

Supporting Information (SI)

Probing Cell Membrane Damage using Molecular Rotor Probe with Membrane-to-Nucleus Translocation

Kang-Nan Wang ^{a,b#}, Guobin Qi ^{b#}, Huiying Chu ^{c#}, Xi-Juan Chao ^a, Liu-Yi Liu ^a, Guohui Li ^{c*}, Qian Cao ^a, Zong-
Wan Mao ^{a*} and Bin Liu ^{b,d*}

Experimental Section

Materials and instruments

All reagents and solvents (analytical grade) were used as received from commercial sources unless otherwise indicated. Solvents were purified by standard procedures. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Aldrich), DMSO (Sigma Aldrich), Hoechst 33342 (2'-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-1H,3'H-2,5'-bibenzimidazole, Sigma Aldrich) DOPC (1, 2-dioleoyl-sn-glycero-3-phosphocholine, Energy Chemical), cholesterol (Energy Chemical), saccharides (Glucose, Energy Chemical), protein (BSA, bovine serum albumin, Energy Chemical) PI (propidium iodide, Sigma Aldrich), Gly (glycerol, J&K Chemical) were used as received. Annexin V-FITC apoptosis detection kit was purchased from Sigma Aldrich. The tested compounds were dissolved in DMSO before the experiments, and the concentration of DMSO was 1% (v/v). ^1H NMR and ^{13}C NMR spectra were recorded on a Mercury Plus 400 or 500 spectrometer. Shifts are referenced relative to the internal solvent signals. ESI-MS were recorded on a Thermo Finnigan LCQ DECA XP spectrometer. Steady-state emission spectra and lifetime measurements were conducted on a combined fluorescence lifetime and steady-state spectrometer FLS 920 (Edinburgh). UV-vis spectra were recorded on a Varian Cary 300 spectrophotometer (USA). ESI-MS were recorded on a Thermo Finnigan LCQ DECA XP spectrometer. The quoted m/z values represent the major peaks in the isotopic distribution. Fluorescence microscopy of cells was performed in Carl Zeiss LSM 710. For MTT assays, the absorbance was quantified using the Infinite M200 microplate reader.

Molecular dynamics simulation

MD simulations were carried out to study the binding and permeation of the **TPAE1/2** across a hydrated 1, 2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) bilayer.

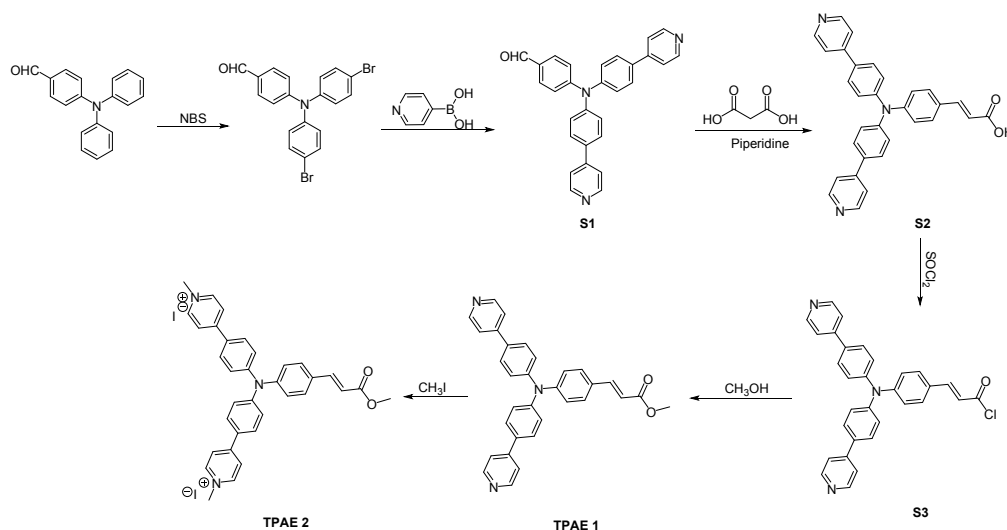
System Preparations. The DOPC bilayer system contains 72 DOPC molecules, in which the thickness of waters is set to 25 Å, and the **TPAE1/2** molecule is put in the center of the water layer. The electronic potential of **TPAE 1** and **TPAE2** was calculated using the Gaussian 09 program with the B3LYP function under 6-311G* basis set.^[1] The partial charges of the substrate molecules were derived using the RESP charge fitted with the antechamber module in AMBER 16.^[2] The other parameters, including vdW, bond, angle, and torsion terms, were obtained with the antechamber module.^[2]

Standard MD simulation. The atomistic MD simulations of the initial model were carried out in the AMBER16 program^[2] using the Lipid17 force field^[3]. Each system was bathed in a 0.15

mM aqueous KCl solution using ~45 TIP3P water molecules/lipids.^[4] Energy minimization was performed by imposing a strong restraint on each DOPC and **TPAE1/2** system and was followed by minimizing the whole system for a few thousand steps. NVT simulations were carried out by heating the whole system slowly from 100 to 303.5 K, and the Berendsen thermostat^[5] was applied to govern the temperature of the whole system. Subsequently, an NVT dynamics of 1 ns was performed, which was followed by a production run. During the production run, all bonds associated with hydrogen atoms were constrained by employing the SHAKE algorithm^[6] as the integration time step of 4 fs could be used. A cutoff value of 12 Å was set for non-bonded interactions. The Particle Mesh Ewald method^[7] was employed for treating electrostatic interactions. For each DOPC and **TPAE1/2** model, five independent MD simulations were implemented using different velocities that were randomly generated at the beginning of the simulations, totally 10 μ s trajectory. The analysis of each MD trajectory was performed using the cpptraj module in AMBER 16.^[8]

Steered MD The potential mean force (PMF) for the **TPAE1/2** molecule translocation across the bilayer was calculated via the steered MD. The steered MD utilizes an additional constraining harmonic potential applied to the distance between mass center of the DOPC bilayer and one chain of **TPAE1/2**. The distance is set to from $-30 \leq z \leq 30$ Å (when z is 0, with **TPAE** model connected to either membrane interface), where z is the one tail's center of mass (COM) relative to the bilayer COM (maintained with a harmonic constraint of 50 kcal/mol/Å²). **TPAE1/2** model were placed in unperturbed bilayers followed by 500 ns for each trajectory.

Synthesis and Characterization



Scheme S1. Synthetic routes to **TPAE1** and **TPAE2**.

4-(Bis(4-(pyridin-4-yl)phenyl)amino)benzaldehyde (S1): The synthesis of compound **S1** was followed literature.^[9,10]

Bis(4-bromophenyl)(4-formylphenyl)amine (light yellow solid, 2.15 g, 69 % yield): ¹H NMR (400 MHz, DMSO) δ 9.82 (s, 1H), 7.77 (d, *J* = 8.8 Hz, 2H), 7.57 (d, *J* = 8.8 Hz, 4H), 7.11 (d, *J* = 8.8 Hz, 4H), 7.00 (d, *J* = 8.7 Hz, 2H). ¹³C NMR (101 MHz, DMSO) δ 191.32 (s), 152.33 (s), 145.27 (s), 133.38 (s), 131.81 (s), 130.18 (s), 128.38 (s), 120.29 (s), 117.91 (s).

S1 (light yellow solid, 0.42 g, 74 % yield): ¹H NMR (500 MHz, CDCl₃) δ 9.88 (s, 1H), 8.67 (s, 4H), 7.77 (d, *J* = 8.7 Hz, 2H), 7.64 (d, *J* = 8.6 Hz, 4H), 7.52 (d, *J* = 5.5 Hz, 4H), 7.29 (d, *J* = 8.6 Hz, 4H), 7.19 (d, *J* = 8.6 Hz, 2H). ¹³C NMR (126 MHz, CDCl₃) δ 190.48 (s), 150.29 (s), 147.29 (s), 147.02 (s), 134.36 (s), 131.41 (s), 130.60 (s), 128.38 (s), 126.09 (s), 121.50 (s), 121.22 (s).

(E)-3-(4-(Bis(4-(pyridin-4-yl)phenyl)amino)phenyl)acrylic acid (S2): 0.76 g (1.78 mmol) **S1** and 0.554 g (3eq.) malonic acid were added to a round bottom flask containing 20 mL of CH₃CN, and 1.23 mL (7eq. 1.058 g) of piperidine was added subsequently. The reaction mixture was heated at 82 °C for 12 h. After the reaction was cooled to room temperature, 100 mL of water was added into the reaction solution. The precipitate of compound **S2** was filtered under reduced pressure and washed with ethanol to yield **S2** as light-yellow solid (0.75g, 92% yield). ESI-MS (*m/z*): calcd. for [M+H]⁺ (C₃₁H₂₄N₃O₂): 470.5440; found: 470.5271 ¹H NMR (400 MHz, DMSO) δ 12.26 (s, 1H), 8.62 (d, *J* = 4.9 Hz, 4H), 7.83 (d, *J* = 7.5 Hz, 4H), 7.66 (d, *J* = 4.7 Hz, 4H), 7.56 (d, *J* = 8.3 Hz, 2H), 7.44 (d, *J* = 15.8 Hz, 1H), 7.22 (d, *J* = 8.6 Hz, 4H), 7.09 (d, *J* = 8.5 Hz, 2H), 6.43 (d, *J* = 15.8 Hz, 1H). ¹³C NMR (101 MHz, DMSO) δ 151.68 (s), 148.81 (s), 147.61 (s), 138.07 (s), 133.33 (s), 131.51 (s), 130.47 (s), 129.61 (s), 125.88 (s), 125.52 (s), 122.12 (s), 119.23 (s).

Methyl (E)-3-(4-(bis(4-(pyridin-4-yl)phenyl)amino)phenyl)acrylate (TPAE1): 0.47 g (1 mmol) of **S2**, 10 mL of thionyl chloride and a few drops of pyridine were added to a round bottom flask with condenser and gas absorption device. The mixture was stirred at -5 °C for 3 h. The reduced hydrochloric acid mist was received by 5% NaOH solution. The precipitate was filtered and CH₃OH (20 mL) was added into a round bottom flask, continue to react for 12 hours. After reaction, NaHCO₃ (1 M) was added to remove the unreacted thionyl chloride and hydrochloric acid. The slight yellow precipitate was filtered and repeatedly washed with 100 mL water and 100 mL diethyl ether three times. The residue was purified by column chromatography over silica gel to yield **TPAE1** as light-yellow solid (0.42 g, 87 % yield). ESI-MS (*m/z*): calcd. for [M+H]⁺ (C₃₂H₂₆N₃O₂): 484.5598; found: 484.5607. ¹H NMR (400 MHz, DMSO) δ 8.62 (s, 4H), 7.83 (d, *J* = 7.6 Hz, 4H), 7.71 (s, 4H), 7.64 (d, *J* = 16.4 Hz, 2H), 7.23 (d, *J* = 7.6 Hz, 4H), 7.09 (d, *J* = 7.7 Hz, 2H), 6.54 (d, *J* = 15.9 Hz, 1H), 3.72 (s, 3H). ¹³C NMR (101 MHz, DMSO) δ 167.31 (s), 150.69 (s),

148.81 (s), 147.59 (s), 146.57 (s), 144.31 (s), 132.71 (s), 130.43 (s), 129.17 (s), 128.68 (s), 125.28 (s), 123.65 (s), 121.17 (s), 116.48 (s), 51.86 (s).

(E)-4-(4-((4-(3-Methoxy-3-oxoprop-1-en-1-yl)phenyl)(4-(1-methyl-1H-pyridin-4-yl)phenyl)amino)phenyl)-1-methylpyridin-1-ium (TPAE2): TPAE2 was obtained as light-yellow solid (540 mg, 81% yield) by treatment of TPAE1 (420 mg) with methyl iodide (617 mg) under 40 °C in ethanol for 72 h. ESI-MS (m/z): calcd. for $[M-2I]^{2+}$ ($C_{34}H_{31}N_3O_2^{2+}$): 513.6227; found: 513.2140 1H NMR (500 MHz, DMSO) δ 8.95 (s, 4H), 8.46 (s, 4H), 8.12 (s, 4H), 7.79 (s, 2H), 7.67 (d, J = 14.3 Hz, 1H), 7.25 (d, J = 41.0 Hz, 6H), 6.61 (d, J = 15.5 Hz, 1H), 4.32 (s, 6H), 3.73 (s, 3H). ^{13}C NMR (126 MHz, DMSO) δ 167.19 (s), 153.53 (s), 149.72 (s), 147.62 (s), 145.84 (s), 144.05 (s), 131.22 (s), 130.69 (s), 130.27 (s), 128.57 (s), 125.93 (s), 124.64 (s), 123.59 (s), 117.73 (s), 51.98 (s), 47.39 (s).

Cell lines and culture conditions: HepG2 cells, A549 cells, and HeLa cells were obtained from Experimental Animal Center of Sun Yat-Sen University (Guangzhou, China). Cells were routinely maintained in RPMI 1640 (Roswell Park Memorial Institute 1640, Gibco BRL) or DMEM (Dulbecco's modified Eagle's medium, Gibco BRL) medium containing 10% FBS (fetal bovine serum, Gibco BRL), 100 μ g/mL streptomycin, and 100 U/mL penicillin (Gibco BRL). The cells were cultured in tissue culture flasks in a humidified incubator at 37 °C in an atmosphere of 5% CO₂ and 95% air. In each experiment, the cells treated with DMSO (1%, v/v) were used as the referent group.

Fluorescence imaging microscopy: Cells were treated with TPAE1/2 (10 μ M) at 37 °C in serum-free media. And then, the cells were subjected to fluorescence microscopy of cells was performed in Carl Zeiss LSM 710, λ_{ex} :405 nm (15 mW cm⁻²), λ_{em} : 580 $\pm$ 20 nm.

Two-photon fluorescence lifetime imaging microscopy (FLIM): HeLa cells were treated with TPAE2 (10 μ M) at 37 °C for 30 min in serum-free media. And then, the cells were subjected to two-photon FLIM (Becker & Hickl, setup is integrated with a Zeiss LSM 710 (Carl Zeiss) laser scanning confocal microscope). The lifetime values were calculated with a professional software SPC Image 5.4 (Becker & Hickl GmbH, Berlin, Germany). λ_{ex} : 810 nm, λ_{em} : 580 $\pm$ 20 nm.

Cellular localization assay: HeLa and A549 cells were seeded into 35 mm dishes (Greiner, Germany) for confocal microscopy. After cultured for two days, the cells were incubated with TPAE1 and TPAE2 at 10.0 μ M for 30 min. The treated cells were observed immediately under a confocal microscope. For colocalization studies, the cells were incubated with TPAE1/2 (10.0 μ M) and BODIPY or DiO (10.0 μ M) and stained for 30 min, and then the cells were washed with PBS for three times. The cells were viewed immediately under a confocal microscope.

Annexin V-FITC staining assay: The assay was performed according to the manufacturer's (Sigma Aldrich, USA) protocol. For confocal microscopy analysis, A549 cells were seeded in 35 mm culture dishes (Corning) for 48 h. After incubated with cisplatin (25 μ M) at 37 °C for 12 h, cells were washed with ice-cold PBS for three times and further incubated with **TPAE2** (10.0 μ M) with 500 μ L annexin-binding buffer, which supplemented with Annexin V-FITC (10.0 μ g/mL) and PI (20.0 μ g/mL) for 15 min in the dark before visualized immediately by confocal microscopy.

Hoechst 33342 staining: HeLa cells were seeded in 35 mm culture dishes (Corning) at 37°C for 48 h. Then the cells were further incubated with Hoechst 33342 and **TPAE2** for 30 min before visualized by confocal microscopy. For **TPAE2**, λ_{ex} : 405 nm; λ_{em} : 600 $\pm$ 20 nm; For Hoechst 33342, λ_{ex} : 405 nm; λ_{em} : 450 $\pm$ 20 nm.

PI staining: HeLa cells were seeded in 35 mm culture dishes (Corning) for 48 h. at 37°C. Then the cells were further incubated with PI and **TPAE2** for the different time before visualized by confocal microscopy. For **TPAE2**, λ_{ex} : 405 nm; λ_{em} : 600 $\pm$ 20 nm; For PI, λ_{ex} : 488 nm; λ_{em} : 610 $\pm$ 20 nm.

ROS Detection in Solution. 9,10-anthracenediyl-bis(methylene) dimalonic acid (ABDA) was used as the indicator to detect ROS generation in the solution. The ABDA solution in H₂O was mixed with the probe and exposed to light irradiation at a power of 40 mW/cm² for different times. The decomposition of ABDA was monitored by the absorbance decrease at 378 nm.

Real-time monitoring of the membrane-to-nucleus process during PDT: the cells were incubated with **TPAE2** (10.0 μ M) or CellMask™ (10.0 μ M) for different times, and then irradiated with a 405 nm light (15 mW cm⁻²), light irradiation for 5 s (0.075 J cm⁻²) at every 2 min.

Cytotoxicity Studies: MTT assays were used to assess the cell viability of HeLa and HepG-2 cells after incubation with the **TPAE2** upon white light irradiation (40 mW/cm²) for 10 min (24 J cm⁻²). Cells cultured in 96-well plates were grown to the confluence with the probe for a designated time in the dark. And then, the cells were washed with PBS and exposed to light irradiation. The cells were further incubated in fresh medium for 24 h and washed with PBS. Then MTT in PBS solution (100 μ L, 0.5 mg/mL) was added into each well. After incubation for 4 h, the supernatant was discarded, and the precipitate was dissolved in DMSO (100 μ L) with gentle shaking. The absorbance at 595 nm was measured using a microplate reader (Infinite M200 Pro, Tecan, Switzerland). The cells without any treatment were used as control.

Statistical analysis: All biological experiments were performed at least twice with triplicates in each experiment. Representative results were depicted in this report, and data were presented as means $\pm$ standard deviations (SD) with statistical significance.

Supporting Figures and Tables

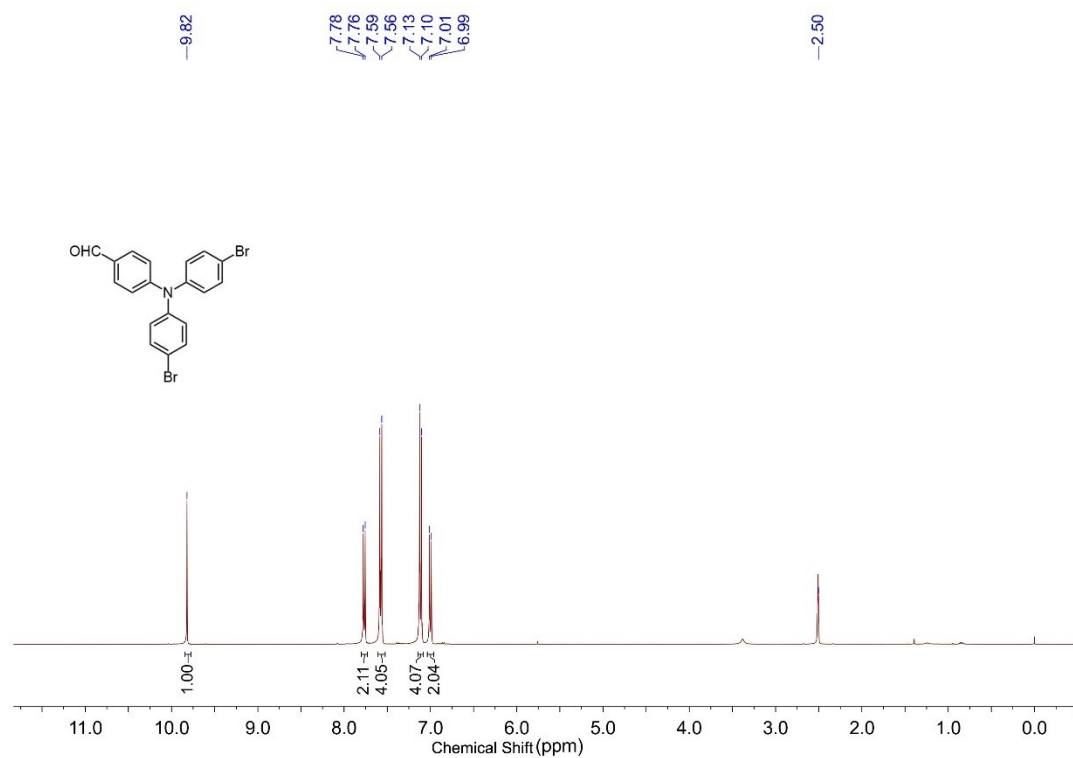


Fig. S1 ¹H NMR spectrum of bis(4-bromophenyl)(4-formylphenyl)amine.

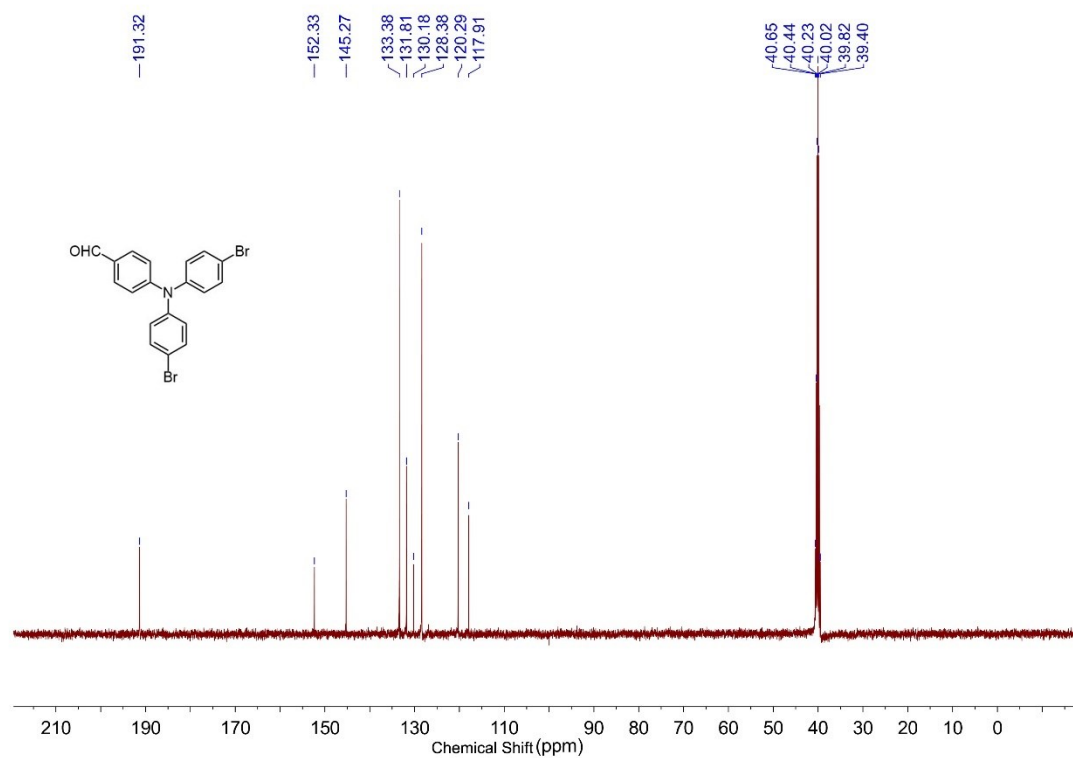


Fig. S2 ¹³C NMR spectrum of bis(4-bromophenyl)(4-formylphenyl)amine.

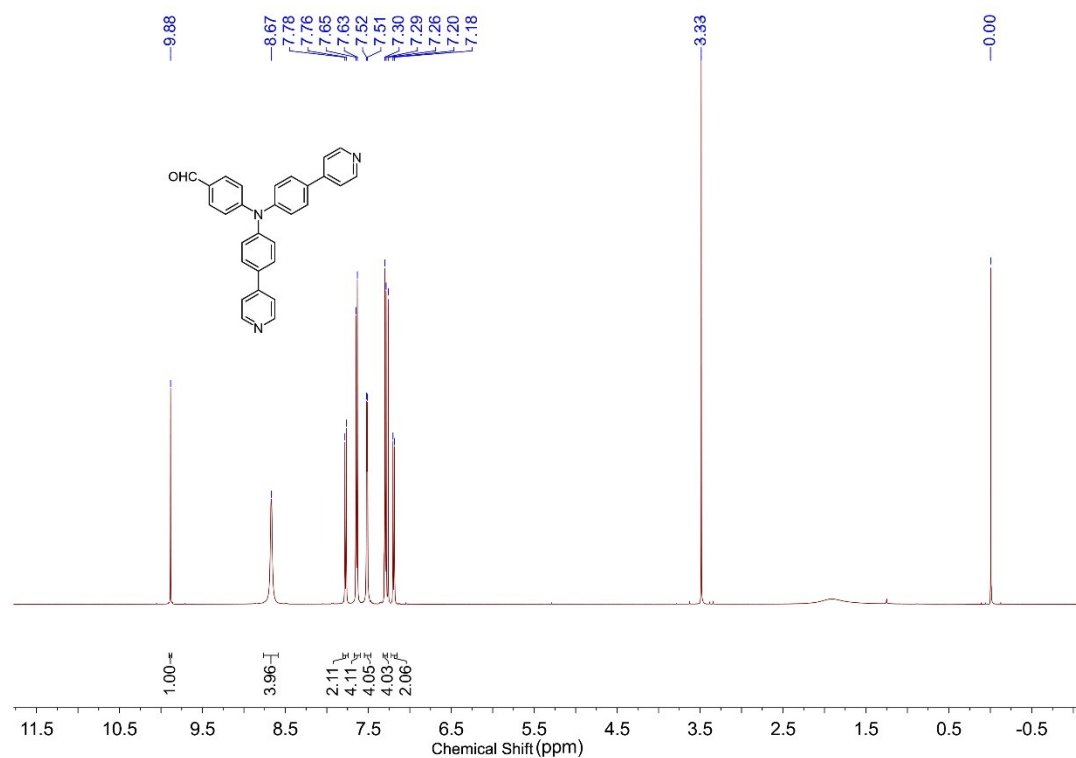


Fig. S3 ¹H NMR spectrum of **S1**.

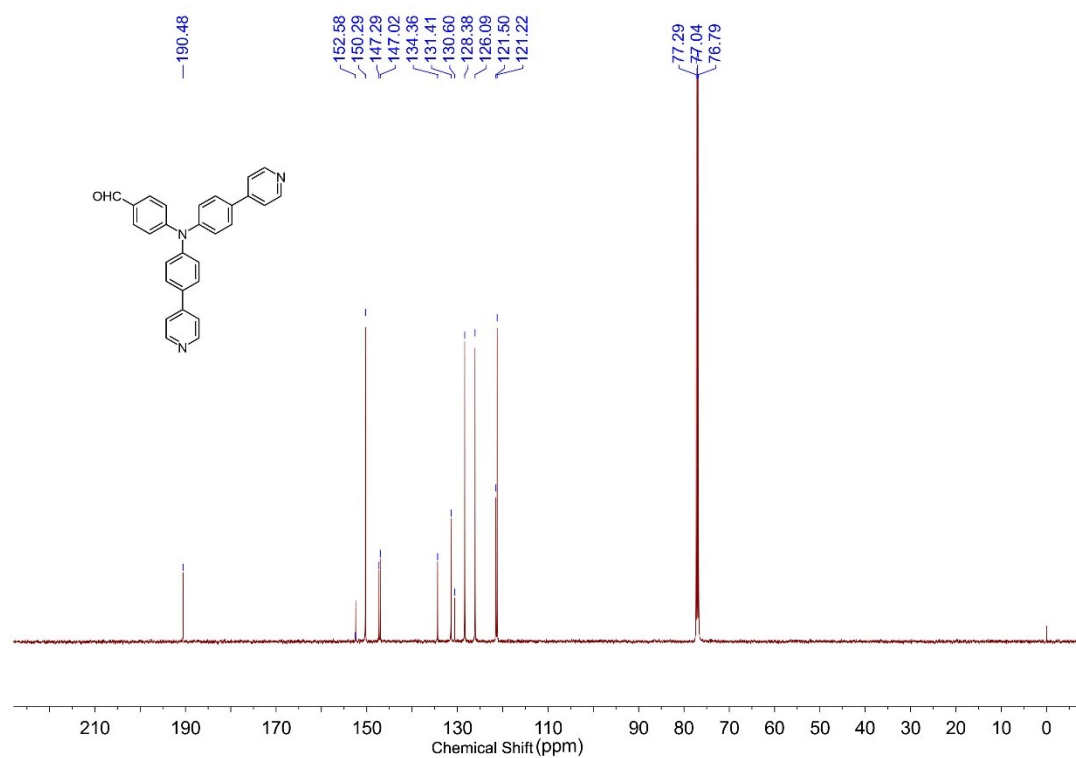


Fig. S4 ¹³C NMR spectrum of **S1**.

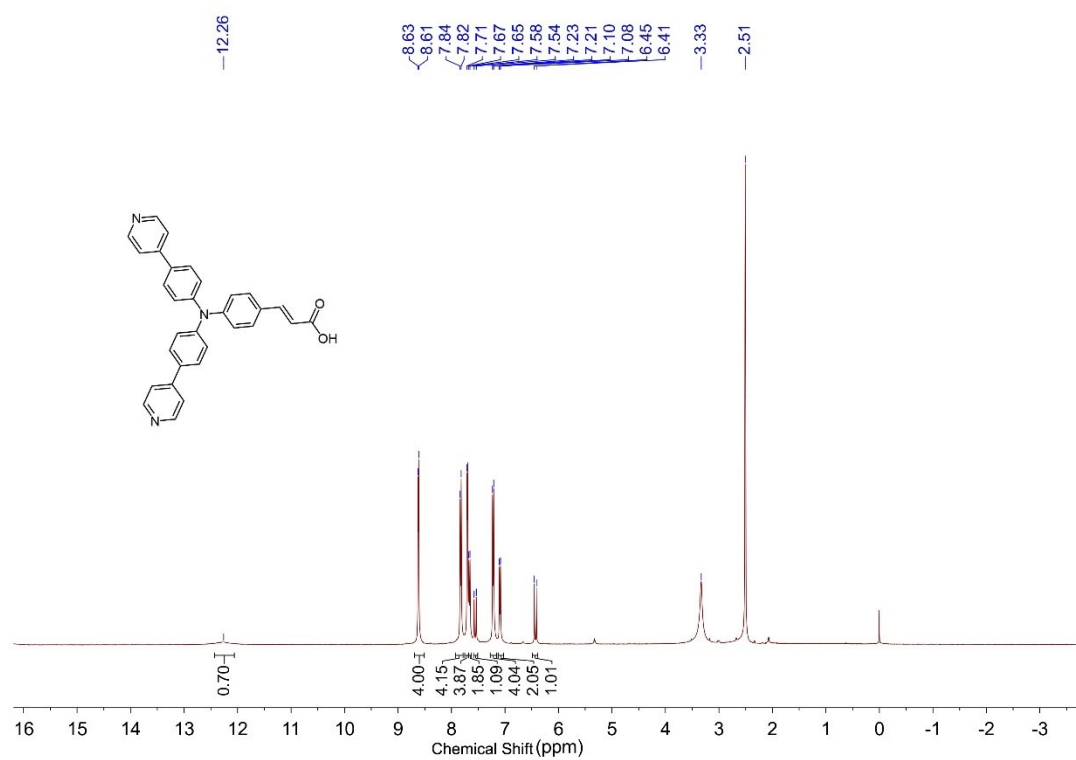


Fig. S5 ¹H NMR spectrum of **S2**.

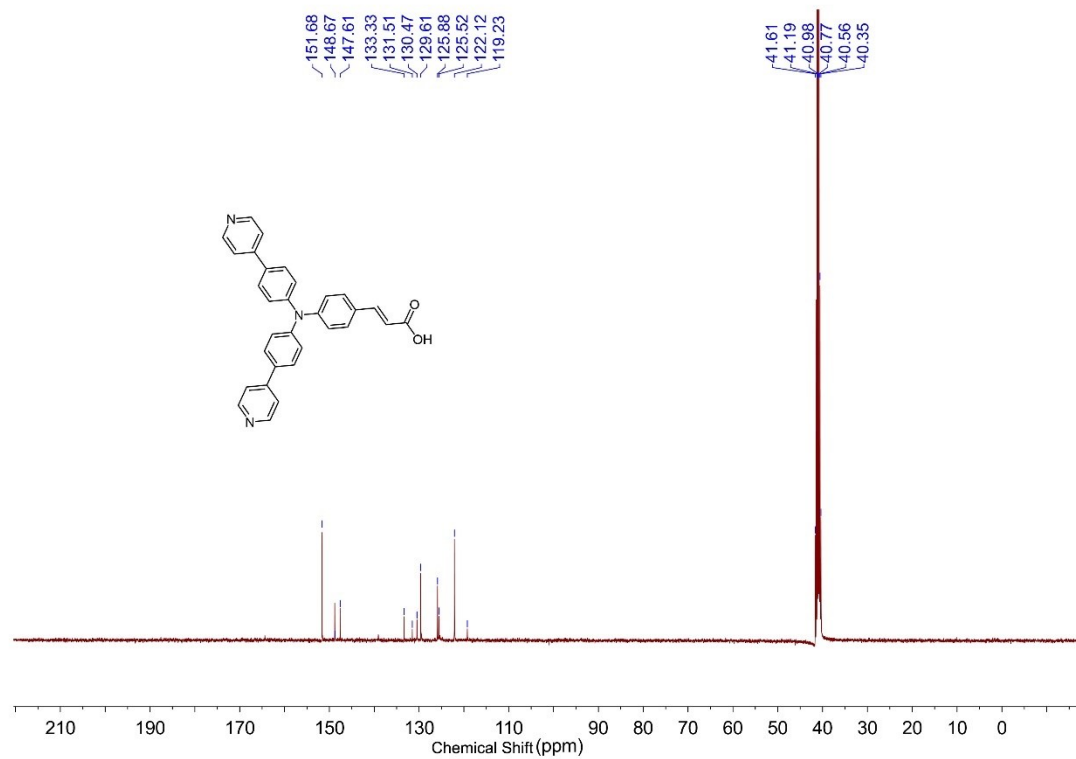


Fig. S6 ¹³C NMR spectrum of **S2**.

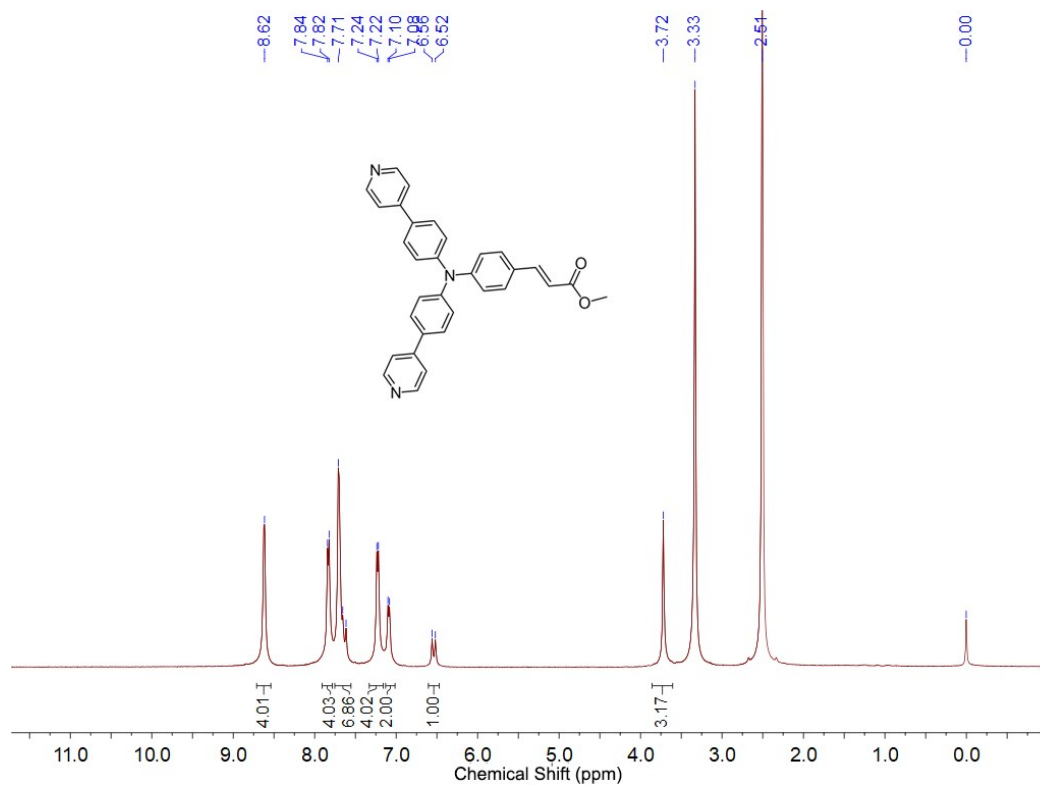


Fig. S7 ¹H NMR spectrum of TPAE1.

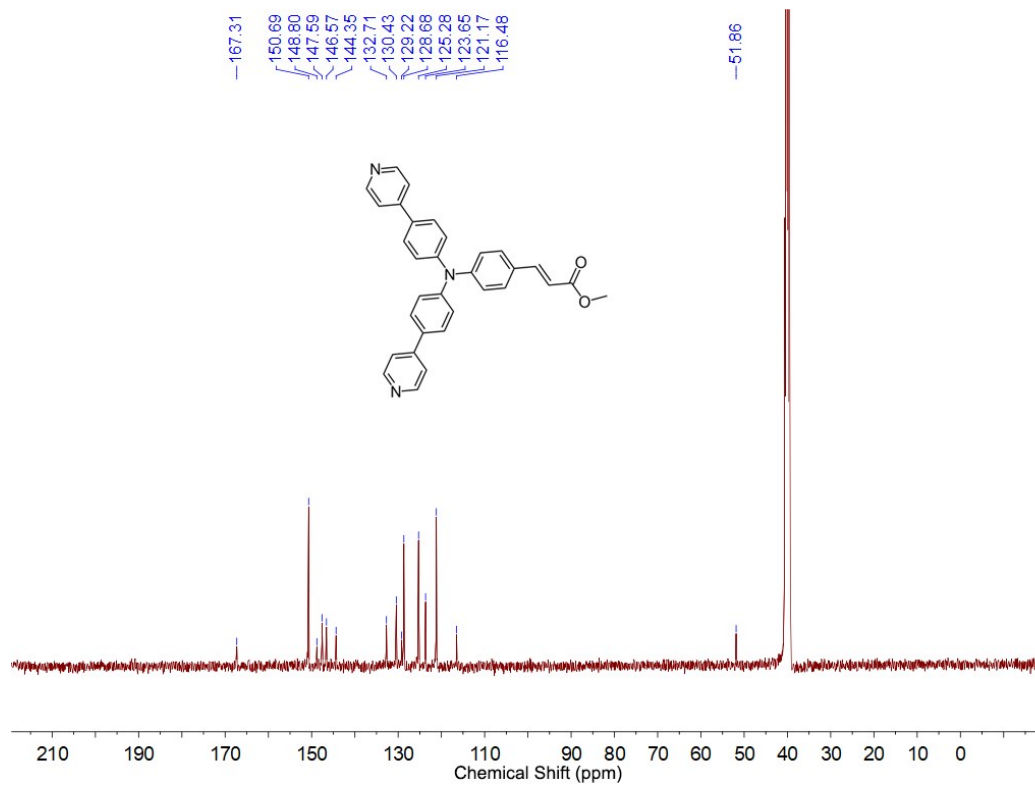


Fig. S8 ¹³C NMR spectrum of TPAE1.

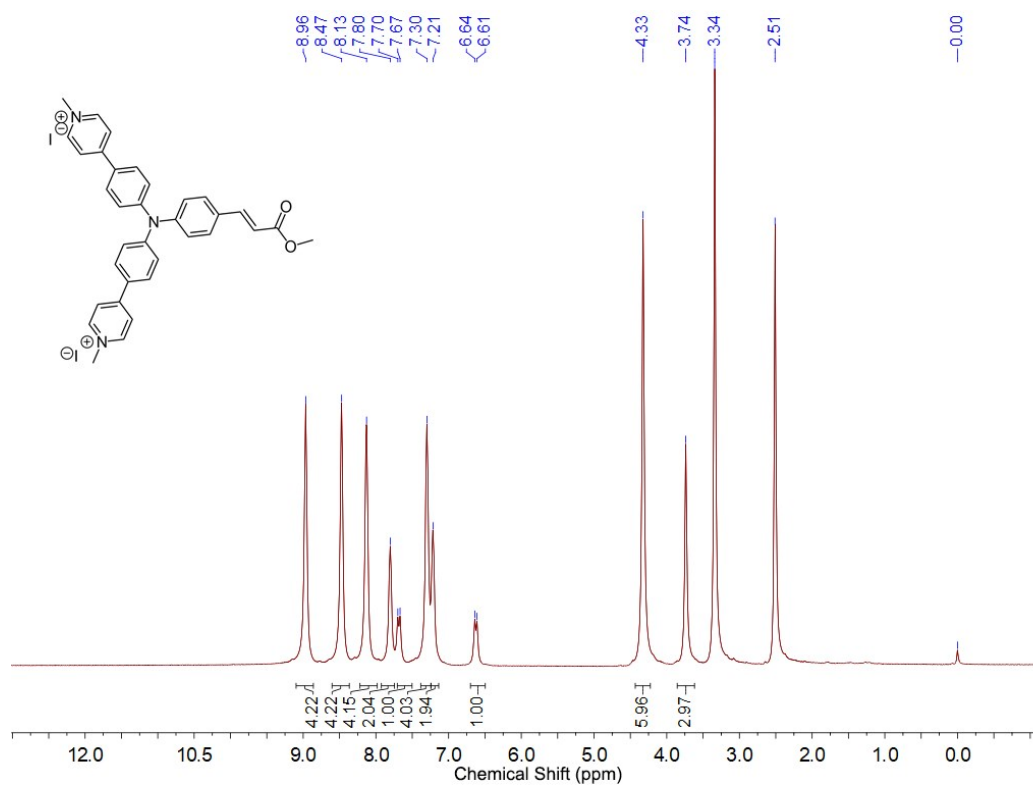


Fig. S9 ¹H NMR spectrum of TPAE2.

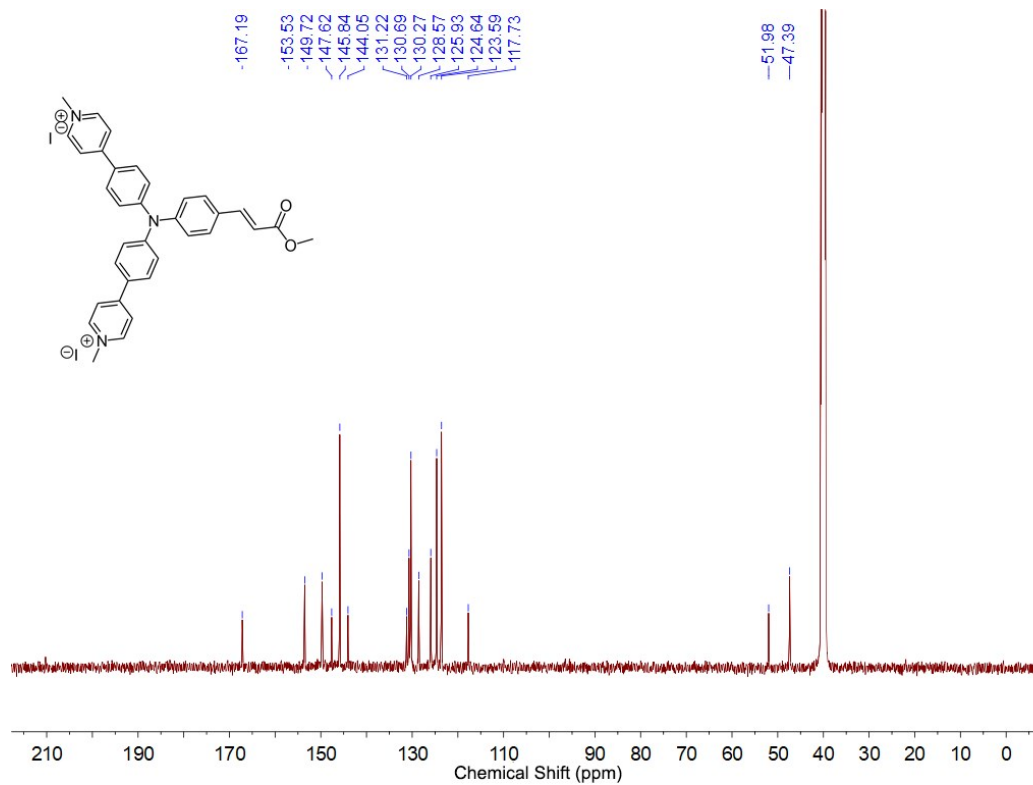


Fig. S10 ¹³C NMR spectrum of TPAE2.

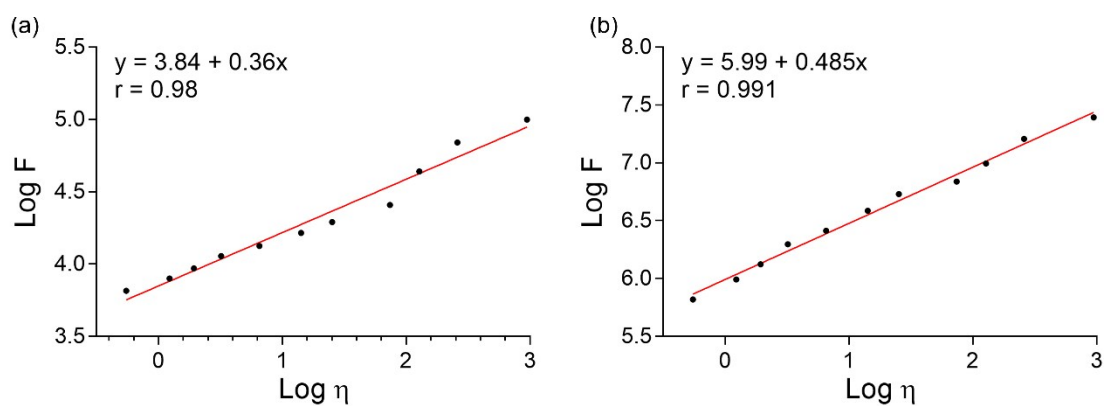


Fig. S11 Linear fit between fluorescence intensity and viscosity of 20.0 μM each of **TPAE1** (a) and **TPAE2** (b) in mixed solvents containing CH_3OH and Gly.

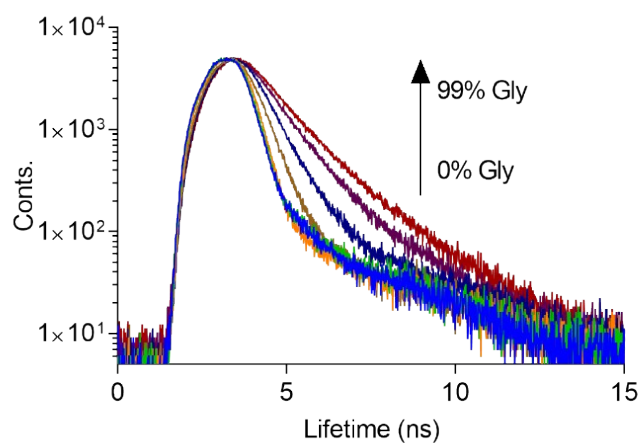


Fig. S12 Lifetime spectra of **TPAE2** (10.0 μM) in mixed solvents containing CH_3OH and glycerol.

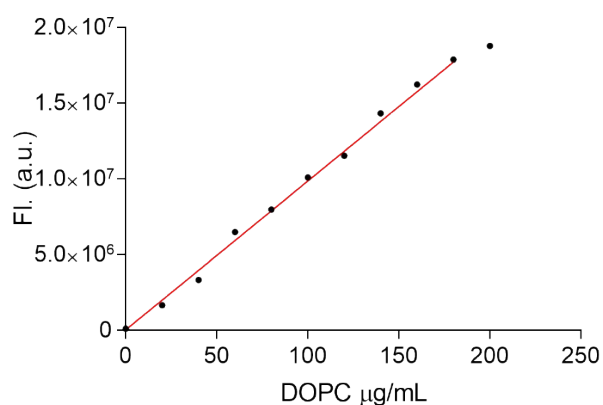


Fig. S13. The linear relationship between the emission of **TPAE2** and the concentration of DOPC (0-200.0 $\mu\text{g/mL}$) in PBS solution (pH = 7.4).

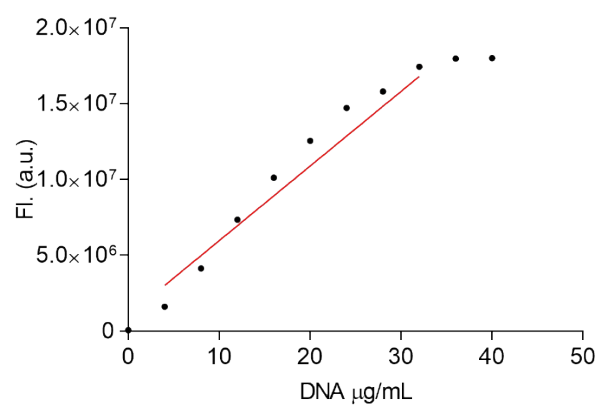


Fig. S14. The linear relationship between the emission of **TPAE2** and the concentration of DNA (0-40.0 $\mu\text{g/mL}$) in PBS solution (pH = 7.4).

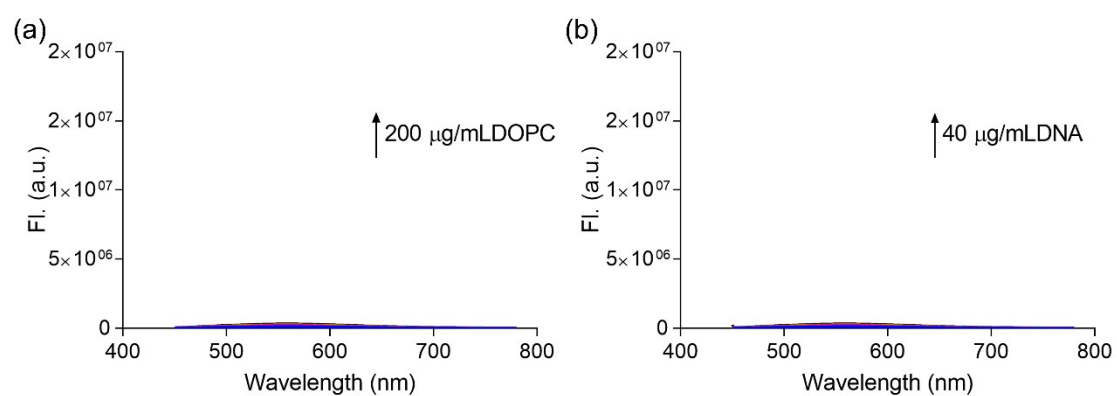
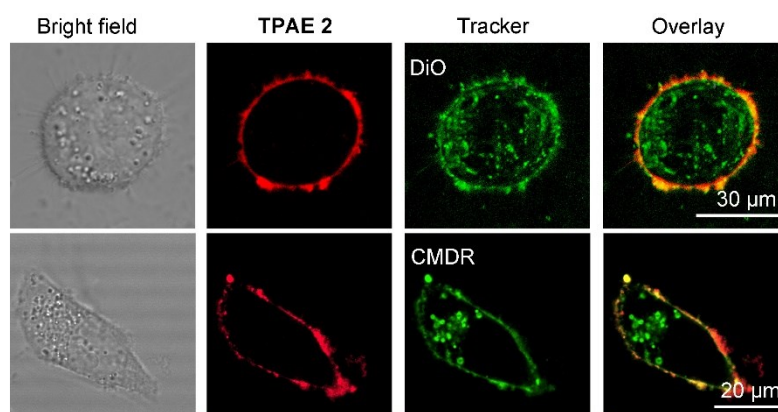


Fig. S15. The emission spectra of **TPAE1** (10.0 μM) upon DOPC (a) and DNA (b) titration in PBS solution



(pH = 7.4), respectively.

Fig. S16. Cells stained by both **TPAE2** (10.0 μM) and commercial dye DiO and CMDR (CellMaskTM Deep Red, pseudo-green color) (10.0 μM) for 2 h. Scale: 20 μm . For **TPAE2**, λ_{ex} : 405 nm; λ_{em} : 600 ± 20 nm; For DiO, λ_{ex} : 488 nm; λ_{em} : 520 ± 20 nm, for CMDR, λ_{ex} : 633 nm; λ_{em} : 650 ± 10 nm.

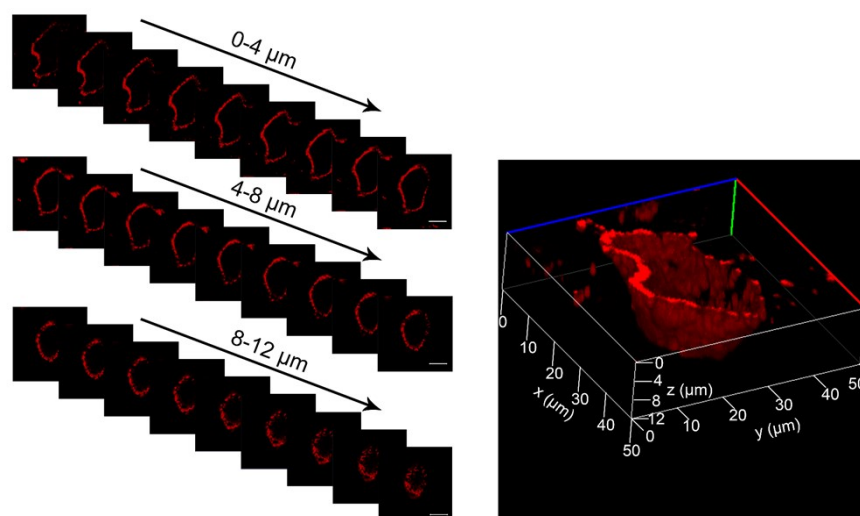


Fig. S17. 3D confocal images of sphere membrane skeleton built by layer-by-layer optical scanning of **TPAE2** (10.0 μM) treated HeLa cells. Scale: 20 μm . For **TPAE2**, λ_{ex} : 405 nm; λ_{em} : 600 ± 20 nm.

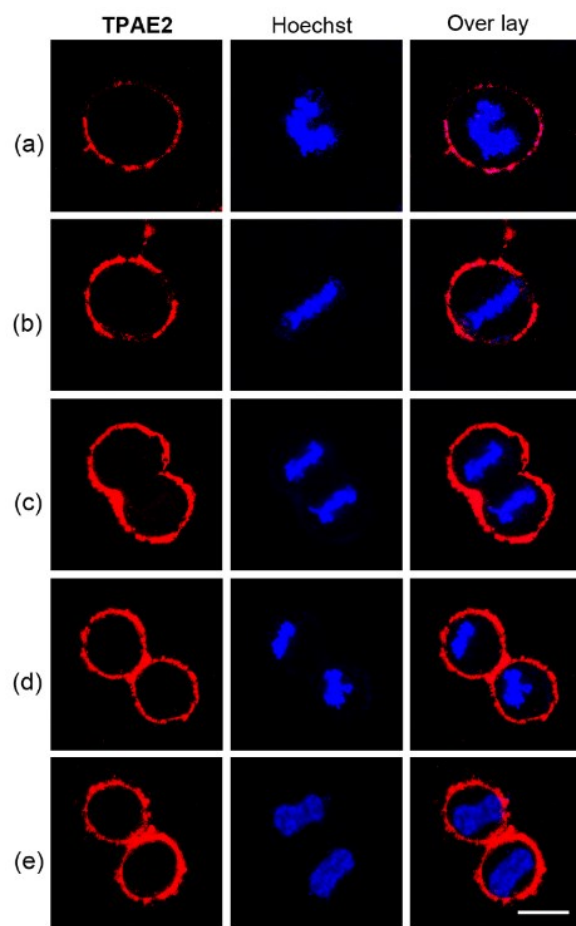


Fig. S18 Dynamic monitoring of cell membrane and chromosomes by **TPAE2** (10.0 μ M) and Hoechst (Hoechst 33342, 5.0 μ g/mL) during cell cycle process. On the Cell division, a clear prophase, metaphase, anaphase, and telophase cycle were observed by cyclic changes of red-emitting membrane and chromosome. No obvious cell morphology or viability change was found during the imaging. For **TPAE2**: λ_{ex} : 405 nm, λ_{em} : 600 $\pm$ 20 nm; For Hoechst, λ_{ex} : 405 nm, λ_{em} : 450 $\pm$ 20 nm. Scale bar: 20 μ m.

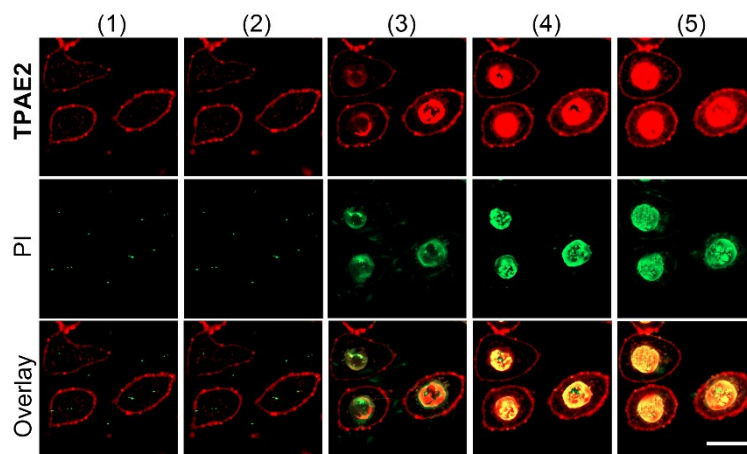


Fig. S19. Cells stimulated with low osmotic solution (water: PBS = 2:1) to induce cell swelling and stained by **TPAE2** (10.0 μM) and PI (20.0 $\mu\text{g/mL}$, pseudo-green color) to monitor the variation of membrane permeability. For **TPAE2**, λ_{ex} : 405 nm; λ_{em} : 600 ± 20 nm; for PI, λ_{ex} : 543 nm; λ_{em} : 610 ± 20 nm. Scale: 20 μm .

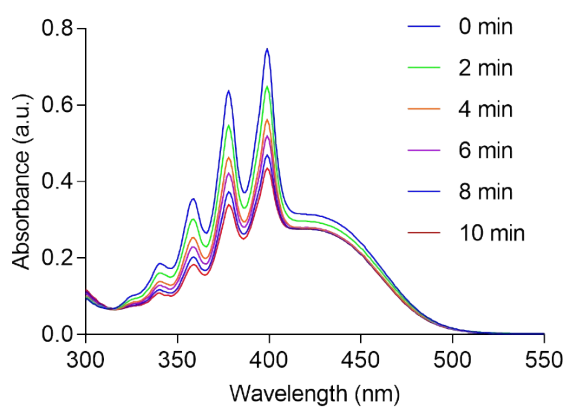


Fig. S20. UV-vis absorption spectra of ABDA (50.0 μM) in **TPAE2** solution (10.0 μM) upon white light irradiation for different time (white light, 40 mW cm^{-2}).

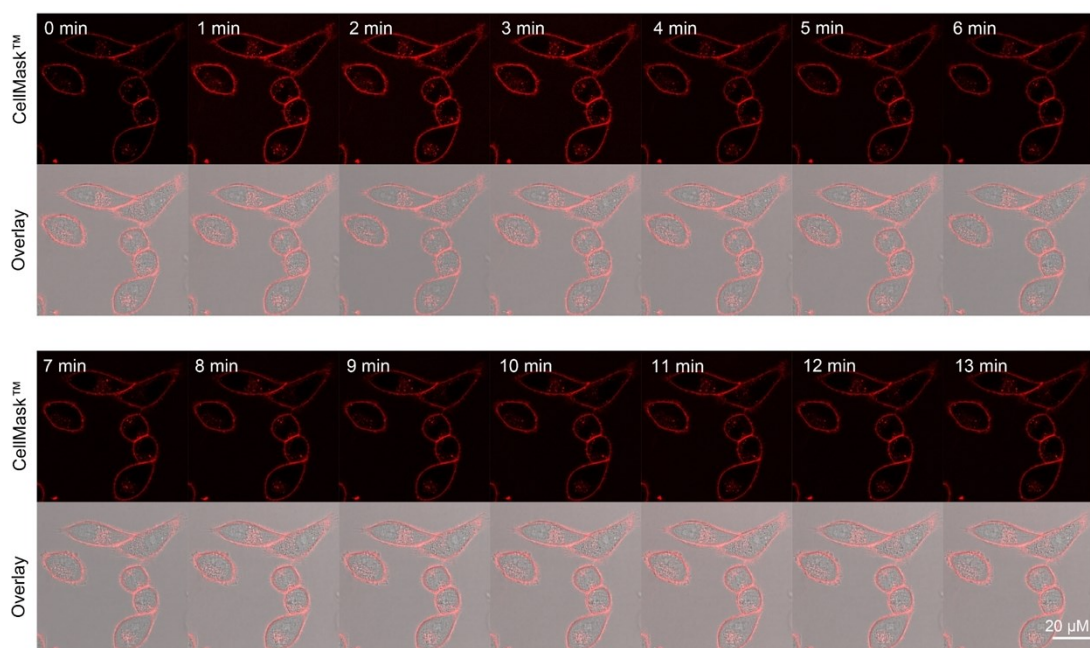


Fig. S21. Control experiments, where HeLa cells were exposed to light irradiation for 5 s immediately. Irradiate every 1 min. HeLa cells stained with CellMask™ (10.0 μ M), in which the morphological changes of plasma membrane and nuclei staining is traced.

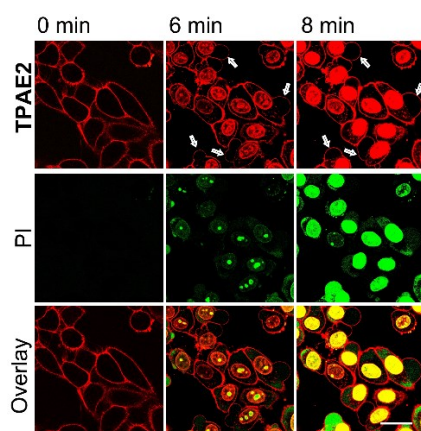


Fig. S22. Real-time monitoring of the membrane-to-nucleus process of **TPAE2** during PDT. HeLa cells co-stained with **TPAE2** (10.0 μ M) and PI (20.0 μ g/mL, pseudo green color) with different times during PDT treatment (0 min, 6min, 8 min). For **TPAE2**, λ_{ex} : 405 nm; λ_{em} : 600 $\pm$ 20 nm; for PI, λ_{ex} : 543 nm; λ_{em} : 610 $\pm$ 20 nm. Scale: 20 μ m.

Table S1. Fluorescence quantum yield of **TPAE1** and **TPAE2** at room temperature.

TPAE1								
	PBS	EA	MeOH	THF	MeCN			
ϕ^a	0.147	0.135	0.059	0.098	0.130			
TPAE2								
	PBS	EA	MeOH	THF	MeCN	PBS (+ DNA)	PBS (+ DOPC)	Gly
ϕ^a	0.007	0.051	0.044	0.017	0.045	0.253	0.216	0.173

a: Quantum yields were calculated according to literature procedures ^[11]. Ethanol solution of rhodamine 6G and coumarin 307 were used as the standard, in which the quantum yields of rhodamine 6G and coumarin 307 are 0.94 and 0.56, respectively.

References

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