Supporting Information

For

A biodegradable fluorescent protein chromophore nanoparticle for hypoxic two-photon photodynamic therapy

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Contents

1. Experimental section

Materials

Phenothiazine, 4-(2-chloroethyl)-morpholine hydrochloride, glycine *tert*-butyl ester hydrochloride, *tert*-butanol, 1-ethoxyethylideneammonium chloride, phydroxybenzaldehyde, ether, KOH, NaOH, $POCl_3$ and K_2CO_3. All biomaterials were purchased from Keygen Biotech Co.Ltd. A-549 cells were used in this work from the American Type Culture Collection. Unless stated otherwise, all chemicals with analytical grade were purchased from commercial sources and used directly as received. All the solvents used to investigate the photophysical behaviors of the fluorophores were spectroscopic grade. Column chromatography was performed on silica gel (200-300 mesh).

Measurements and characterization

NMR (^1H and ^{13}C) measurements were recorded on a Bruker ADVANCE III HD 600 NMR spectrometer. UV-vis spectra are recorded on the Shimazdu 2450 UV-visible spectrophotometer. High-resolution mass spectra (HRMS) were acquired on an Ultra flextreme MALDI-TOF/TOF and Agilent Technologies 6530 Accurate-Mass equipment. Steady-state fluorescence excitation and emission spectra were obtained by a time-correlated single photon counting fluorimeter (Fluoromax-4/plus) with a xenon lamp as the light source. The EPR (Electron Paramagnetic Resonance) spectra were recorded on a Bruker Magnettech ESR5000. The power of the customized 460 nm blue LED lamp was 23 $mW \cdot cm^{-2}$. The of singlet oxygen yield (Φ_Δ) and its applications in cells were both performed under the condition of irradiation at 23 $mW \cdot cm^{-2}$ (the irradiation was tested with an Aicevoos-V10 irradiation meter). One-photon fluorescent images of cells were acquired from FLUOVIEW FV3000/FLUOVIEW FV1000MPE OLYMPUS (CLSM) and two-photon fluorescent images of cells were acquired from Olympus multiphoton microscope (FVMPE-RS) equipped with an InSight DS-OL pulsed IR laser system (Spectra-Physics, 80 MHz, 120 fs). The Z-scan two-photon absorption cross section is measured from NLO-Z. The microscope used for 2PE-PDT was an upright

UV Visible and Fluorescence Spectral Characterization

Equip 1 mM of DPFP-SS-FA H_2O mother liquor and 1 mM DMSO mother liquor of PFP-SS-PFP (*note*: DMSO uses biological grade reagents to reduce damage to biological samples in subsequent experiments). The UV visible absorption spectrum has a scanning range of 350-

700 nm, and the z-light emission spectrum has a scanning range of 600-800 nm (excitation wavelength of 490 nm, slit width of 5 nm).[1]

Fluorescence lifetime measurement

Using a nanosecond transient absorption spectrometer, test and fit the fluorescence lifetime of DPFP-SS-FA and DPFP-SS-FA+GSH. Test concentration: 10 μM.[2]

Singlet oxygen quantum yield (Φ_Δ) detection

The Φ_Δ of the photosensitizers were detected by using DPBF (1,3-diphenylisobenzofuran) as the capture agent for $^1O_2$ and the $Ru(bpy)_3Cl_2$ as the reference (Φ_Δ=0.87 in methanol solution).[3] Keeping the absorbance of the photosensitizers and DPBF were about 0.2 and 1 respectively. The mixed solution was irradiated under 460 nm light, and recorded the absorbance value of the DPBF solution to get the decrease slop. Finally, the Φ_Δ was calculated on the basis of the formula below:[1]

$$\Phi_{\Delta[PS]} = \Phi_{\Delta\left[Ru(bpy)_3cl_3\right]} \times \frac{k_{[PSs]}}{k_{\left[Ru(bpy)_3cl_3\right]}} \times \frac{F_{\left[Ru(bpy)_3cl_3\right]}}{F_{[PSs]}}$$

Where, the k is the decrease slope between the absorbance and its corresponding time. F is absorption correction factor ($F=1-10^{-OD}$), and OD is its maximum absorbance. PSs is DPFP-SS-FA.

Detection of $O_2^{\bullet-}$ generation ability in aqueous solution

The probe dihydrorhodamine 123 (DHR 123), can convert $O_2^{\bullet-}$ produced in the solution into rhodamine 123 and emit strong green fluorescence at 424 nm, is used to detect $O_2^{\bullet-}$ in solution. The DPFP-SS-FA (10 μM) were mixed with DHR 123 (20 μM) in cuvette. The fluorescence intensity of the mixture at 424 nm (λ_{ex} = 500 nm) were measured every 15 s under blue LED light irradiation (460 nm, 23 $mW \cdot cm^{-2}$).

Theoretical calculations

Molecular structural parameters of the compounds were studied theoretically by quantum chemical computational technique based on the DFT method. The quantum-chemical methods at the B3LYP/6-31G (d) level of theory using Gaussian 16 were employed to calculate the HOMO and LUMO energies of the synthesized compounds.

Cumulative release of DPFP-SS-FA

The release of FPOH (m/z = 639.2521) from nanoparticles was investigated under shaking (100 rpm) at 37 °C in different media, 10 mM PBS 7.4 containing 10 mM GSH. Typically, 1 mL of DPFP-SS-FA dispersions (2 mg/mL) in dialysis bag (MWCO 1000 D) was immersed in 20 mL of release media. The release profiles were measured at different time intervals: 1 h, 2 h, 3 h and 4 h. At desired time points, 1 mL sample was analyzed and replaced with the equal fresh media. FPOH (m/z = 639.2521) content was determined by LC-MS and HPLC.

MTT cytotoxicity assay

L-O_2 cells, MCF-7 cells and A-549 cells (5000 cells/well) were seeded into the 96-well plate and incubated for 24 h at 37 °C, respectively. Then prepared different concentrations (1 μM, 2 μM, 3 μM, 4 μM, 5 μM, 6 μM and 7 μM) photosensitizers DPFP-SS-FA were added to the 96-well plate incubated for 24 h in hypoxic microenvironment (2% O_2) and normoxia microenvironment (21% O_2), respectively.

Dark toxicity: Dripping 100 μL/well MTT (5 mg/mL) into the 96-well plate and incubated for 4 h in the dark in hypoxic microenvironment (2% O_2) and normoxia microenvironment (21% O_2), respectively. Then adding 100 μL/well DMSO solution into well and incubated for 2 h. Finally, the cell viability in hypoxic microenvironment (2% O_2) and normoxia microenvironment (21% O_2), respectively, were calculated by the absorbance tested by a microplate reader.

Phototoxicity test: Irradiating the 96-well plate in hypoxic microenvironment (2% O_2) and normoxia microenvironment (21% O_2), respectively, under 460 nm light for 15minutes and incubated for 12 h. Adding 100 μL/well MTT solution (5 mg/mL) into 96-well plate and incubated for 4 h. Then 100 μL/well of DMSO solution were added to 96-well plate and incubated for 2 h. Finally, the cell viability Dark toxicity: Dripping 100 μL/well MTT (5 mg/mL) into the 96-well plate and incubated for 4 h in the dark in hypoxic microenvironment (2% O_2) and normoxia microenvironment (21% O_2), respectively. Then adding 100 μL/well DMSO solution into well and incubated for 2 h. Finally, the cell viability in hypoxic microenvironment (2% O_2) and normoxia microenvironment (21% O_2), respectively, were calculated by the absorbance tested by a microplate reader

AO/EB staining

The prepared confocal culture dish containing 2 μM photosensitizer DPFP-SS-FA was irradiated for 5 minutes and 15 minutes under 460 nm light in hypoxic microenvironment (2% O_2) and normoxia microenvironment (21% O_2), respectively. And then placed in the incubator for 2 h. The diluted acridine orange (AO)/ ethidium bromide (EB) stain was added and continued to incubate for 30 minutes. The culture dish was taken out, washed three times with PBS buffer solution. In the end, placed under the confocal laser microscope for imaging. All of the above procedures were done in the dark.

2. Photophysical properties of the photosensitizers

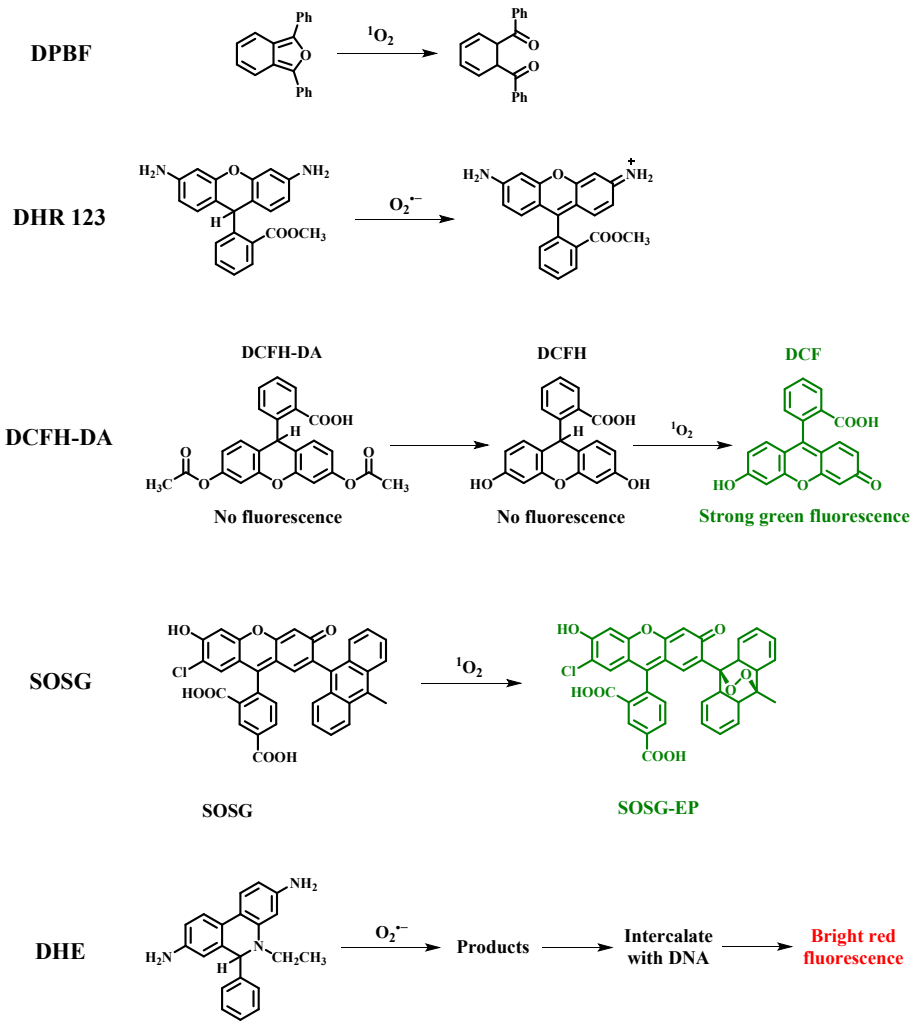

Figure S1. Reaction mechanisms of DPBF, DHR123, DCFH-DA, SOSG and DHE for the

detection of general $^1O_2$, $O_2^{\bullet-}$ and ROS.

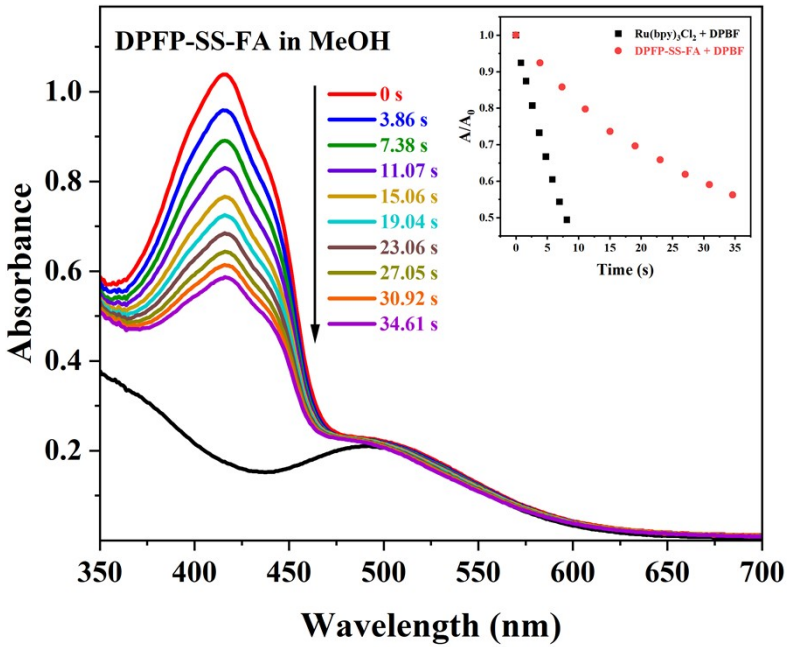

Figure S2. The $^1O_2$ detection of photosensitizer DPFP-SS-FA in MeOH solution.

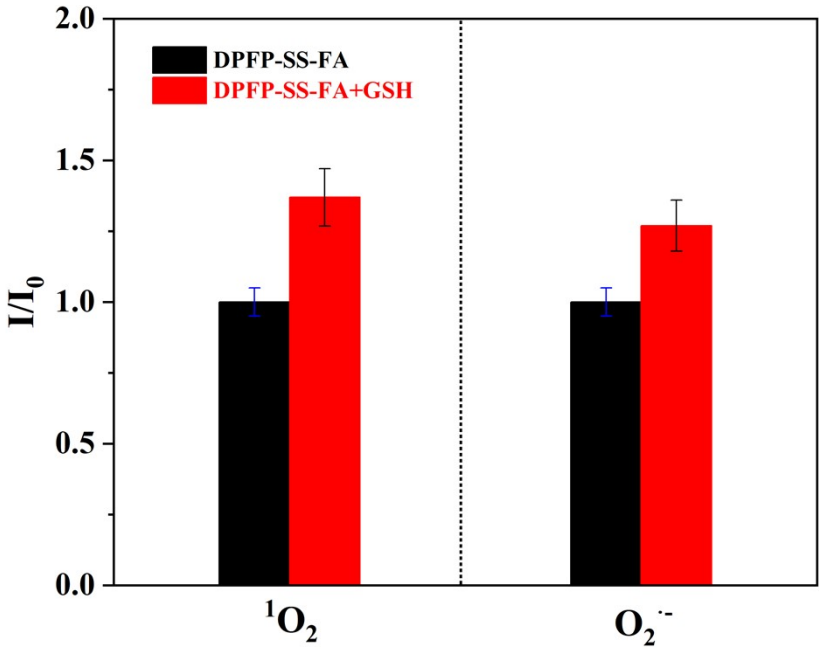

Figure S3. Comparison of $^1O_2$ and $O_2^{\bullet-}$ generation efficiencies of DPFP-SS-FA and DPFP-SS-FA+GSH under the same conditions.

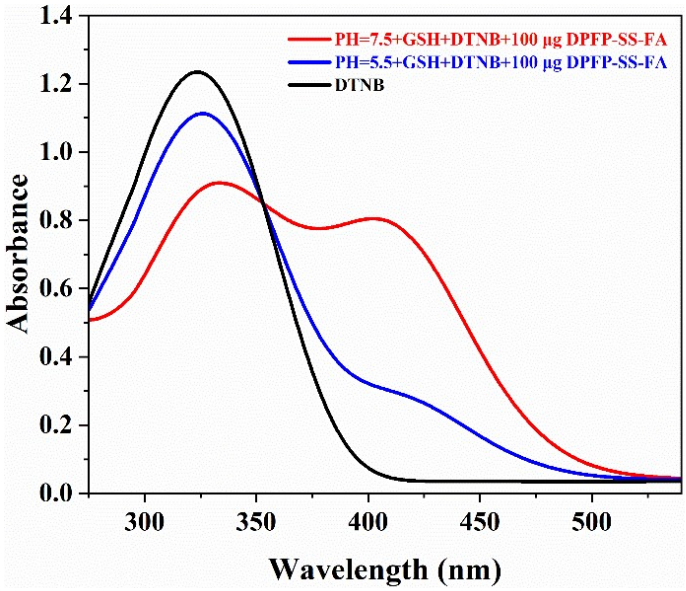

Figure S4. The ability of GSH consumption by DPFP-SS-FA at different pH values.

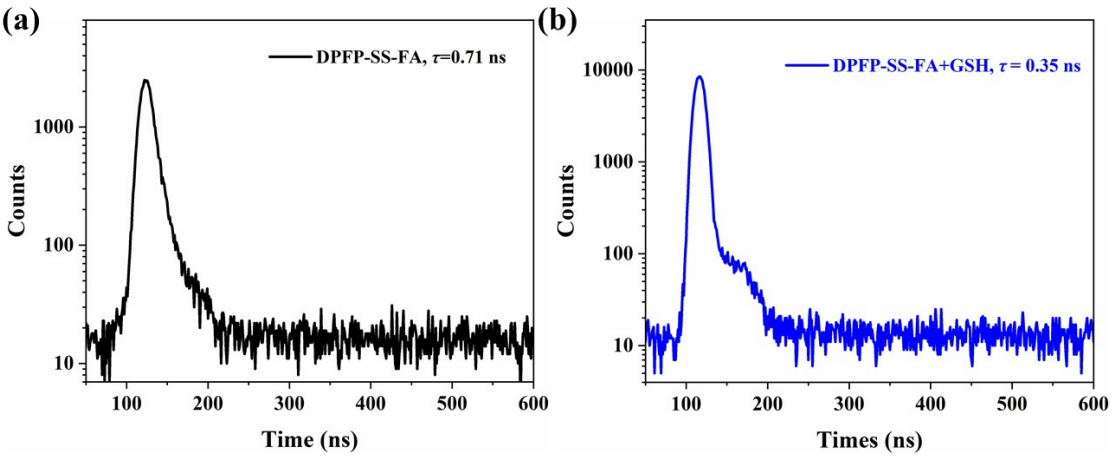

Figure S5. (a) The lifetime decay curve of DPFP-SS-FA. (b) The lifetime decay curve after adding GSH for 24h.

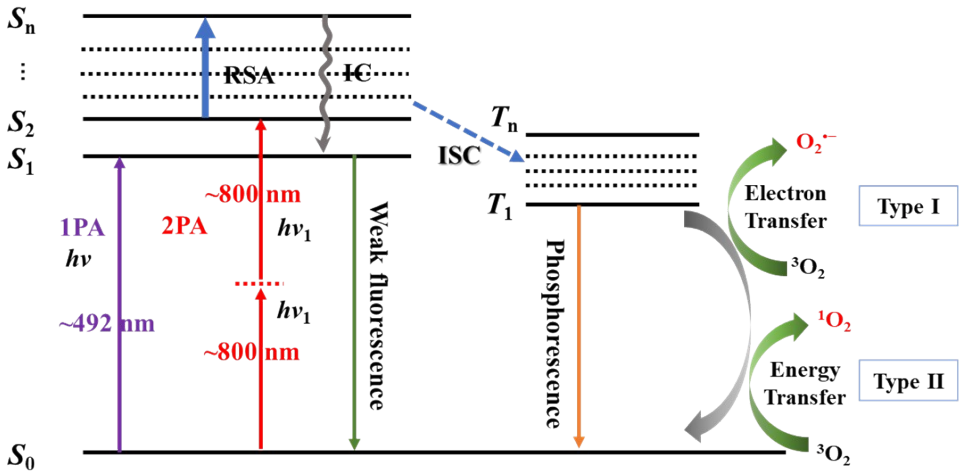

Figure S6. Jablonski energy level diagram for two-photon photodynamic therapy.

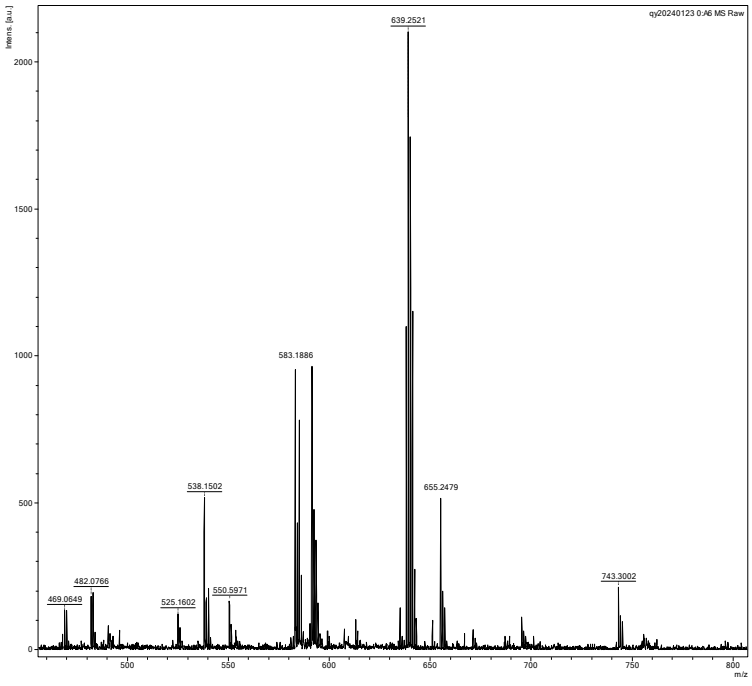

Figure S7. HRMS spectrum of the products from the reaction of DPFP-SS-FA with GSH.

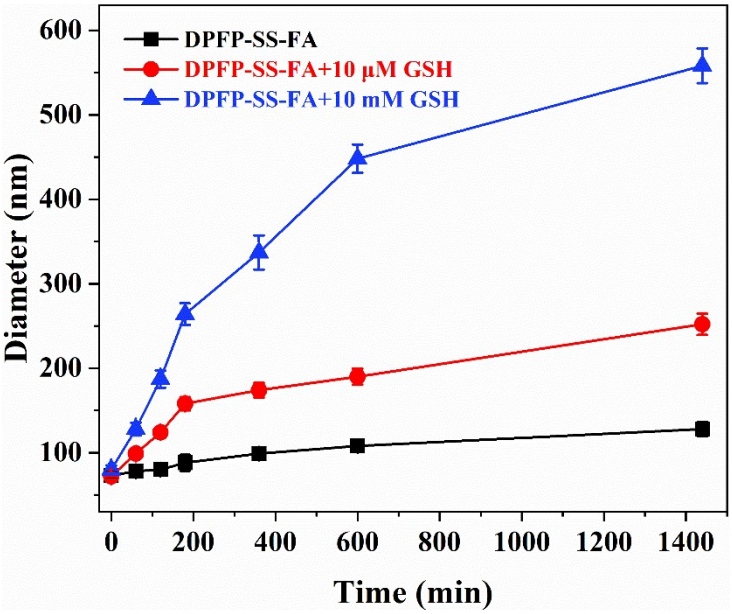

Figure S8. Particle size changes of DPFP-SS-FA during 24 h of 0 or 10 μM or 10 mM GSH treatment in PBS (0.02 M, pH = 7.4) at 37 °C measured by DLS. Data were expressed as mean ± SD (n = 3).

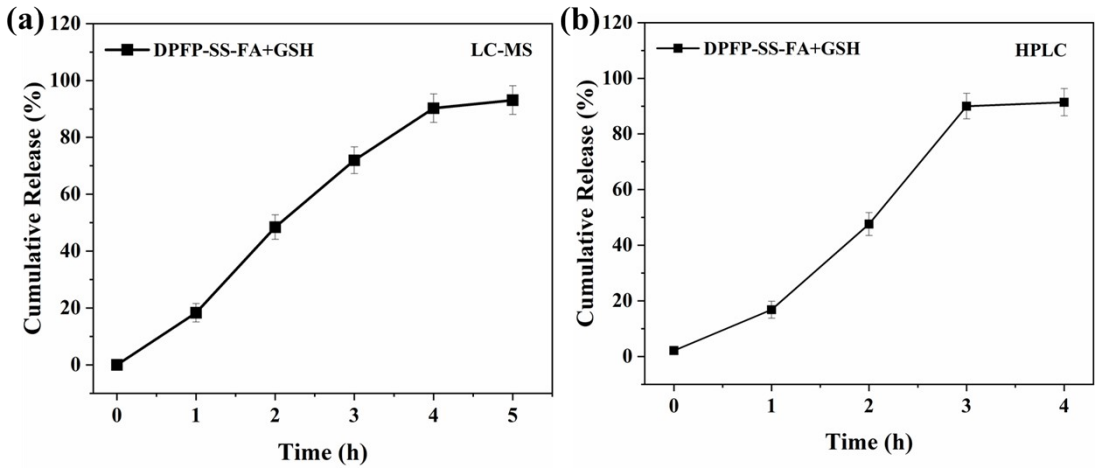

Figure S9. Cumulative release profiles of DPFP-SS-FA in 10 mM GSH determined by LC-MS (a) and HPLC (b).

3. The dark/light cytotoxicity test in A-549 cells of photosensitizers

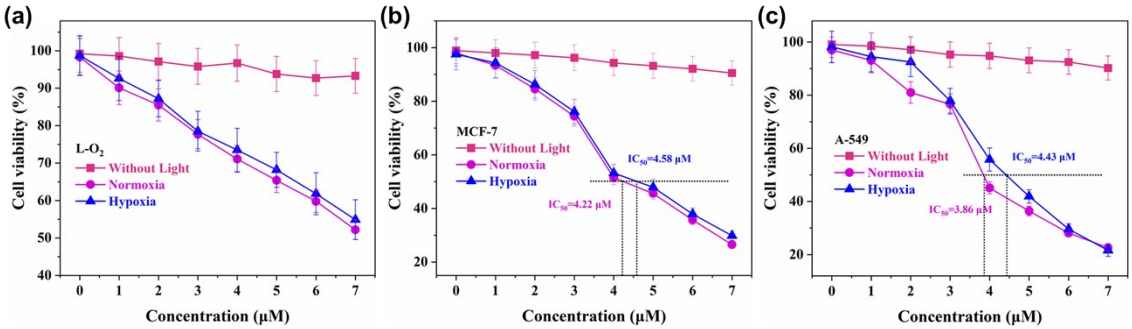

Figure S10. Cell growth curve of L-O_2 cells, MCF-7 cells, and A-549 cells treated with **DPFP-SS-FA** under light irradiation for 10 min (460 nm, 23 mW·cm^{-2}).

4. NMR spectra of the compounds

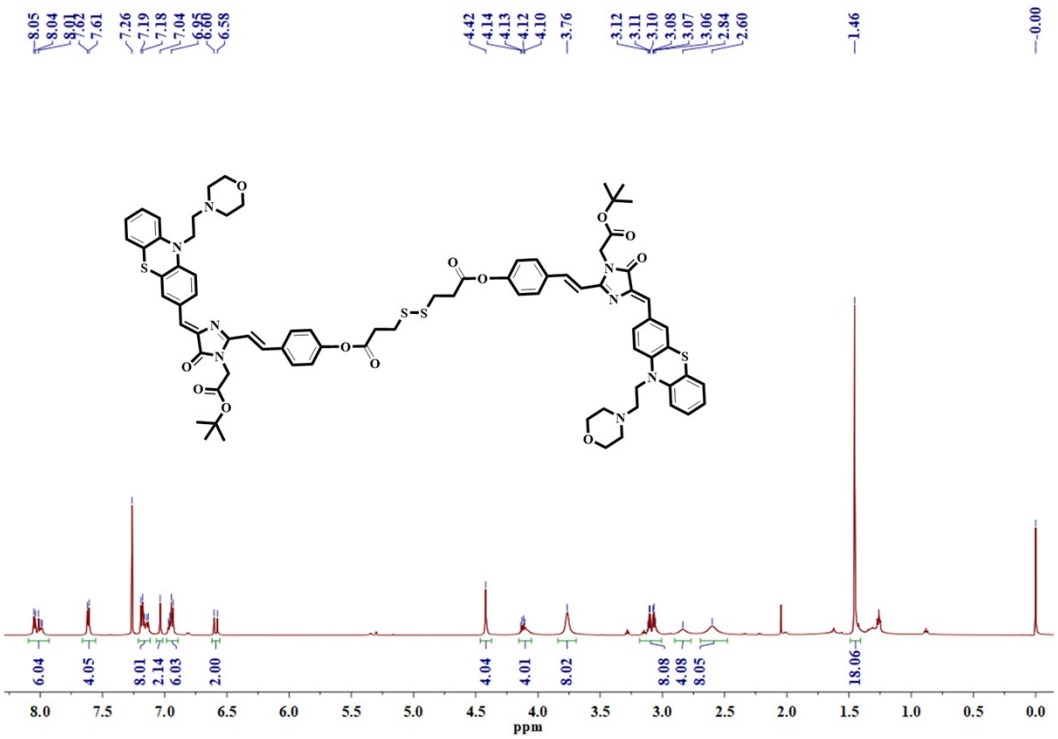

Figure S11. ^{1}H NMR spectrum of compound **PFP-SS-PFP**.

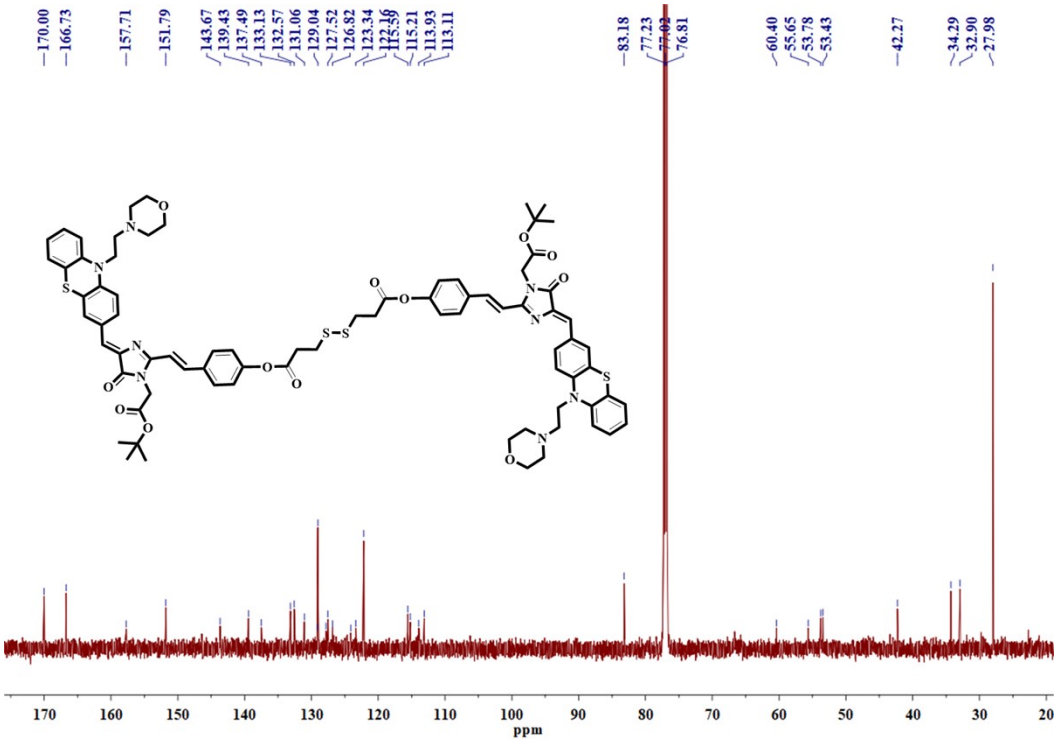

Figure S12. ^{13}C NMR spectrum of compound **PFP-SS-PFP**.

5. HRMS spectra of the compounds

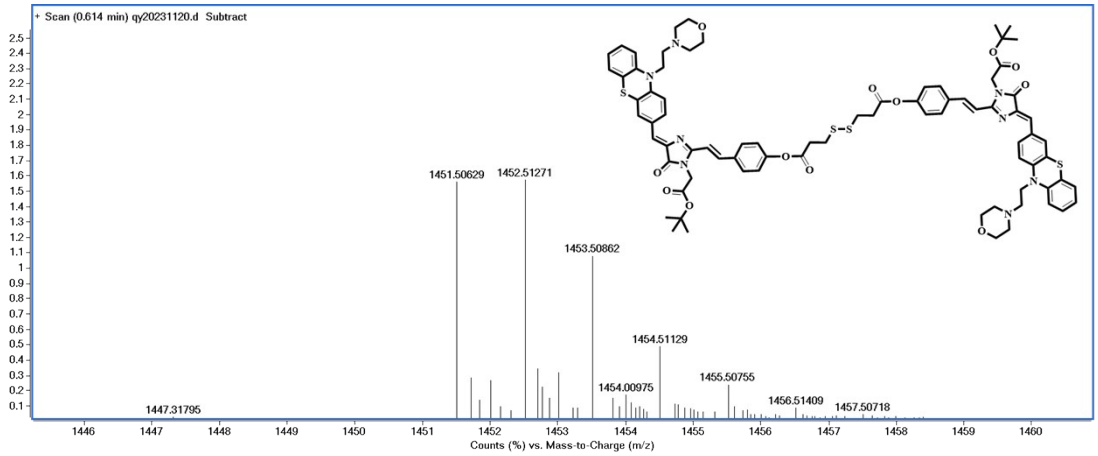

Figure S13. HRMS spectrum of compound **PFP-SS-PFP**.

References

(1) Jia, H.; Hu, G.; Shi, D.; Gan, L.; Zhang, H.; Yao, X.; Fang, J. *Anal. Chem.*, **2019**, *91*, 8524.
(2) Ke, L.; Wei, F.; Xie, L.; Karges, J.; Chen, Y.; Ji, L.; Chao, H. *Angew. Chem. Int. Ed.*, **2022** *61*, e202205429.
(3) Zhi, X.; Xiang, W.; Qian, Y. *J. Lumin*, **2021**, *240*,118424-118432.