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A mitochondria-targeting supramolecular photosensitizer based on pillar[5]arene for photodynamic therapy

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1. Materials and Methods

1.1 Materials.

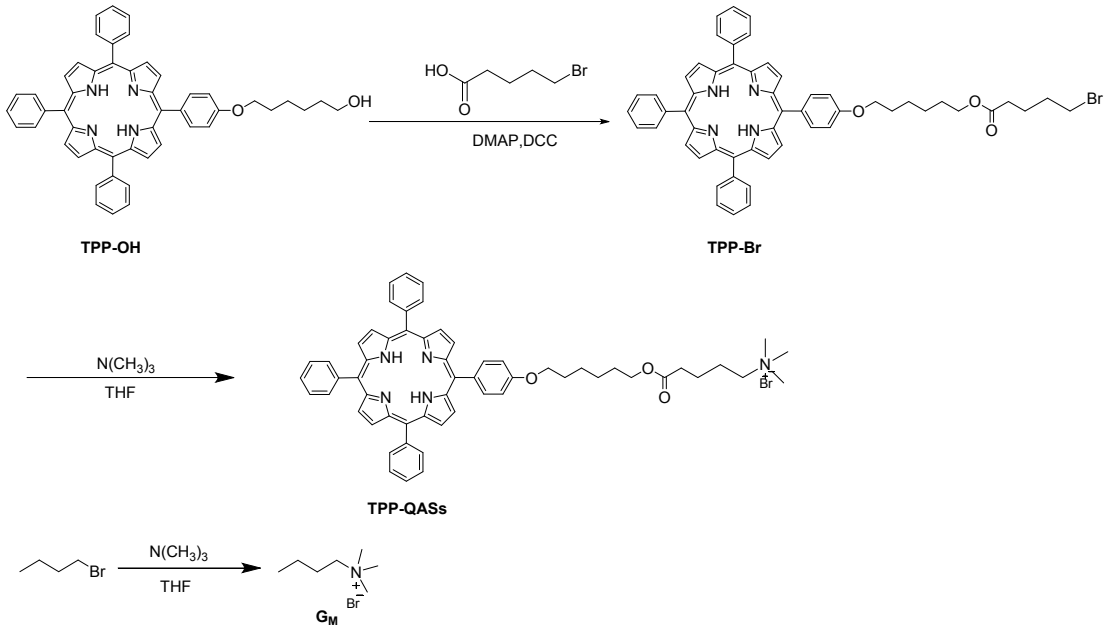
WP5, free porphyrin (TPP-OH) and G_m was synthesized according to the previous literature procedure, respectively.[1-3] Tetrahydrofuran (THF) was refluxed to remove water over sodium. Dichloromethane (DCM) were dried over calcium hydride and distilled before use. 5-bromovaleric acid, 1-bromobutane, 1-bromododecane, dodecyltrimethylammonium bromide (DTAB), 4-dimethylaminopyridine (DMAP), dicyclohexylcarbodiimide (DCC), and trimethylamine were all purchased from Aladdin Reagents of China and used directly as received. Rhodamine 123 (Rh123), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 4',6-diamidino-2-phenylindole (DAPI), and 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) were purchased from Beyotime and used as received. Other common reagents and solvents were purchased from Sinopharm Chemical Reagent Co. Ltd (SRC) and purified by standard procedure.

1.2 Methods

^1H NMR spectra was recorded at 400 MHz, using a BRUKER AV400 Spectrophotometer, in D_2O or $CDCl_3$ with tetramethylsilane (TMS) as an internal reference. Absorption spectra were determined on a SHIMADZU UV-2550 UV spectrophotometer using in quartz Cuvette with 1 cm beam path length, and fluorescence spectra measurements were performed on a Varian's Cary Eclipse fluorescence spectrophotometer at room temperature with excitation and emission slit width of 10 nm and 5 nm, respectively. Dynamic light scattering (DLS) and zeta potential measurements were carried out with a BECKMAN COULTER Delasa Nano C particle analyzer. All the measurements were carried out at room temperature. Transmission electron microscopy (TEM) analysis was performed on a JEOL JEM1400 electron microscope operated at 100 kV. Samples for TEM were prepared by dropping the micelle solution onto a carbon-coated copper grid and then dried at room temperature. TOF-MS was performed by using an AB Sciex4800 Plus MODI TOF/TOF analyzer; a Vario EL III Element analyzer was used for elemental analysis.

2. Syntheses of compounds and Characterization

Scheme S1. Synthesis of quaternary ammonium salts terminal porphyrin (**TPP-QASs**) and the model compound G_M



Synthesis of TPP-Br

6-(5-(4-phenoxyl)-10, 15, 20-triphenylporphyrin)-1-hexanol (365 mg, 0.50 mmol), DMAP (90 mg, 0.70 mmol), and 5-bromovaleric acid (181 mg, 1.0 mmol) were dissolved in anhydrous dichloromethane (10 mL). The flask was cooled to 0 °C in ice-water bath. Then DCC (194 mg, 0.59 mmol) in 4 mL of anhydrous dichloromethane was added dropwise and stirred for 24 h at room temperature. After filtration, the filtrate was concentrated and further purified by silica gel column chromatography using dichloromethane. After drying two days in a vacuum oven at room temperature, a purple solid was obtained (0.502 g, yield: 91.9%). ^{1}H NMR (400 MHz, $CDCl_3$, δ): 8.87 (m, 8H,*β-H*), 8.21 (m, 6H, 10, 15, 20-Ar-*o-H*), 8.11 (m, 2H, 5-Ar-*o-H*), 7.76 (m, 9H, 10, 15, 20-Ar-*m*- and *p-H*), 7.28 (m, 2H, 5-Ar-*m-H*), 4.26 (t, 2H, Ar-O-CH_2-CH_2-), 4.17 (t, 2H, -CH_2-OCO-CH2-), 3.44 (t, 2H, -CH_2-Br), 2.39 (t, 2H, -CH_2-OCO-C*H2*-), 2.00-1.59 (m, 12H, -CH_2-(CH_2)$_4$-CH_2-OH, -CH_2-(CH_2)$_2$-CH_2-Br), -2.77 (s, 2H,-N*H*-).

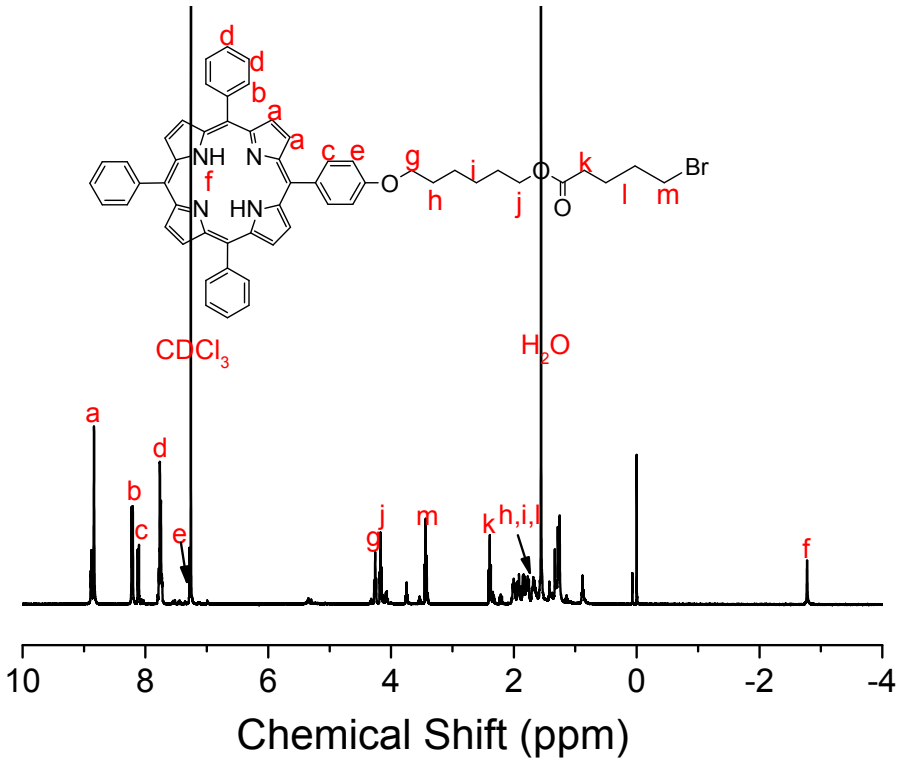


Fig. S1 ^{1}H NMR spectrum of TPP-Br.

Synthesis of TPP-QAS.

The solution of **TPP-Br** (446 mg, 0.5 mmol) and excess trimethylamine were dissolved in 10 mL of THF and refluxed for 24 h. After being cooled to room temperature, the solvent and unreacted trimethylamine were removed under vacuum to produce the **TPP-QAS** in yield of 96% (420 mg, 0.48 mmol). ^{1}H NMR (400 MHz, $CDCl_3$, δ): 8.87 (m, 8H,*β-H*), 8.21 (m, 6H, 10, 15, 20-Ar-*o-H*), 8.11 (m, 2H, 5-Ar-*o-H*), 7.76 (m, 9H, 10, 15, 20-Ar-*m*- and *p-H*), 7.28 (m, 2H, 5-Ar-*m-H*), 4.26 (t, 2H, Ar-O-C*H*$_2$-CH$_2$-), 4.16 (t, 2H, -C*H*$_2$-OCO-CH2-), 3.41 (t, 2H, -C*H*$_2$N$^+$(CH$_3$)$_3$), 3.35 (s, 9H, -CH$_2$N$^+$(C*H*$_3$)$_3$), 2.47 (t, 2H, -CH$_2$-OCO-C*H2*-), 2.00-1.59 (m, 12H,-CH$_2$-(C*H*$_2$)$_4$-CH$_2$-OH, -CH$_2$-(C*H*$_2$)$_2$-CH$_2$-Br), -2.78 (s, 2H,-N*H*-); elemental analysis calcd (%) for $C_{58}H_{58}BrN_5O_3$ (951.3723): C 73.10, H 6.13, N 7.35; found: C 72.69, H 6.64, N 6.86; MALDI-TOF-MS m/z calcd for $C_{58}H_{58}N_5O_3$, 872.4534;found 872.4979 (**Fig. S4** in the Supporting Information).

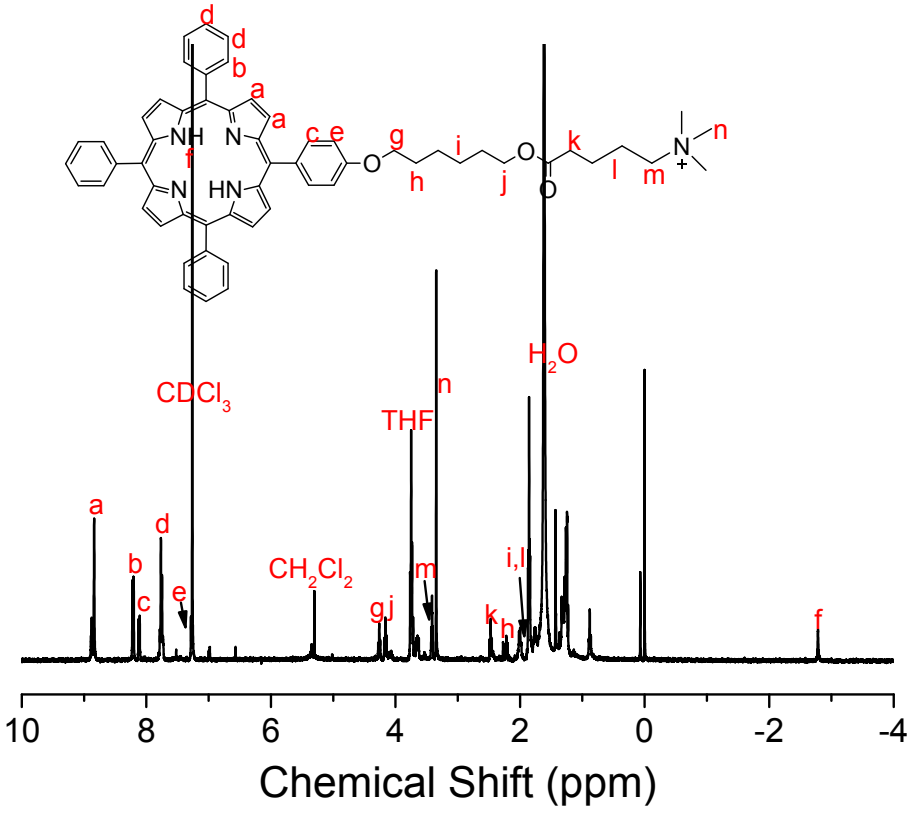


Fig. S2 ^{1}H NMR spectrum of **TPP-QAS**.

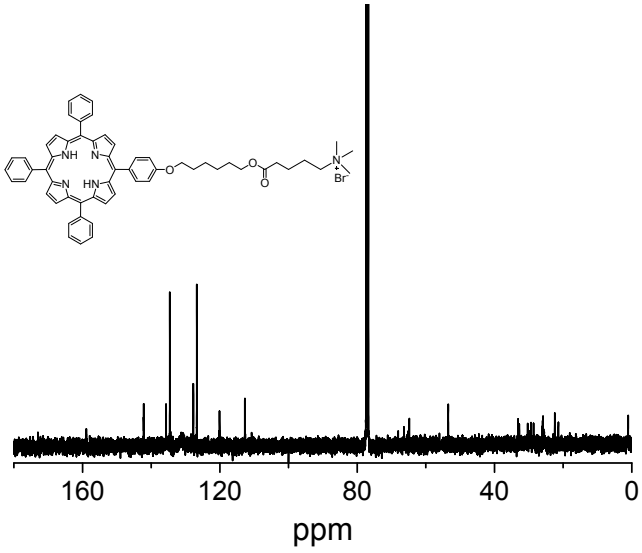


Fig. S3 ^{13}C NMR spectrum of **TPP-QAS** in $CDCl_3$.

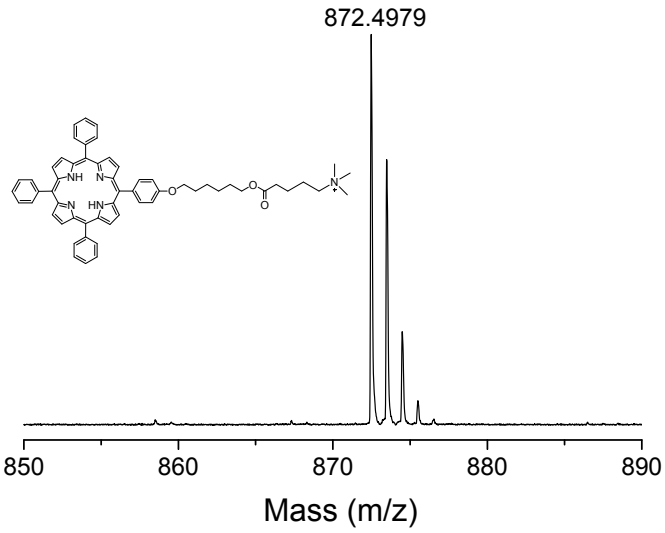


Fig. S4 MALDI-TOF-MS spectrum for **TPP-QAS**, calcd for $C_{58}H_{58}N_5O_3$: 872.4534; found: 872.4979.

3. Investigation of the interactions between WP5 and TPP-QASs.

To investigate association constant for the complexation between **WP5** and **TPP-QASs**, fluorescence titration experiments were performed to estimate the association constant (K_a) of **WP5⊃TPP-QASs** in aqueous solution, which had a constant concentration of **WP5** (2.50×10^{-5} M) and varying concentrations of **TPP-QASs**. By a non-linear curve-fitting method, the K_a of **WP5⊃TPP-QASs** was estimated to be $(1.42 \pm 0.36) \times 10^5$ M^{-1}

The non-linear curve-fittings were based on the following equation:

$$\Delta F = (\Delta F_\infty/[H]_0)(0.5[G]_0 + 0.5([H]_0+1/K_a) - (0.5([G]_0^2 + (2[G]_0(1/K_a - [H]_0)) + (1/K_a + [H]_0)^2)^{0.5}))$$

Where, ΔF is the fluorescence intensity changes at 330 nm at $[H]_0$; ΔF_∞ is the fluorescence intensity changes at 323 nm when **WP5** is completely complexed; $[H]_0$ is the fixed initial concentration of **WP5** and $[G]_0$ is the initial concentration of **TPP-**

QASs.[4-6]

4. Critical aggregation concentration (CAC) of the TPP-QASs/WP5/DTAB.

The CAC of **TPP-QASs/WP5/DTAB** was estimated by a fluorescence spectra using pyrene as a hydrophobic fluorescent probe. A predetermined amount of pyrene in acetone was added into a series of volumetric flasks and then allowed to evaporate at room temperature. **TPP-QASs/WP5/DTAB** solution was diluted to a series of concentrations ranging from 0.25 to 20 μg/mL by ultrapure water and then sonicated 30 min at room temperature. The ratio of pyrene fluorescence intensities excited at 383 and 372 nm (I_{382}/I_{372}) was plotted as a function of the logarithm of the concentration of **TPP-QASs/WP5/DTAB** spherical nanoparticles. The CAC was estimated as the cross-point when extrapolating the intensity ratio I_{382}/I_{372} at low and high concentration regions. The CAC of **TPP-QASs/WP5/DTAB** in aqueous solution is about 4.7×10^{-3} mg/mL.

5. Self-assembly of the TPP-QASs/WP5/DTAB in aqueous solution.

TPP-QASs (2 mg, 0.0023 mmol) and **DTAB** (0.3 mg, 0.0010 mmol) were separately dissolved in 0.15 mL of DMF and then stirred and maintained at 25 °C with a water bath for 2 h in the dark. Subsequently, the DMF solution was injected into a **WP5** aqueous solution (10 mL, C = 0.072 mM). After stirring for another 2 h, DMF was removed by dialysis (MWCO 12 kDa) against deionized water for 2 days (renew fresh water for 6 times). Finally, the concentration of the assembled solution was 0.135 mg/mL.

6. Determination of drug release properties based on the TPP-QASs/WP5/DTAB spherical nanoparticles.

0.05 M PBS buffer solutions (pH = 7.4), 0.05 M PBS buffer solutions (pH = 6.8), and 0.05 M citrate buffer solutions (pH = 5.0) were used as drug release media to simulate normal physiological conditions and the intracellular conditions of tumor. The pH typical release experiment was further performed as following: 2 mL of appropriate

release medium was added into 8 mL of **TPP-QASs/WP5/DTAB** supramolecular aggregates solution. At predetermined time intervals, 2 mL of the release media was taken out for measuring the released porphyrin fluorescence intensity by the fluorescence technique, and then it was returned to the original release media after measuring. The fluorescence intensity of porphyrin was determined by measuring emission intensity at 655 nm. A nearly 100% release of porphyrin from calcein-loaded vesicles could be obtained by adding very low pH solutions (the solution of HCl, pH = 1) to the supramolecular aggregates solution.

7. Cell Culture.

A549 cells (human lung adenocarcinoma cell line) were routinely incubated in Dulbecco's modified Eagle's medium (DMEM) supplied with 50 units/mL (penicillin and streptomycin) and 10% fetal bovine serum (FBS) in a humidified standard atmosphere of 5% CO_2 at 37 °C.

8. Cellular Uptake and Colocalization Imaging.

The cellular uptake and colocalization experiments were carried out using confocal laser scanning microscope (CLSM). In brief, A549 cells were cultured in 2 mL culture medium on polylysine-coated glass slides inside 30 mm glass culture dishes and allowed to grow to 50−70% at 37 °C. Afterward, cells (on glass slides) were washed with PBS, and reincubated in DMEM medium containing the spherical nanoparticles sample (**TPP-QASs/WP5/DTAB** spherical nanoparticles) at 37 °C under 5% CO_2 for 24 h. After predetermined time, the culture medium was removed and washed with PBS three time. Subsequently, the rhodamine 123 (2.0 ug/mL) was added to stain the mitochondria for 30 min. The cells were imaged directly via CLSM after repeated washing with PBS. Imaging of **TPP-QASs/WP5/DTAB** spherical nanoparticles and Rhodamine 123 were achieved through excitation at 405 nm and 488 nm respectively.

9. Mitochondrial Membrane Potential.

CLSM and flow cytometric analysis were performed to study the mitochondrial

membrane potential in A549 cells using a fluorescent probe JC-1.

For flow cytometry, after 10 μg mL^{-1} **TPP-QASs/WP5/DTAB** spherical nanoparticles was internalized by A549 in 6-well plates for 24 h, the cells were washed three times with warm PBS. Then light irradiation was performed for preset time with light intensity at 400 mW/cm^2. 2 h later, the cells were collected and resuspended in 1 mL JC-1 solution (2.5 μg mL^{-1}) for 30 min at 37 °C in the dark. The samples were washed twice with PBS and detected by Beckman Flow Cytometer.

For CLSM, The cells were seeded in polylysine-coated glass slides inside 30 mm glass culture dishes. After incubated for 24 h at 37 °C, the medium was extracted and the cells were washed twice with PBS. Then, 10 μg mL^{-1} **TPP-QASs/WP5/DTAB** spherical nanoparticles in DMEM was added. After 24 h, the light irradiation was performed (band pass: 420 nm and 400 mW cm^{-2}) for preset time. 2 h later, the cells were incubated for 30 min with JC-1 (2.0 μg mL^{-1}) for 30 min at 37 °C. Cells were then washed twice with warm PBS and examined immediately on a Confocal Laser Scanning Microscope. The JC-1 was excited at 488 nm and light emissions were collected at 510-540 nm (green) and at 570-600 nm (red).

Here, the mitochondrial membrane potential was monitored using JC-1 dye as the sensor. The decrease of mitochondrial membrane potential is a crucial indicator to evaluate the dysfunction of mitochondria since the damage of mitochondria causes the depolarization of mitochondria with a decrease in the membrane potential.[7] According to the previous reports,[8,9] the high mitochondrial membrane potential of the cells facilitates the formation of JC-1 aggregates, whereas JC-1 dyes could well disperse in cells with the low mitochondrial membrane potential. Meanwhile, the JC-1 dye could undergo a reversible change in fluorescence emission where aggregated JC-1 fluoresces red and monomeric JC-1 emits green. Therefore, the red/green fluorescence ratio can be used to discriminate the status of mitochondria.

10. Dark cytotoxicity of TPP-QASs/WP5/DTAB spherical nanoparticles.

The cytotoxicity of various samples in A549 cells was assessed via MTT assay. The cells were seeded in 96-well plates at the cell population of about 5000 cells/well in

200 μL DMEM. After 24 h of incubation in 96-well plates at 37 °C, samples with various concentrations were added to each well. And the samples were then subject to incubation for 24 h. Thereafter, the wells were incubated for another 4 h with RPMI 1640 medium containing 5 mg mL^{-1} MTT. After discarding the culture medium, 150 μL of DMSO was added to each well to extract the formazan products with gentle agitation for 10 min. The absorbance at 492 nm was measured using a spectrophotometric microplate reader (Thermo Multiskan MK3 spectrometer). The cell viability was calculated as follows: cell viability (%) = $(OD_{test})/(OD_{control}) \times 100$, where OD_{test} is the absorbance in the presence of sample solutions and $OD_{control}$ is the absorbance without treatment.

11. Phototoxicity of TPP-QASs/WP5/DTAB spherical nanoparticles.

The in vitro phototoxicity of **TPP-QASs/WP5/DTAB** spherical nanoparticles was performed using the above similar procedure for dark cytotoxicity. After 24 h of incubation, the cells were carried out under subdued light. The plate with cells was exposed to light from a visible light emitting diode (LED) lamp (400 mW/cm^2, 420 nm) for 10 min. The cells were then incubated at 37 °C for 24 h, and the cell viability was also assayed using the MTT assay as described above.

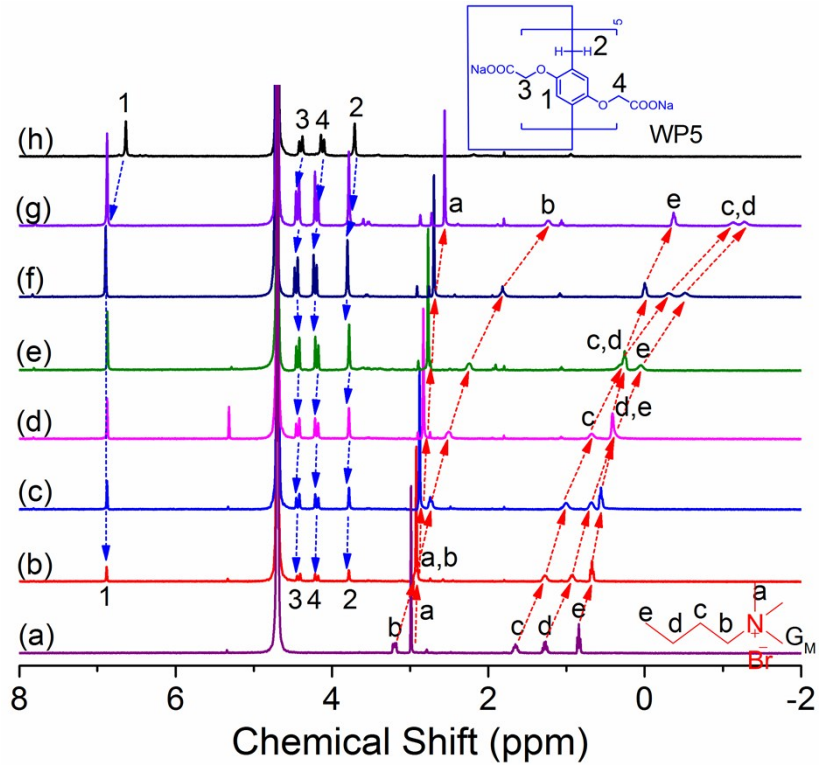


Fig. S5 ^{1}H NMR spectra (400 MHz, D_2O, 298 K) of G_M at a constant concentration of 2.00 mM with different concentrations of **WP5** (mM): (a) 0.00, (b) 0.50, (c) 1.00, (d) 1.50, (e) 2.00, (f) 3.00, (g) 4.00, and (h) individual **WP5** (2.00 mM).

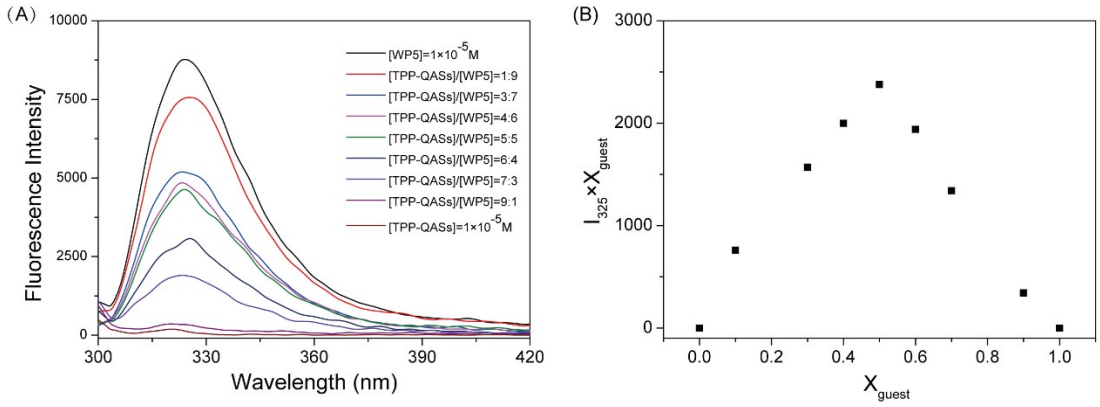


Fig. S6 (A) Fluorescence spectra of the mixture of **WP5** and **TPP-QASs** in water (containing 1% DMSO to improve the solubility of **TPP-QASs**) at different molar ratios (from [**TPP-QASs**]/[**WP5**] =1:9 to [**TPP-QASs**]/[**WP5**] = 9:1) with a constant concentration of [**WP5**] + [**TPP-QASs**] = 1×10^{-5} M. (B) Job plot showing the 1:1 stoichiometry of the complex between **WP5** and **TPP-QASs** by plotting the difference in fluorescence intensity at 325 nm (a characteristic fluorescence spectra of

WP5) against the mole fraction of **TPP-QASs** at an invariant total concentration of 1×10^{-5} M in aqueous solution.

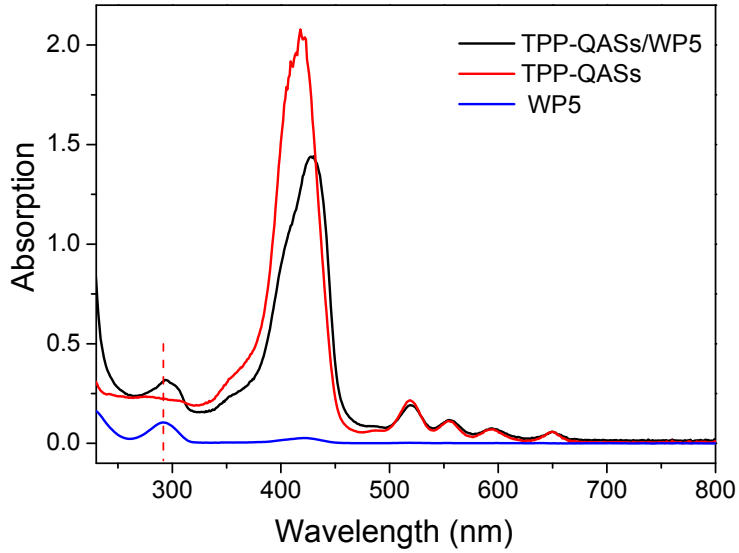


Fig. S7 UV-vis absorption spectra of **TPP-QASs** (0.02 mM), **TPP-QASs**:**WP5** = 5:1 with **TPP-QASs** (0.02 mM), and **WP5** (0.02 mM) at room temperature.

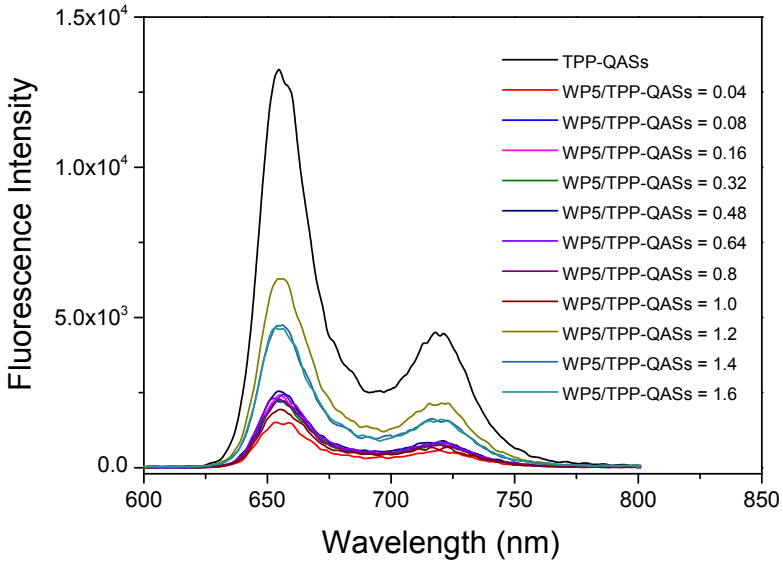


Fig. S8 Fluorescence spectra of **TPP-QASs** (1×10^{-5} M) in aqueous solution at room temperature with different concentrations of **WP5**: 0, 0.04, 0.08, 0.16, 0.32, 0.48, 0.64,

0.8, 1.0, 1.2, 1.4, and 1.6× 10^{-5} M in aqueous solution at room temperature.

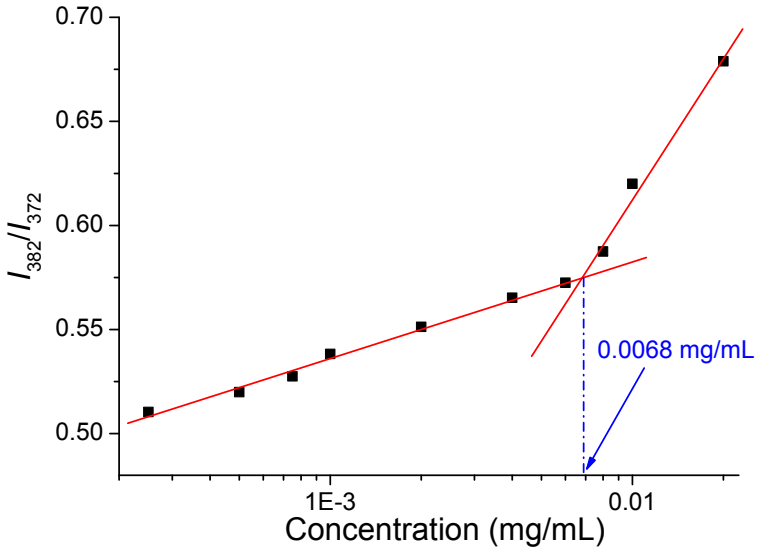


Fig. S9 Plots of fluorescence intensity ratios (I_{382}/I_{372}) from pyrene emission spectra as a function of the concentration of **TPP-QASs/WP5** in aqueous solution at 25°C. Pyrene concentration was fixed at 6.0 × 10^{-7} mol/L.

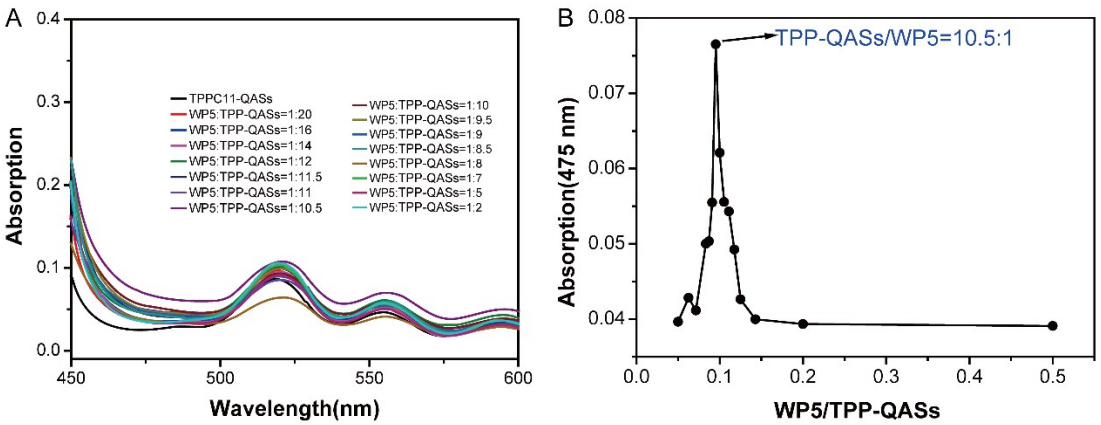


Fig. S10 (A) UV-vis absorption of a mixture of **WP5** and **TPP-QASs** in water with constant **TPP-QASs** concentration (0.002 mm) on increasing the concentration of **WP5** (0.05-0.33 equiv) at 25°C. (B) Dependence of the relative absorption intensity at 475 nm on the **WP5** concentration with a fixed concentration of **TPP-QASs** (0.002 mm) at 25°C.

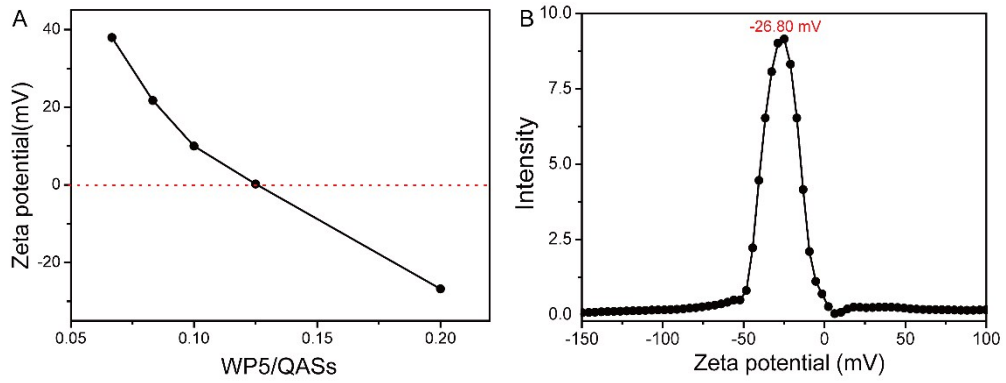


Fig. S11 (A) The zeta potentials of the aggregates formed by **TPP-QASs/WP5** at different molar ratio of [**WP5**]/[**QASs**] by increasing the concentration of **WP5**. (B) The zeta potentials of **TPP-QASs/WP5/DTAB** spherical nanoparticles.

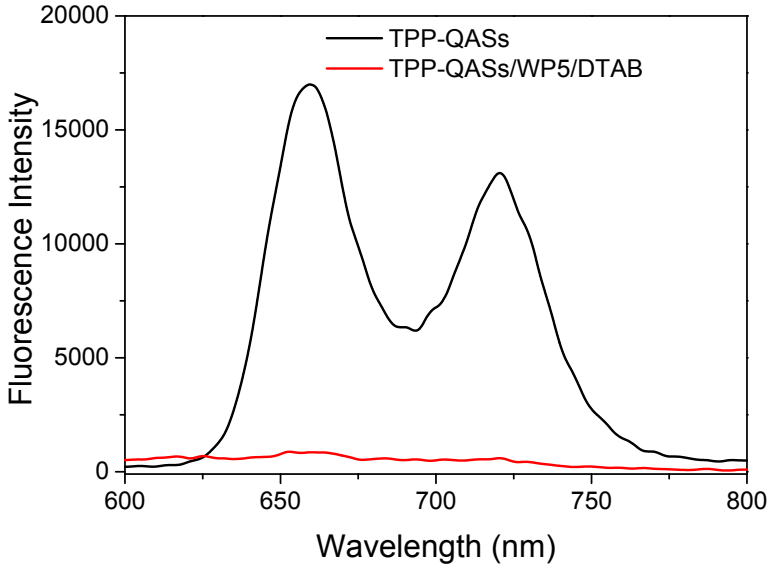


Fig. S12 Fluorescence spectra of **TPP-QACs** (2.1×10^{-4} M) and **TPP-QASs/WP5/DTAB** supramolecular spherical nanoparticles (the concentration of **TPP-QACs** is 2.1×10^{-4} M) in aqueous solution at room temperature.

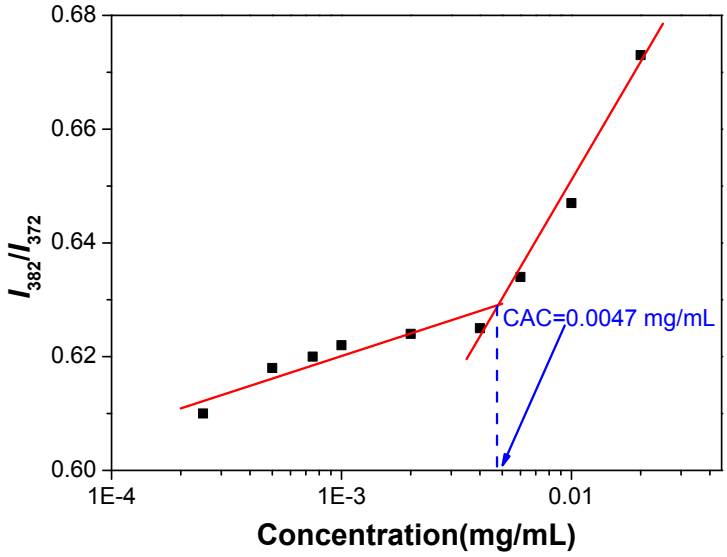


Fig. S13 Plots of fluorescence intensity ratios (I_{382}/I_{372}) from pyrene emission spectra as a function of the concentration of **TPP-QASs/WP5/DTAB** in aqueous solution at 25°C. Pyrene concentration was fixed at 6.0×10^{-7} mol/L.

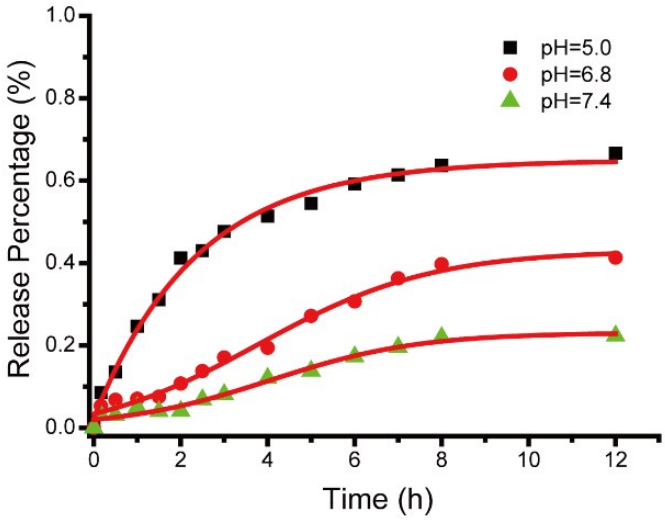


Fig. S14 The pH-responsive **TPP-QASs** release profiles of **TPP-QASs/WP5/DTAB** spherical nanoparticles in the release media at different pH values.

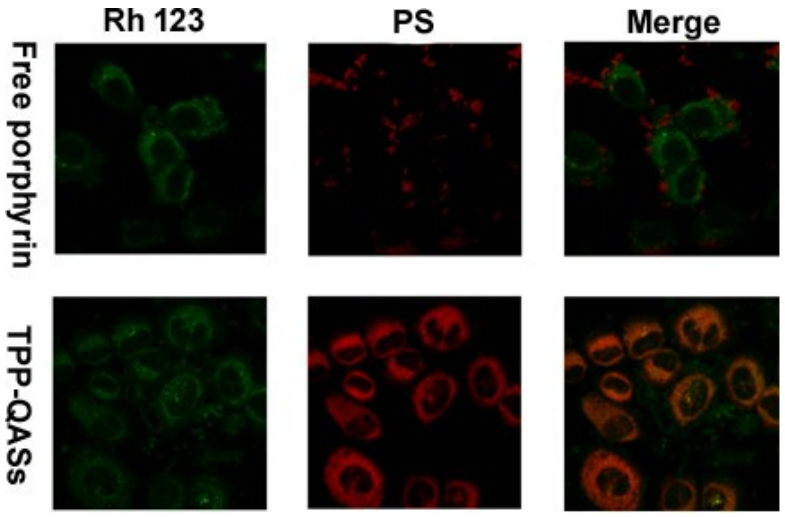


Fig. S15 CLSM of A549 cells incubated with porphyrins (**TPP-QASs** or free porphyrin) and rhodamine 123 for 4 hours.[9]

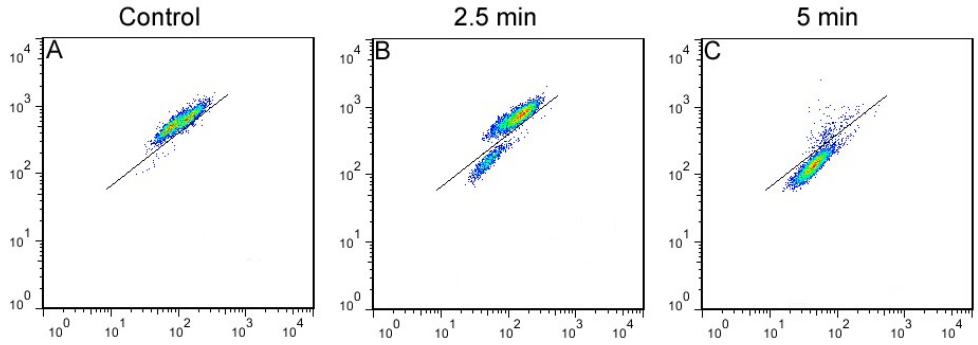


Fig. S16 Representative flow cytometric analyses of mitochondrial membrane potential (using JC-1 as indicator) for A549 cells incubated with 10 μg/mL of **TPP-QASs/WP5/DTAB** (B,C) for 24 h and then exposed to 420 nm LED irradiation for 2.5 min (B) and 5 min (C), respectively.

References

(1) Li, H.; Chen, D.-X.; Sun, Y.-L.; Zheng, Y. B.; Tan, L.-L.; Weiss, P. S.; Yang, Y.-W. *Journal of the American Chemical Society* **2013**, *135*, 1570.

(2) Xu, L.; Liu, L.; Liu, F.; Cai, H.; Zhang, W. *Polymer Chemistry* **2015**, *6*, 2945.

(3) Meng, L.-B.; Zhang, W.; Li, D.; Li, Y.; Hu, X.-Y.; Wang, L.; Li, G. *Chemical Communications* **2015**, *51*, 14381.

(4) Ackermann, T. *Berichte der Bunsengesellschaft für physikalische Chemie* **1987**, *91*, 1398.

(5) Ashton, P. R.; Ballardini, R.; Balzani, V.; Bělohradský, M.; Gandolfi, M. T.; Philp, D.; Prodi, L.; Raymo, F. M.; Reddington, M. V.; Spencer, N.; Stoddart, J. F.; Venturi, M.; Williams, D. J. *Journal of the American Chemical Society* **1996**, *118*, 4931.

(6) Zhang, J.; Huang, F.; Li, N.; Wang, H.; Gibson, H. W.; Gantzel, P.; Rheingold, A. L. *The Journal of Organic Chemistry* **2007**, *72*, 8935.

(7) Han, K.; Lei, Q.; Wang, S.-B.; Hu, J.-J.; Qiu, W.-X.; Zhu, J.-Y.; Yin, W.-N.; Luo, X.; Zhang, X.-Z. *Advanced Functional Materials* **2015**, *25*, 2961.

(8) Xu, J.; Zeng, F.; Wu, H.; Hu, C.; Wu, S. *Biomacromolecules* **2014**, *15*, 4249.

(9) Xu, J.; Zeng, F.; Wu, H.; Yu, C.; Wu, S. *ACS Applied Materials & Interfaces* **2015**, *7*, 9287.