# Perphenazine Modified Pillar[5]arene Based Nano-Assemblies for Synergistic Photothermal and Photodynamic Cancer Therapy

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# Supporting Information (15 pages)



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# 1. Materials and methods

## Materials

All reagents were commercially available and used as supplied without further purification. Solvents were either employed as purchased or dried according to procedures described in the literature.

## Measurements

**NMR spectroscopy**. $^{1}H$ and $^{13}C$ NMR spectra were recorded on a Brucker AV400 spectrometer.

**Fluorescence spectroscopy**. Steady-state fluorescence spectra were recorded in a conventional quartz cell (light path 10 mm) on a Varian Cary Eclipse equipped with a Varian Cary single-cell peltier accessory to control temperature.

**UV/Vis spectroscopy**. UV/Vis spectra and the optical transmittance were recorded in a quartz cell (light path 10 mm) on a Shimadzu UV-3600 spectrophotometer equipped with a PTC-348WI temperature controller.

**ESI-MS spectroscopy**. Electrospray ionization mass spectra (ESI-MS) were measured by Agilent 6520 Q-TOF-MS.

**Preparation of PeP5⊃Por NPs**: $10^{-3}$ M of **PeP5-Por** solution (1 mL, DMF) was dropped into 100 mL water, stirred the mixture quickly to disperse it evenly. Then, the mixture solution was transferred into dialysis tube (MWCO 3500 Da) to dialyze against distilled water. At last, the aqueous solution of **Por/Pe5** NPs was lyophilized to obtain nanomedicine powder, which was then dispersed in PBS (7.4) for biological studies.

**Cytotoxicity experiments**. HeLa cells were incubated in Dulbecco's modified Eagle's medium (DMEM). The medium was supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin. Cells were seeded in 96-well plates ($5 \times 10^4$ cell $mL^{-1}$, 0.1 mL per well) for 4 h at 37ºC in 5% $CO_2$. Then the cells were incubated with different groups for 4 h. The relative cellular viability was determined by the MTT assay.

**Confocal laser scanning microscopy.** Cells were seeded in 6-well plates ($5 \times 10^4$ cell $mL^{-1}$, 2 mL per well) for 24 h at 37ºC in 5% $CO_2$. The cells were incubated with the corresponding solution for 4 h. Then the medium was removed, and the cells were washed with phosphate buffer solution for three time. Finally, the cells were subjected to observation by a confocal laser scanning microscope.

**TEM microscopy.** High-resolution Transmission electron microscopy (TEM) images were acquired using a Tecnai 20 high-resolution transmission electron microscope operating at an accelerating voltage of 200 keV. The sample for high-resolution TEM measurements was prepared by dropping the solution onto a copper grid. The grid was then air-dried.

**DLS spectroscopy.** Solution samples were examined on a laser light scattering spectrometer (BI-200SM) equipped with a digital correlator (TurboCorr) at 636 nm at a scattering angle of 90°. The hydrodynamic diameter (Dh) was determined by DLS experiments at 25°C.

**ROS detection**. HeLa cells were seeded into 12 mm sterile coverslips in a 6-well plate and maintained for 12 h. The cells were incubated with **Por**, **PeP5⊃Por**, and **P5⊃Por** for 4h, then irradiated with 808 nm (10 mM/$cm^2$) laser for 10 min. Afterward, DCFH-DA/SOSG/APF/DHE was added to incubate together with cells and imaged, respectively.

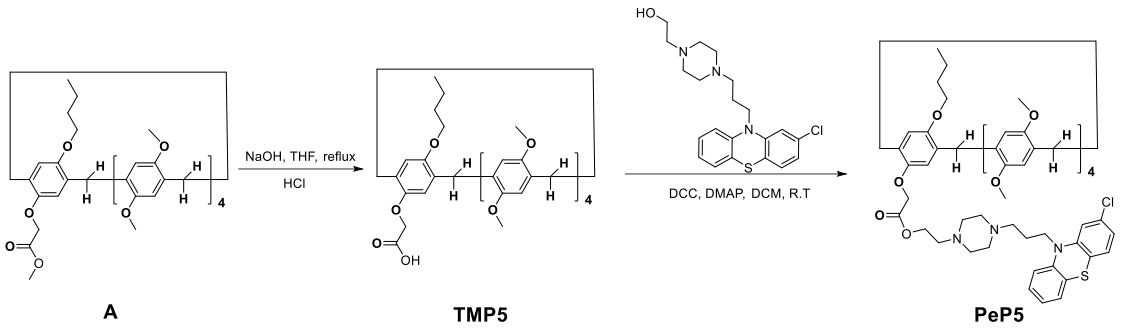
**Live-Dead Cell Staining**. The same density of HeLa cells ($3 \times 10^5$ cell $mL^{-1}$) were distributed into three confocal dishes (35 mm) for 12 h. Then the 2-plate cells were cultured with new DMEM containing particles. After 5 h, the cells were subjected to dark or laser irradiation (808 nm, 1 W/$cm^2$, 10 min). After 48 h, the cells were stained with a calcein AM/propidium iodide mixture for 30 min and washed twice using PBS. The fluorescence images were eventually acquired via a confocal laser scanning microscope.

**Simulation methods**:

In this work, the density functional theory (DFT) calculation was performed using the Dmol$^3$ code [S1]. The exchange-correlation interaction was treated by the generalized gradient approximation (GGA) with PBE functional [S2]. A double numerical quality basis set with d-type polarization function (DNP[S3]) was utilized for all the geometric optimizations, total energy calculations. The core electrons were modeled using effective core pseudopotentials (ECP) by Dolg[S4] and Bergner[S5]. Grimme's semi-empirical DFT-D[S6] was introduced in the computations to guarantee a better description of the electron interaction in a long range. All calculations were spin unrestricted. The positions of all the atoms were fully relaxed until the following convergence criterion are met respectively: 0.002 Ha/Å for force, $10^{-5}$ Ha for total energy and 0.005 Å for displacement. The real space cutoff radius was 4.1 Å. The self-consistent field computations criterion was chosen to be $10^{-6}$ Ha.

S1. Delley B (2000) From molecules to solids with the DMol$^3$ approach. J Chem Phys 113 (18):7756-7764
S2. Perdew JP, Burke K, Ernzerhof M (1996) Generalized Gradient Approximation Made Simple. Phys Rev Lett 77 (18):3865-3868
S3. Delley B (1990) An all-electron numerical method for solving the local density functional for polyatomic molecules. J Chem Phys 92:508-517
S4. Dolg M, Wedig U, Stoll H, Preuss H (1987) Energy-adjusted abinitio pseudopotentials for the first row transition elements. J Chem Phys 86 (2):866-872
S5. Bergner A, Dolg M, Küchle W, Stoll H, Preuß H (1993) Ab initio energy-adjusted pseudopotentials for elements of groups 13–17. Mol Phys 80 (6):1431-1441
S6. S. Grimme, J. Antony, S. Ehrlich, H. Krieg, J. Chem. Phys. 2010,132, 154104.

## 2. Synthesis of perphenazine modified pillar[5]arene (**PeP5**)




Scheme S1. Synthetic route to **PeP5**.

Synthesis of **TMP5**: Compound **A** [S7](0.3 mmol, 0.255g) was dissolved in 20 mL THF and added to a 50 mL round-bottomed then reflux at 65 ℃. After reflux dissolution, 15 mmol (0.60g) NaOH was added to the reaction solution for 10 hours. After the end of the reaction, the organic solution was removed by spin evaporation, and the reaction liquid was adjusted to acidity (pH = 1), the reaction was left to precipitate solid, and finally the product **TMP5** was extracted and filtered.

Synthesis of **PeP5**: Compound TMP5 (0.25 mmol, 0.21g), 2-(4-(3-(2-chloro-10H-phenothiazin-10-yl)propyl)piperazin-1-yl)ethan-1-ol (0.3 mmol, 0.12g), DCC (0.625 mmol, 0.128g) and DMAP (0.125 mmol, 0.015g) were added into 20 mL DCM solution and stirred at room temperature for 24 hours. After the reaction, the organic solvent was removed by rotary evaporation and **PeP5** was obtained by column chromatography.

**TMP5**:

White solid, 49%, m.p.213-214℃; $^{1}$H NMR (400 MHz, $CDCl_3$) δ 6.81 – 6.77 (m, 4H, ArH), 6.75 – 6.70 (m, 4H, ArH), 6.67 (s, 1H, ArH), 6.58 (s, 1H, ArH), 4.28 (s, 2H, $CH_2$), 3.92 – 3.88 (m, 2H, $CH_2$), 3.81 – 3.76 (m, 10H, $CH_2$), 3.68 – 3.60 (m, 24H, $CH_3$), 1.78 (t, *J* = 7.4 Hz, 2H, $CH_2$), 1.55 – 1.50 (m, 2H, $CH_2$), 0.96 (t, *J* = 7.4 Hz, 3H, $CH_3$). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 170.97, 151.41, 150.96, 150.82, 150.77, 150.75, 150.71, 150.68, 150.66, 150.13, 148.28, 129.19, 128.86, 128.47, 128.36, 128.25, 128.23, 128.19, 128.17, 127.85, 127.69, 124.78, 115.34, 115.15, 114.54, 114.28, 114.09, 114.04, 114.02, 113.95, 113.91, 113.73, 68.56, 65.72, 56.87, 55.98, 55.88, 55.86, 55.76, 55.74, 55.71, 31.82, 30.19, 30.03, 29.79, 29.58, 28.61, 19.50, 13.98; MS (m/z): HRMS (ESI) Calcd. for $C_{49}H_{56}O_{12}$([M + Na]$^{+}$): 859.3669, found: 859.36377.

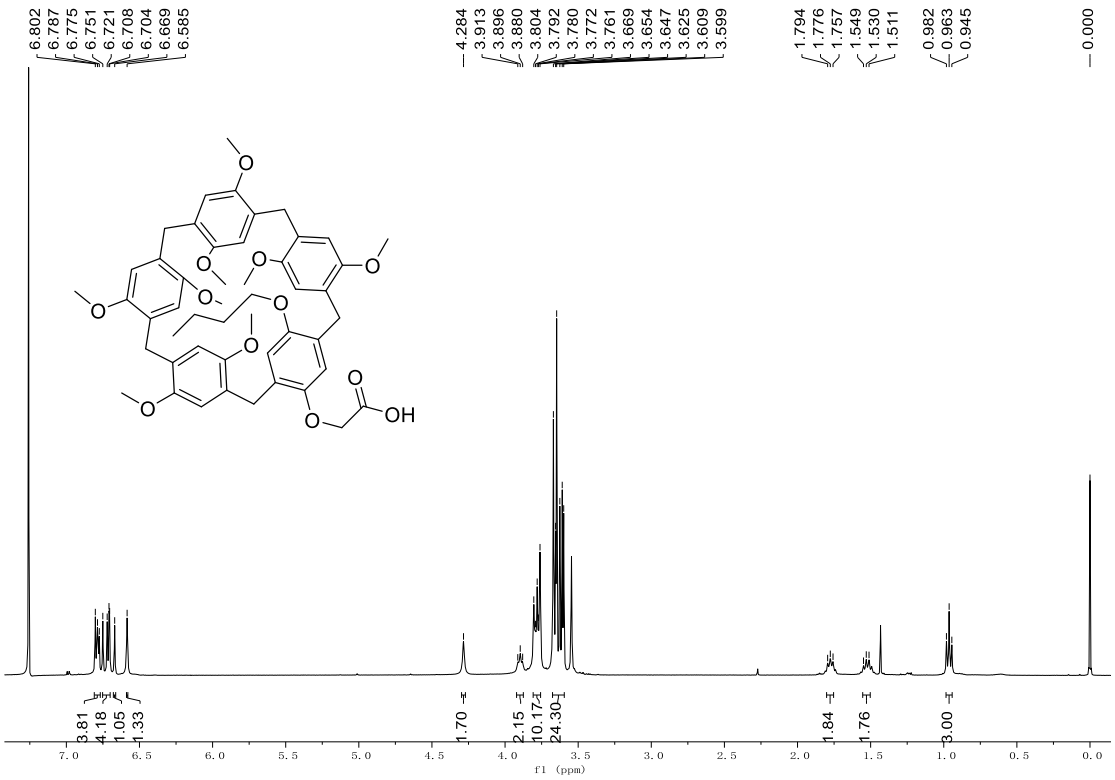



Figure S1. $^{1}$HNMR (400 MHz, 298K, $CDCl_3$) spectrum of **TMP5**.

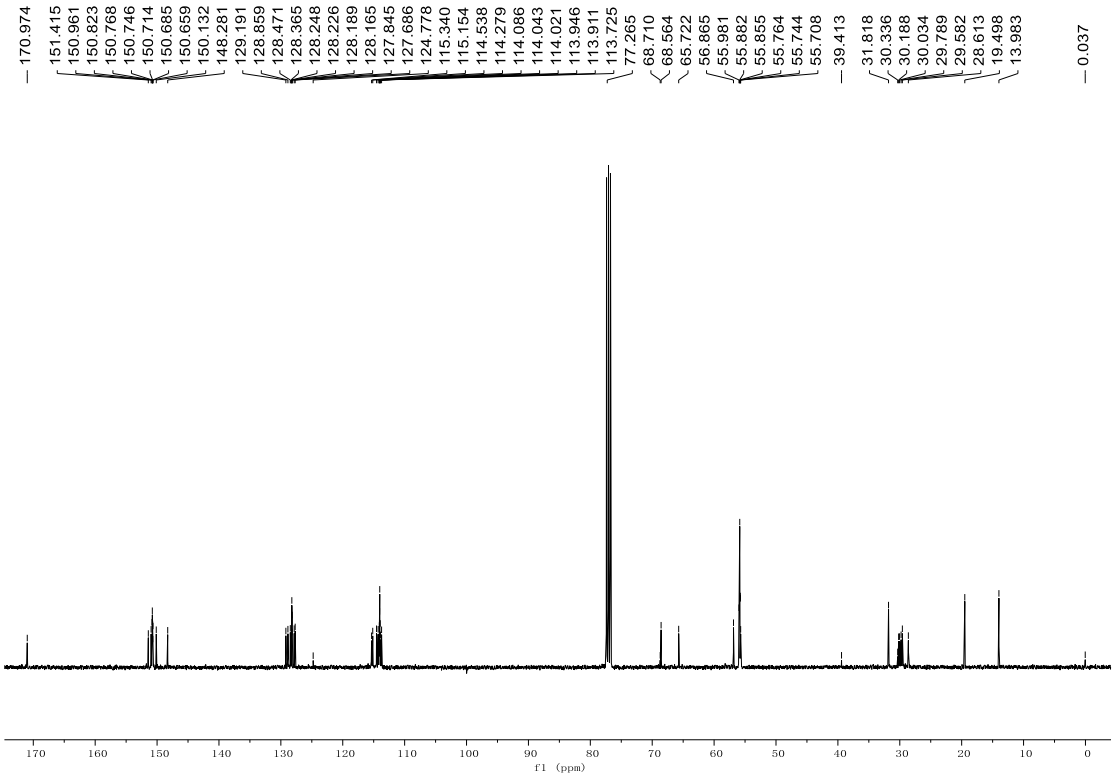



Figure S2. $^{13}$C NMR (100 MHz, 298K, $CDCl_3$) spectrum of **TMP5**.

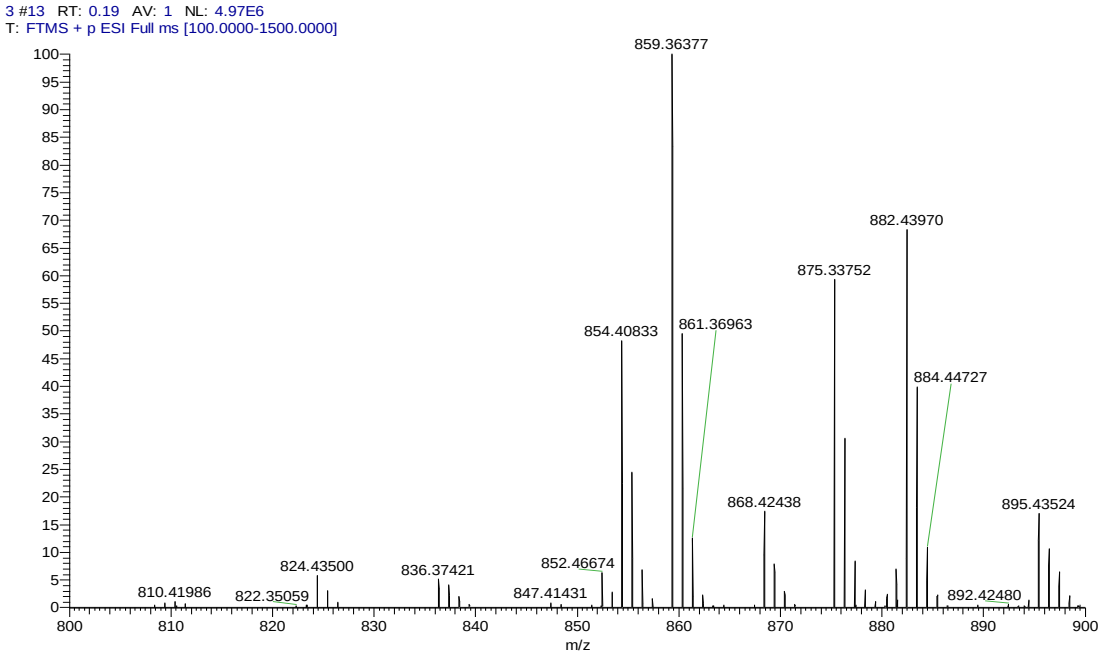



Figure S3. HR-MS (ESI) of **TMP5**. Calcd. for $C_{49}H_{56}O_{12}$([M + Na]$^+$): 859.3669, found: 859.36377.

**PeP5:**

Gray solid, 40%, m.p.200-202 ℃; $^1$H NMR (400 MHz, $CDCl_3$) δ 7.17 – 7.10 (m, 2H, ArH), 7.01 (d, *J* = 8.1 Hz, 1H, ArH), 6.96 – 6.84 (m, 5H, ArH), 6.82 – 6.72 (m, 9H, ArH), 4.48 (s, 2H, $CH_2$), 4.31 (d, *J* = 7.6 Hz, 2H, $CH_2$), 3.89 (t, *J* = 6.8 Hz, 2H, $CH_2$), 3.85 – 3.81 (m, 4H, $CH_2$), 3.78 – 3.64 (m, 34H, $8CH_3$, $5CH_2$), 2.64 (t, *J* = 6.0 Hz, 2H, $CH_2$), 2.47 (d, *J* = 7.0 Hz, 4H, $CH_2$), 1.95 – 1.91 (m, 2H, $CH_2$), 1.76 – 1.70 (m, 2H, $CH_2$), 1.54 – 1.47 (m, 2H, $CH_2$), 1.37 – 1.28 (m, 2H, $CH_2$), 1.13 – 1.06 (m, 2H, $CH_2$), 0.95 (t, *J* = 7.4 Hz, 3H, $CH_3$). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 169.89, 169.36, 150.97, 150.93, 150.89, 150.83, 150.78, 150.76, 150.74, 150.73, 150.70, 150.64, 150.50, 150.47, 150.41, 150.21, 148.85, 146.51, 144.53, 133.22, 128.84, 128.74, 128.56, 128.51, 128.38, 128.29, 128.21, 128.18, 128.16, 128.14, 128.10, 128.08, 128.06, 127.99, 127.92, 127.80, 127.54, 127.46, 124.76, 123.51, 122.93, 122.26, 115.87, 115.85, 115.34, 114.85, 114.74, 114.35, 114.15, 114.10, 114.04, 113.97, 113.84, 113.71, 113.65, 77.35, 67.95, 66.52, 62.40, 57.70, 56.55, 55.94, 55.90, 55.85, 55.84, 55.79, 55.78, 55.73, 55.71, 55.67, 55.49, 53.46, 53.30, 53.21, 52.89, 49.11, 45.33, 34.01, 31.89, 31.87, 30.88, 29.88, 29.73, 29.65, 29.46, 29.21, 25.69, 25.05, 24.25, 19.57, 19.55, 14.07, 14.03; MS (m/z): HRMS (ESI) Calcd. for $C_{70}H_{80}ClN_3O_{12}S$([M + 2H + 2Na]$^+$): 1269.5103, found: 1269.61267.

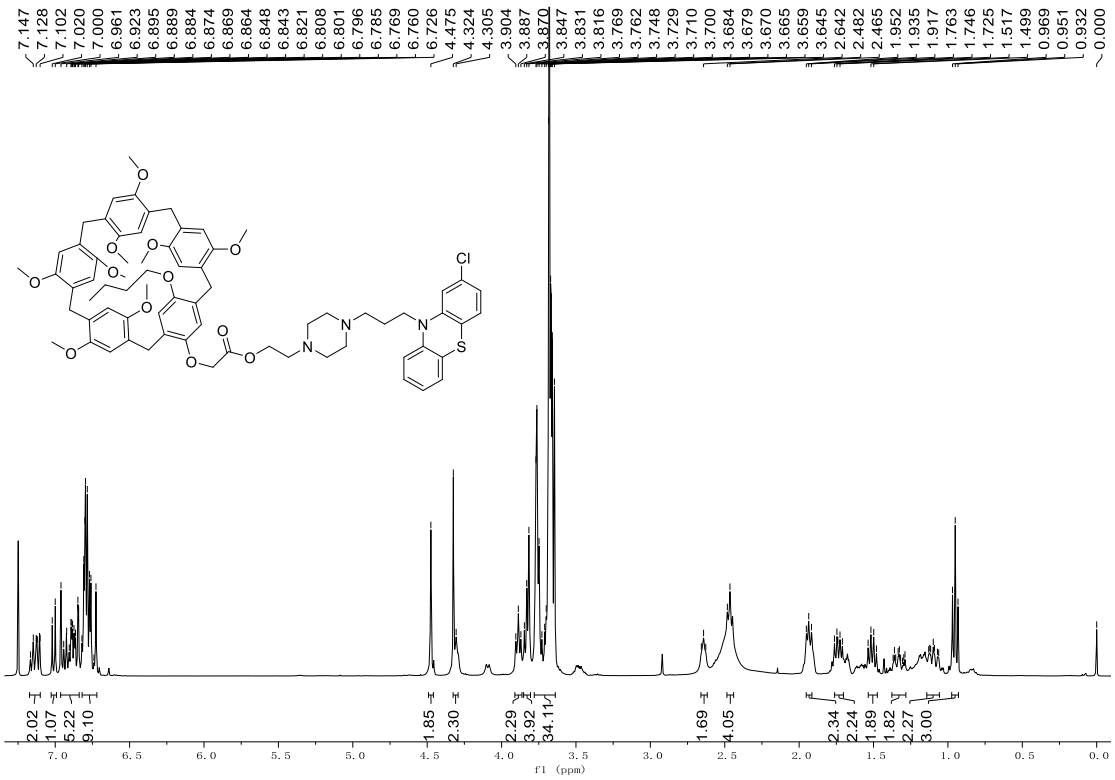



Figure S4. $^{1}$HNMR (400 MHz, 298K, $CDCl_3$) spectrum of **PeP5**.

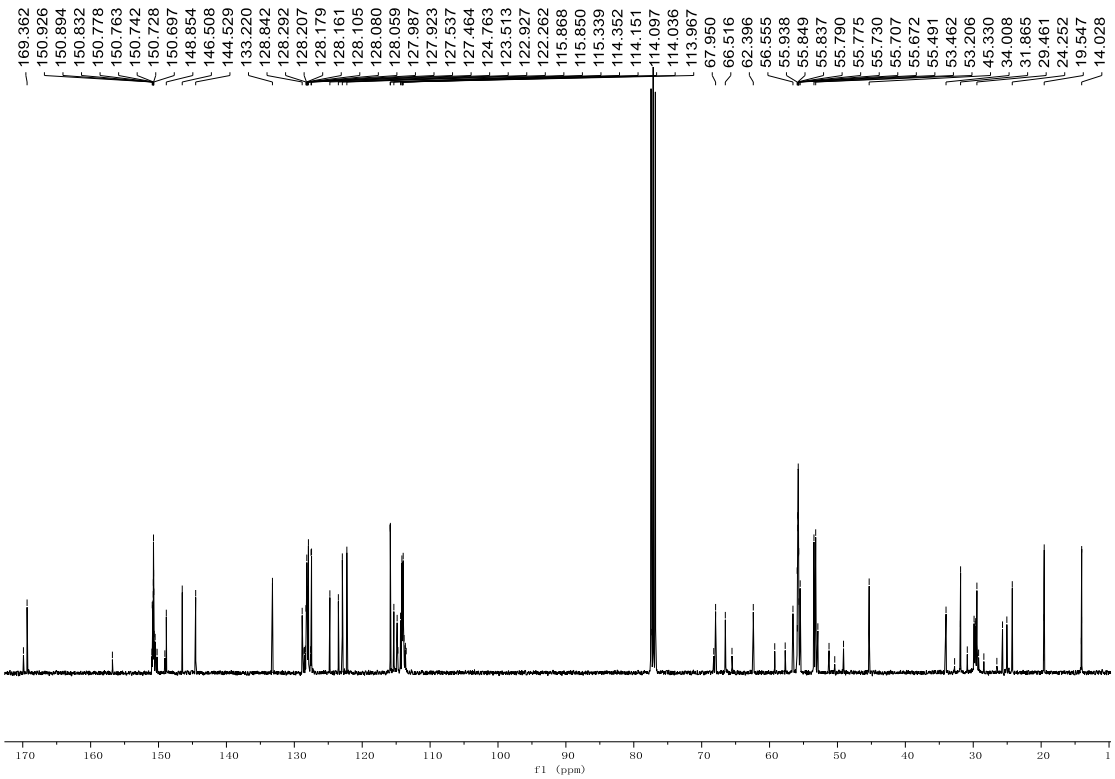



Figure S5. $^{13}$C NMR (100 MHz, 298K, $CDCl_3$) spectrum of **PeP5**.

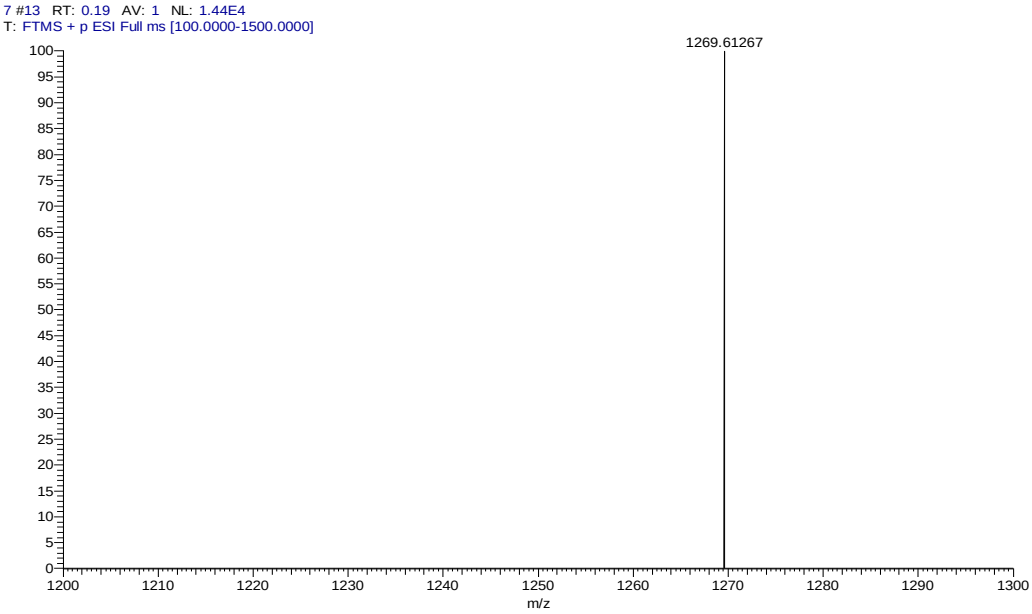
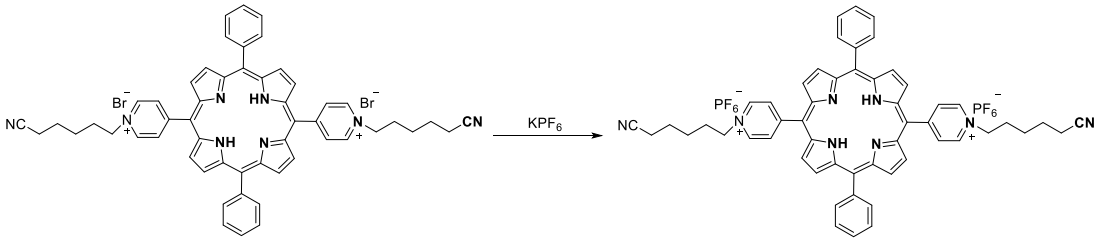



Figure S6. HR-MS (ESI) of **PeP5**. Calcd. for $C_{70}H_{80}ClN_3O_{12}S([M + 2H + 2Na]^+)$: 1269.5103, found: 1269.61267.

## 3. Synthesis of **Por**




**Br-Por** **Por**

Scheme S2. Synthetic route to **Por**.

Synthesis of **Por**: The compound **Br-Por**[S8] (0.3 mmol, 0.29g) was fully dissolved in methanol, then added to the saturated potassium hexafluorophosphoric acid solution by drops, and then precipitated into solid. The solid was collected by centrifugation and washed with methanol (3 × 10 mL) and water (3 × 10 mL) to obtain purple solid **Por**.

**Por**:

Purple solid, 70%, m.p.245-247℃; $^1$H NMR (400 MHz, $CD_3CN$) δ 9.10 (d, *J* = 6.5 Hz, 4H, ArH), 9.05 (d, *J* = 5.7 Hz, 4H, CH), 8.93 (d, *J* = 15.3 Hz, 4H, CH), 8.84 (d, *J* = 6.7 Hz, 4H, ArH), 8.25 – 8.22 (m, 4H, ArH), 7.86 (m, 6H, ArH), 4.88 (t, *J* = 7.5 Hz, 4H, $CH_2$), 2.57 (t, *J* = 6.9 Hz, 4H, $CH_2$), 2.33 (m, 4H, $CH_2$), 1.86 (m, 4H, $CH_2$), 1.81 – 1.74 (m, 4H, $CH_2$), -2.86 (s, 2H, NH). $^{13}$C NMR (101 MHz, $CD_3CN$) δ 143.0, 134.2, 133.0, 126.7, 48.26, 48.08, 48.04, 47.89, 47.83, 47.63, 47.62, 47.40, 47.37, 47.19, 47.13, 46.98, 34.0, 30.3, 25.0, 24.6; MS (m/z): HRMS (ESI) Calcd. for $C_{54}H_{48}F_{12}N_8P_2([M]^+)$: 404.1995, found: 404.19925.

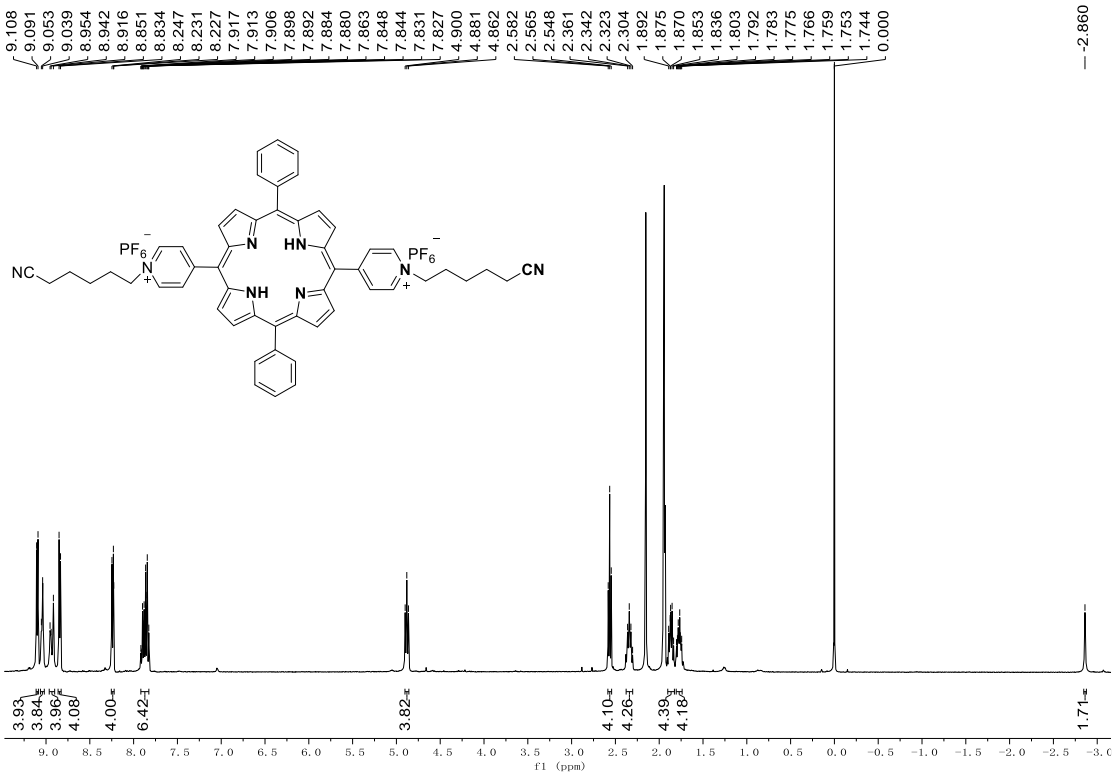



**Figure S7.** $^{1}$H NMR (400 MHz, 298K, $CD_3CN$) spectrum of **Por**.

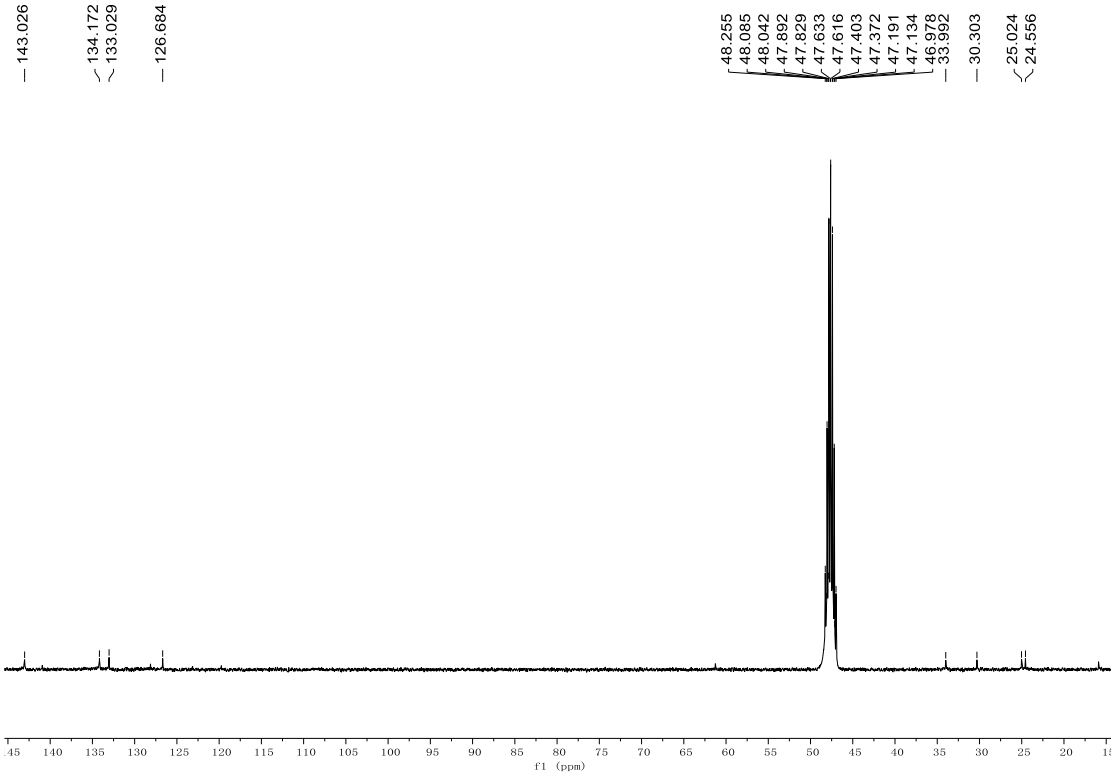



Figure S8. $^{13}$C NMR (100 MHz, 298K, $CD_3CN$) spectrum of **Por**.

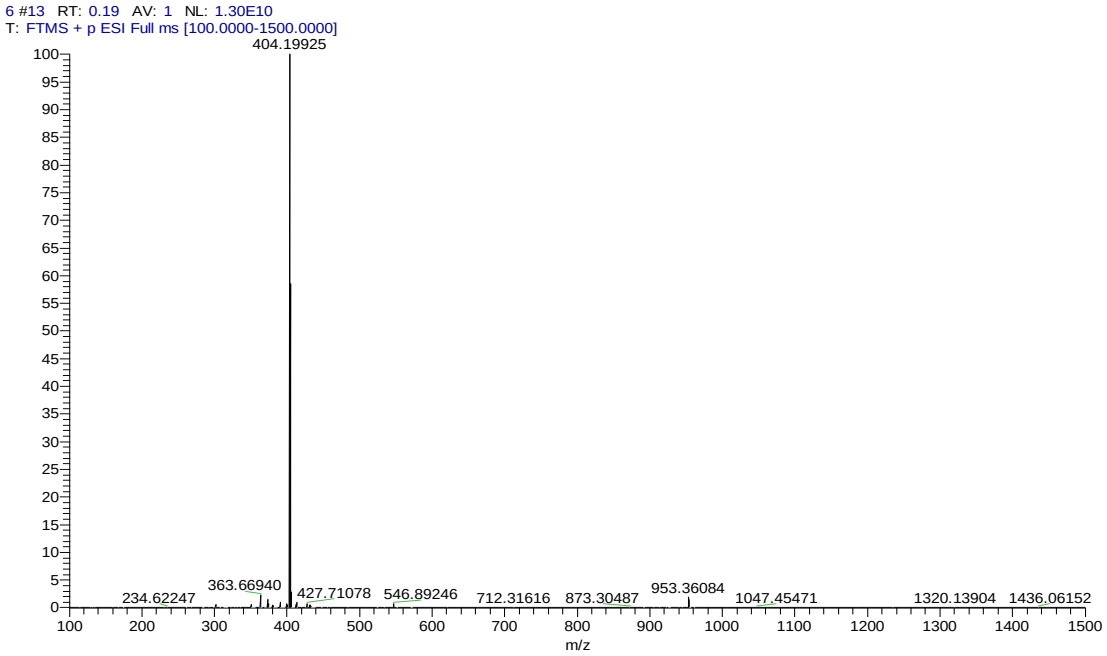



Figure S9. HR-MS (ESI) of **Por**. Calcd. for $C_{54}H_{48}F_{12}N_8P_2([M]^+)$: 404.1995, found: 404.19925.

## 4. Host-guest interaction between **PeP5** and model **guest**

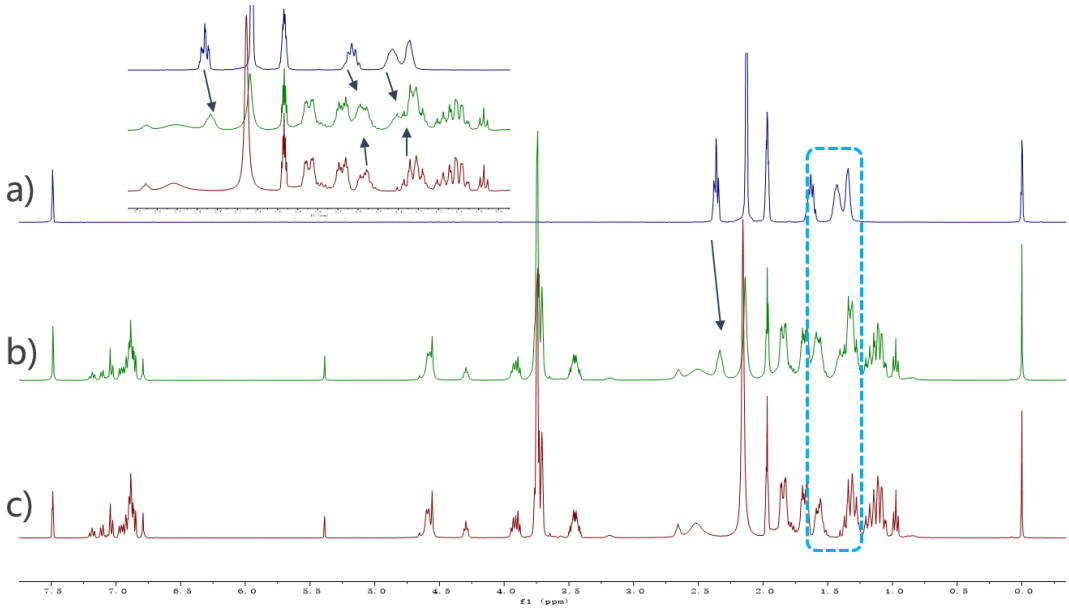



Figure S10. $^1$H NMR (400 MHz, 298 K, $CDCl_3$ + $CD_3CN$) of (a) sebaconitrile (10.0 mM), (b) sebaconitrile/**PeP5** (sebaconitrile = 10.0 mM, **PeP5** = 10.0 mM) and (c) **PeP5** (10.0 mM).

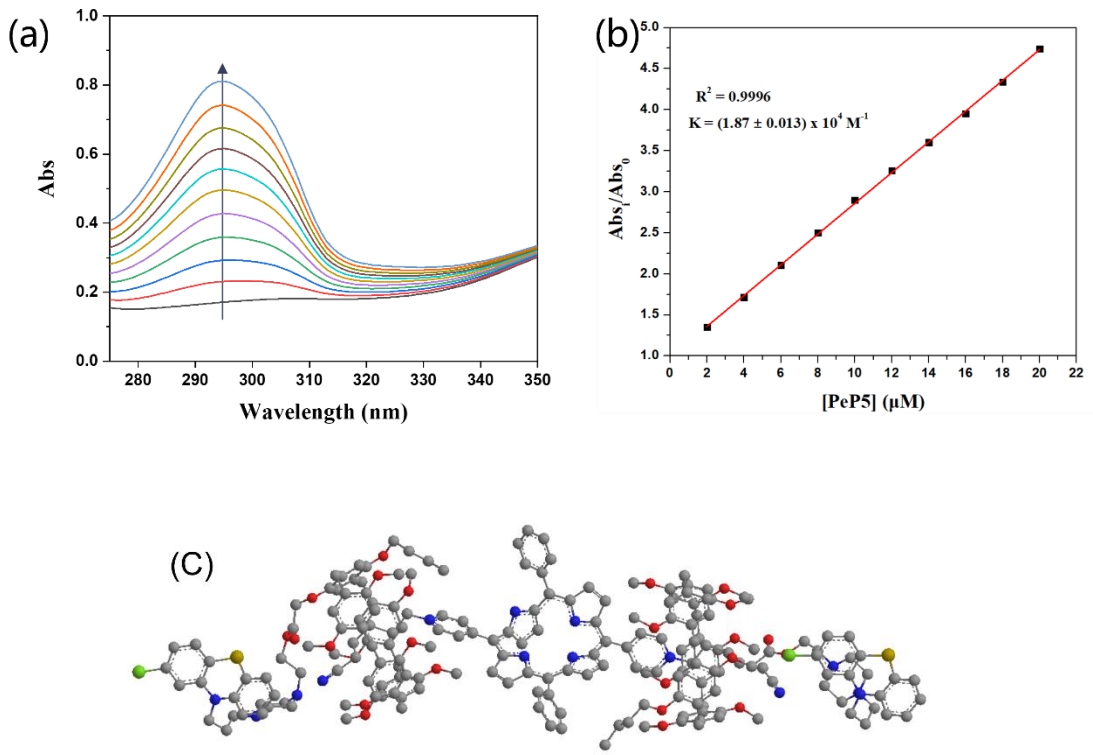



Figure S11. (a) UV-vis spectra of **Por** (10 μM) after **PeP5** (concentrations were 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 μM, respectively) were added (b) Complexation constant curves of host-guest interactions between **PeP5** and **Por** by using Stern-Volmer curve. (c) Calculated structure of **PeP5** ⊃ **Por**.

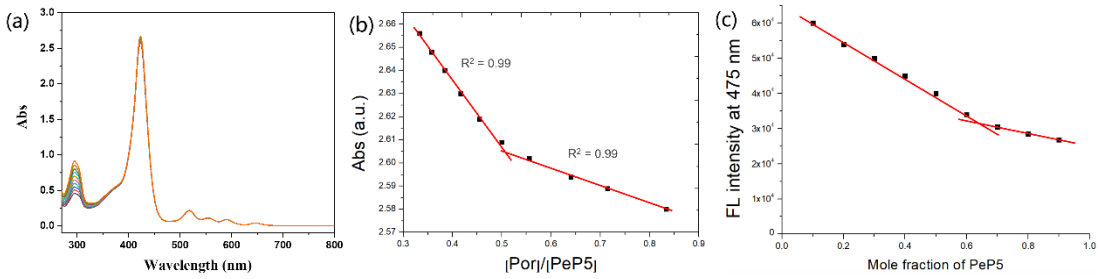



Figure S12. **(a)** UV titration of **Por** (10 μM) in acetonitrile solution with increasing **PeP5** concentration. **(b)** Plot the work of the **PeP5** ⊃ **Por** complex by plotting the UV absorbance at 423 nm. Working diagram of composite **PeP5** ⊃ **Por** showing a stoichiometric ratio of 2:1 between **PeP5** and **Por**. (c) Job's plot for **Por** upon addition of **PeP5** ([**Por**] + [**PeP5**] = 10 μM]).

## 5. ROS generation ability PeP5⊃Por NPs

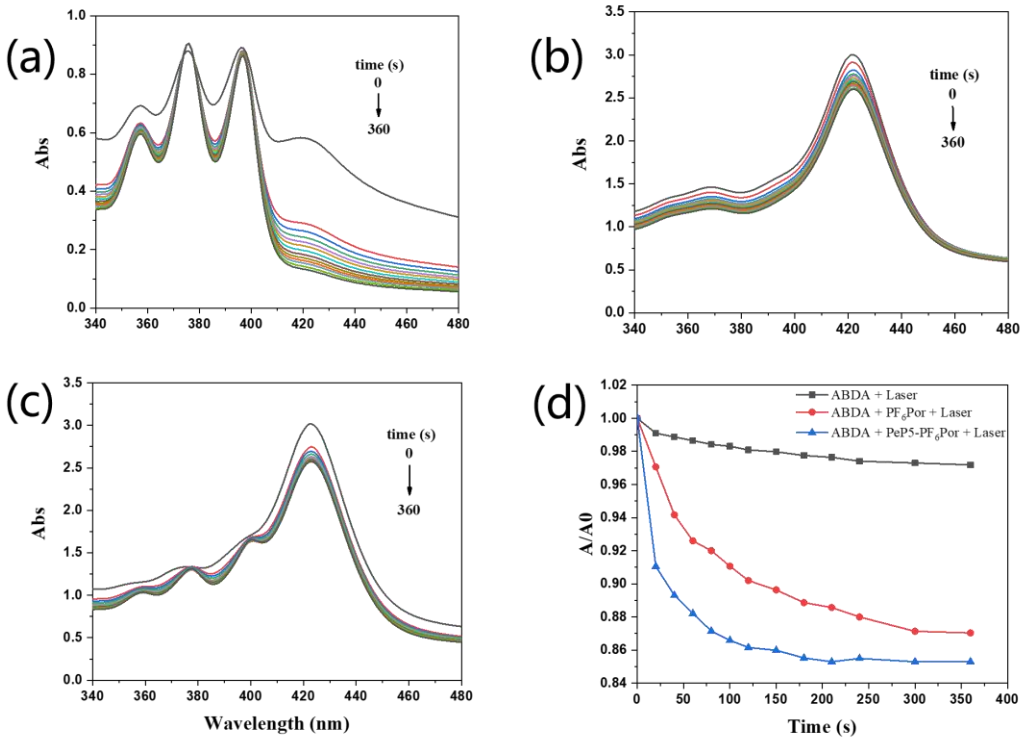



Figure S13. Time-dependent UV-vis spectra of **(a) ABDA** and **(b)** with **Por (c)** with **PeP5-Por** after irradiation with 808 nm light (1 mW $cm^{-2}$) in acetonitrile solution **(d)** Comparison of A/A0 rate of ABDA solution with **Por** and **PeP5** under irradiation. A0 is the initial absorbance, and A is the immediate absorbance.

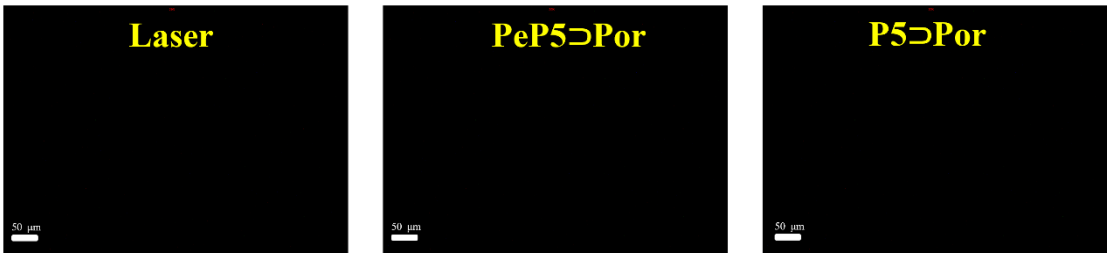



Figure S14 CLSM images of ROS species generated in HeLa cells after 808 nm (10 mW/$cm^2$) light irradiation with different treatment.

## 6. In vitro study

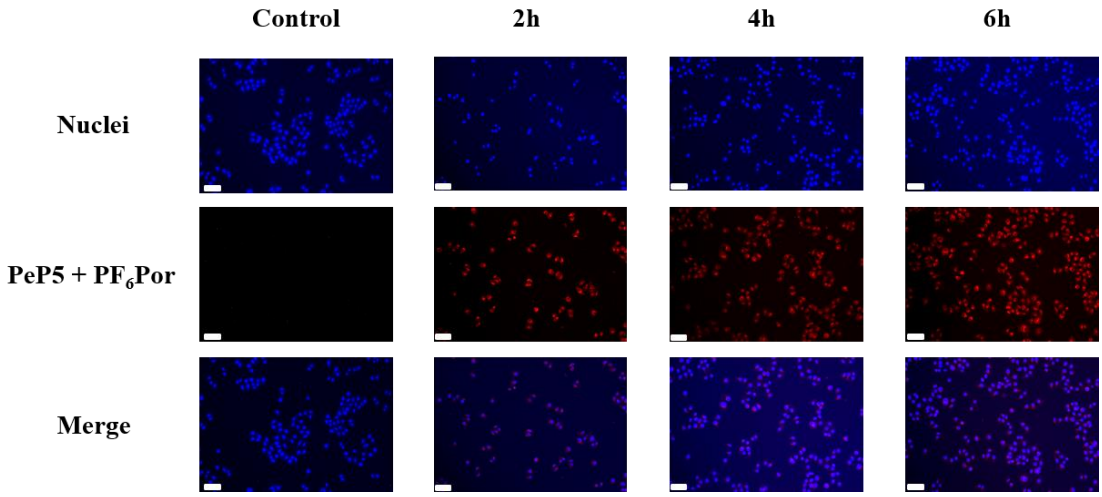



Figure S15. Fluorescence images of HeLa cells incubated with **PeP5/Por** for 0h, 2 h, 4h, and 6 h. The cell nuclei were stained as blue by Hoechst 33342, red was the fluorescence of **PeP5/Por**.

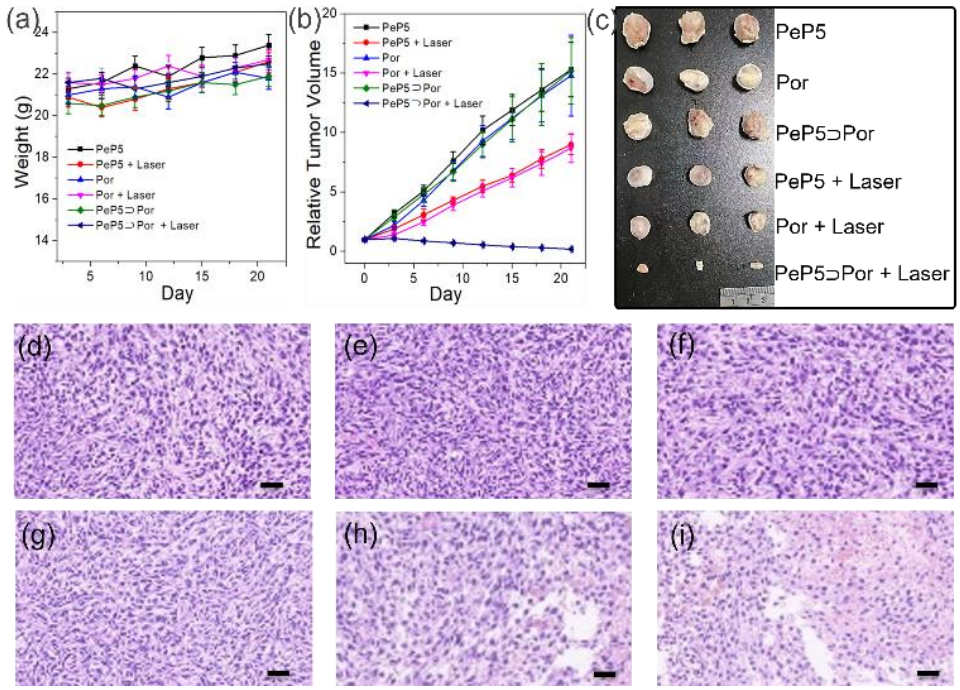



Figure S16. (a) Body weight change of mice. (b) Relative tumour volume change after treatment with different treatment. (c) Photographs of the tumour after incubation with different groups. (d)-(i) H&E assays of the dissected tumours with PeP5, Por, PeP5⊃Por, PeP5 + Laser, Por + Laser, PeP5⊃Por + Laser groups, respectively. Scale bar = 50 μm.

S7. R. Zhang, X. Yan, H. Guo, L. Hu, C. Yan, Y. Wang, and Y. Yao, *Chem. Commun.*, **2020**, *56*, 948-951.

S8. Y. Zhang, Y. Wang, T. Chen, Y. Han, C. Yan, J. Wang, B. Lu, L. Ma, Y. Ding, and Y. Yao, *Chem. Commun.*, **2023**, *59*, 8266-8269.