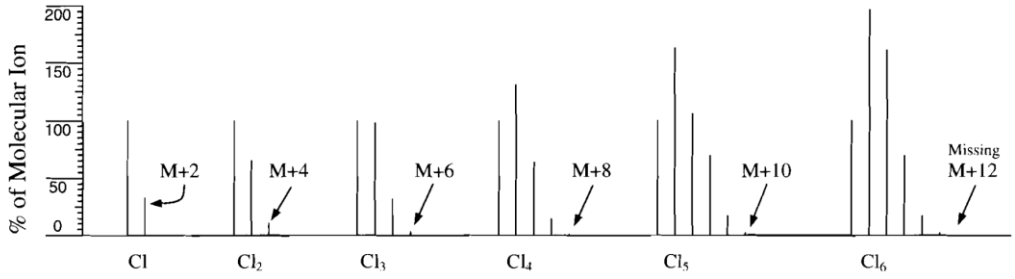


Figure S17. HR-MS (ESI) analyses for the prodrug (10 μM) and the PS(5 μM) incubated with one-photon light (500 nm and 30 $mW \cdot cm^{-2}$) for 60 minutes.

Some peaks are resulted from the contribution of isotopes of chlorine and other elements, since ^{37}Cl account for considerable ratio in isotopes (namely the natural abundance of chlorine isotopes are relatively high). The following figure (from R. M. Siverstein, et al. Spectrometric Identification of Organic Compounds, 7th Edition, John Wiley & Sons, 2005) provides the predicted MS patterns for compounds with various amounts of chlorine combinations.


% of Molecular Ion
200
150
100
50
0
M+2
M+4
M+6
M+8
M+10
Missing
M+12
Cl
Cl2
Cl3
Cl4
Cl5
Cl6

3.2 DNA crosslink

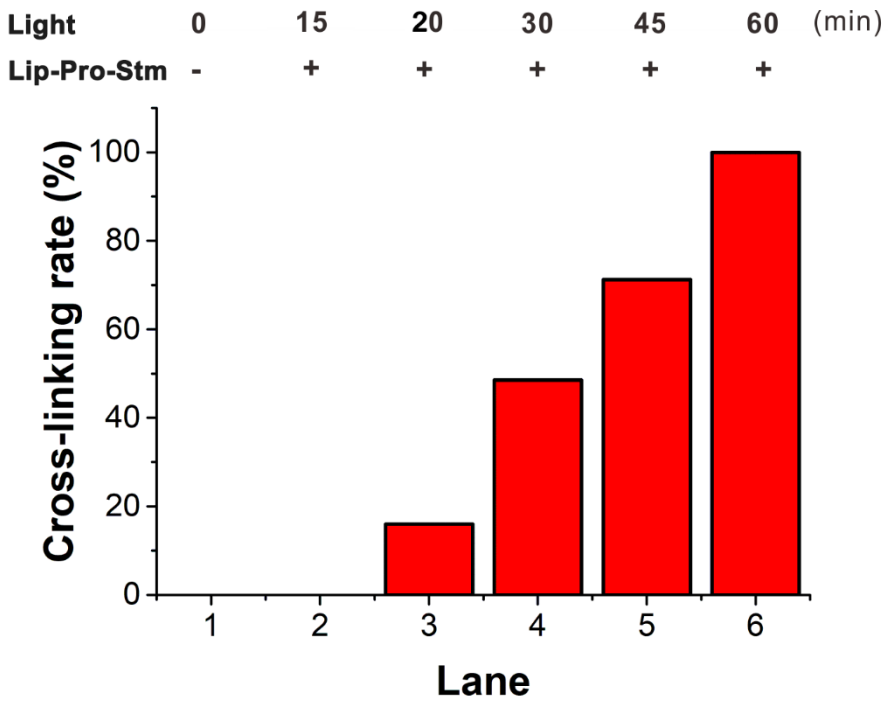


Figure S18. Quantification of Figure 4b obtained by processing acquired images using software ImageJ from the National Institute of Health (http://rsb.info.nih.gov/ij).

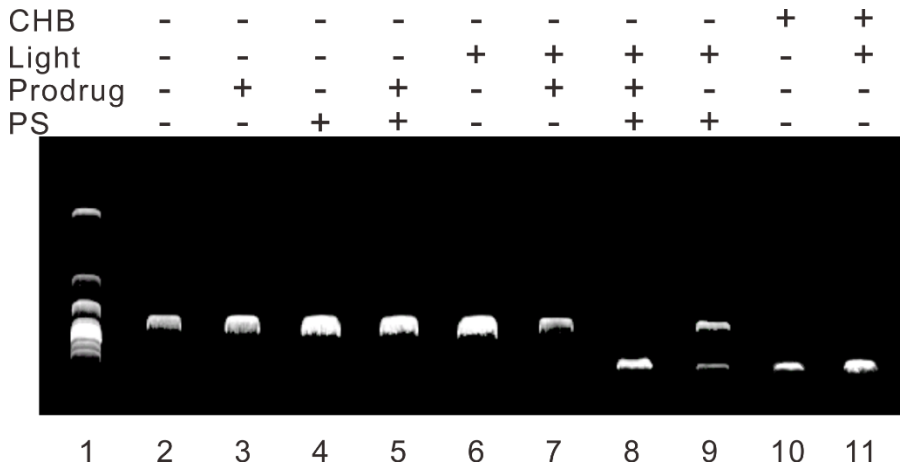


Figure S19. Agarose gel electrophoresis assay for linear DNA (0.5 μg pBR322) crosslinking formation upon treatment with different formulations; the DNA were incubated in culture media with --- lane 1: DNA ladder, lane 2: buffer, lane 3-11: prodrug represents 6 μM of the prodrug, PS represents 4 μM of the PS, CHB represents 6 μM of the CHB for 2 h after 60 min of 500 nm light radiation or in the dark.

3.3 Cell cytotoxicity

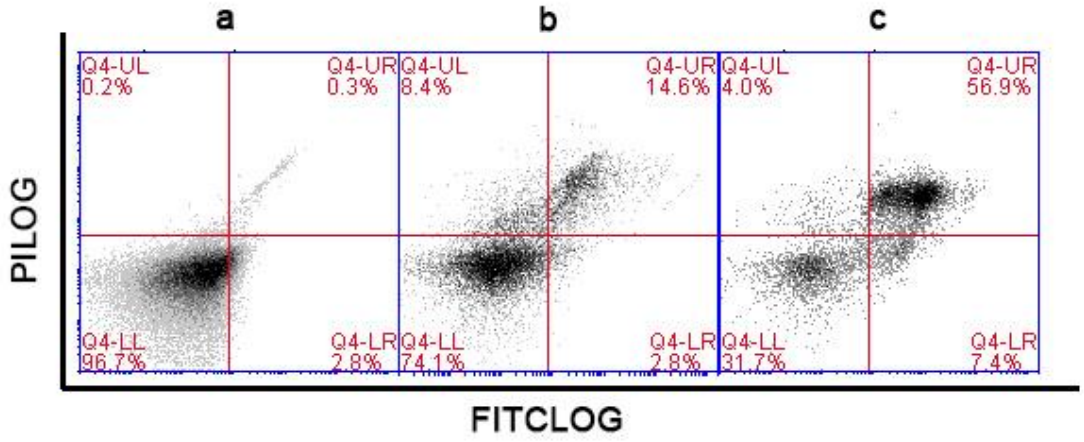


Figure S20. Annexin V-FITC/Propidium Iodide (PI) dual staining of HeLa cells for detecting the time-related apoptosis by flow cytometry. The HeLa cells were incubated in culture media for 3 h with (a) buffer; (b) 13 μM CHB; (c) 50 μg/mL (equivalent to 12.7 μM of the prodrug, 4.07 μM of the PS) LIP-Pro-Stm after 60 min of 500 nm light radiation.

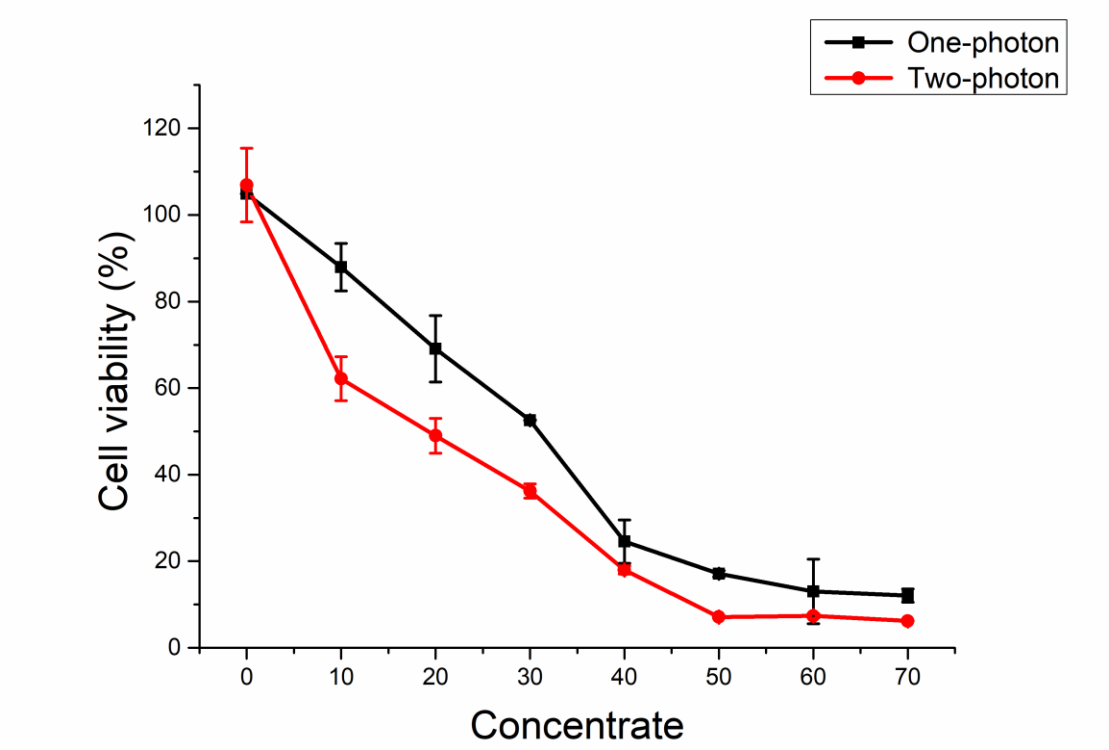


Figure S21. Viabilities of HeLa cells treated with different formulations of varied concentrations of Lip-Pro-Stm upon one-photon (500 nm, 30mW·cm^{-2}) and two-phonton (840nm 55 mW·cm^{-2} femtosecond laser) light irradiation for 60 min, as evaluated with MTT assay.

4. Notes and references

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2 M. Fischer and J. Georges, Chem. Phys. Lett., 1996, 260, 115-118.
3 N. S. Makarov, M. Drobizhev and A. Rebane, Opt. Express, 2008, 16, 4029-4047.