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Amphiphilic fluorescent probe self-encored in plasma to detect pH fluctuation in cancer cell membrane

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Author Contributions

‡ A. P. and M. M. J. equally contributed to this work

1. Experimental Section

Materials and Methods

N-(2-bromoethyl) phthalimide (Sigma), dihexylamine (Sigma), hydrazine hydrate (Sigma), N, N-dimethylethylenediamine (DMEN) (Sigma), ethylene glycol (TCI), K_2CO_3 (Rankem), EtOH (Merck), acetonitrile (ACN) (Avra), dichloromethane (Sigma) were purchased commercially and used without further purification. Flash column chromatography was performed using silica gel (100-200 mesh) and analytical thin layer chromatography was performed using silica gel 60 (pre-coated sheets with 0.25 mm thickness). Mass spectra were recorded on anion SpecHiResESI mass spectrometer. NMR spectra were collected on a 400 MHz and 500 MHz spectrometer (Bruker, Germany).

***Synthesis of 1*:** To a stirred solution of N-(2-bromoethyl) phthalimide (6 g, 23.72 mmol) in acetonitrile, dihexylamine (6.6 g, 35.58 mmol) and K_2CO_3 (13.1 g, 94.88 mmol) were added and stirred for 12 h at 70 °C. After completion of the reaction, the reaction mixture was cooled to room temperature and diluted with water (50 mL) and then extracted with ethyl acetate (3 × 50 mL). Then combined organic layer was washed with brine, then dried over anhydrous $MgSO_4$ and concentrated under reduced pressure to get crude. The crude compound was passed through the silica gel (100-200 mesh) column chromatography using ethyl acetate in hexane (1:9) as an eluent. The pure compound was dried in vacuum to afford compound **1** (6.2 g, yield 63.36%) as pale brown liquid. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.76 (q, J = 5.27 Hz, 2H), 7.62 (q, J = 5.47 Hz, 2H), 3.66 (t, J = 6.75 Hz, 2H), 2.62 (t, J = 6.7 Hz, 4H,), 2.36 (t, J = 7.27 Hz, 2H), 1.28 -1.16 (m, 16H), 0.80 (t, J = 6.7 Hz, 6H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 168.26, 133.73, 132.38, 123.22, 54.06, 51.42, 31.84, 29.33, 27.25, 22.66, 14.11. ESI-MS m/z ($M+H^+$): calcd. 359.2620, found 359.2790.

***Synthesis of 2*:** Phthalimide protected compound **1** (6 g, 16.76 mmol) was dissolved in acetonitrile, then hydrazine hydrate (3.35 g, 67.03 mmol) was added slowly and stirred for 4 h at room temperature. The solvent was evaporated, and water was added. The aqueous phase was extracted three times with chloroform and the combined organic layer was dried with anhydrous $MgSO_4$. Further evaporation under reduced pressure afforded pure amines as red-brown oil (3.8 g, yield 92.2%). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.25 (s, 2H), 2.71-2.57 (m, 2H), 2.45-2.34 (m, 6H), 1.25 (s, 16H), 0.86 (t, J = 4.62 Hz, 6H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 59.58, 56.80, 39.58, 31.83, 29.40, 27.10, 22.62, 14.28. ESI-MS m/z ($M+H^+$): calcd. 229.2565, found 229.2635.

***Synthesis of 3*:** Compound 3 was prepared according to the literature.[1]

***Synthesis of 4*:** Compound **3** (500 mg, 1.44 mmol) and methyl iodide (5 mL) were taken in a sealed tube and heated at 70 °C for 2 h. Then reaction mass was cooled to room temperature and concentrated and purified by column chromatography (E.A: Hexane = 1:1) to get pure compound **4** (563 mg, yield 80%) as white solid. ^{1}H-NMR (500 MHz, DMSO-d_6): δ 8.61 (t, *J* = 6.5 Hz, 2H), 8.38 (d, *J* = 7.5 Hz, 1H), 8.27 (d, *J* = 7.5 Hz, 1H), 8.041 (t, *J* = 7.5 Hz, 1H), 4.46 (s, 2H), 3.22 (s, 11H). ^{13}C-NMR (125 MHz, DMSO-d_6): δ 163.22, 133.27, 132.05, 130.07, 129.13, 128.60, 122.75, 121.96, 62.13, 52.76, 33.94. ESI-MS m/z ($M+H^+$): calcd. 362.0546, found 362.0755.

***Synthesis of P_1CS*:** Compound **2** (1.58 g, 6.90 mmol) and compound **4** (500 mg, 1.38 mmol) were dissolved in ethylene glycol (10 mL) in a single neck round bottom flux and stirred at 150 °C for 12 h. TLC was checked (50% E.A in hexane) and then reaction mass was dissolved in 50 mL water and extracted with ethyl acetate (3 x 30 mL). Then total organic layer was dried over $MgSO_4$ and concentrated under reduced pressure to get crude compound. Then crude was purified by column chromatography (60% E.A in hexane). **P_1CS** was obtained as semiliquid compound (554 mg, yield 85%). ^{1}H-NMR (500 MHz, DMSO-d_6): δ 8.26 (s, 2H), 7.96 (s, 1H), 7.66 (d, *J* = 3.5 Hz, 2H), 4.86 (s, 1H), 4.24 (t, *J* = 7.0 Hz, 4H), 3.32 (s, 11H), 3.06 (t, *J* = 6.0 Hz, 4H), 2.70 (s, 2H), 1.314 (s, 16H), 0.85 (t, *J* = 6.5 Hz, 6H). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 169.54, 154.93, 139.85, 131.05, 128.02, 126.69, 124.69, 122.11, 116.85, 110.01, 62.79, 53.90, 52.93, 47.77, 37.11, 31.05, 26.69, 26.08, 22.31, 14.08 ppm. ESI-MS m/z ($M+H^+$): calcd. 510.3850, found 510.2790.

***Synthesis of P_2CS*:** Compound **4** (500 mg, 1.38 mmol) and DMEN (610 mg, 6.90 mmol) were dissolved in ethylene glycol (10 mL) in a single neck round bottom flux and stirred at 150 °C for 12 h. TLC was checked (70% E.A in hexane) and then reaction mass was dissolved in 50 mL water and extracted with ethyl acetate (3 x 30 mL). Then total organic layer was dried over $MgSO_4$ and concentrated under reduced pressure to get crude compound. Then crude was purified by column chromatography (75% E.A in hexane) to afford pure **P_2CS** as solid compound (406 mg, yield 80%). ^{1}H-NMR (500 MHz, DMSO-d_6): δ 8.74 (t, *J* = 8.0 Hz, 2H), 8.49 (d, *J* = 7.5 Hz, 1H), 8.32 (d, *J* = 8.5 Hz, 1H), 8.04 (d, *J* = 7.5 Hz, 1H), 4.45 (q, *J* = 10.5 Hz, 4H), 4.30 (t, *J* = 6.0 Hz, 1H), 3.32 (s, 11H), 2.76 (s, 8H). ^{13}C-NMR (125 MHz, DMSO-d_6): δ 161.22, 152.45, 139.01, 133.27, 131.68, 129.82, 128.60, 122.75, 121.96, 116.22, 62.13, 52.45, 47.15, 36.35 ppm. ESI-MS m/z ($M+H^+$): calcd. 370.2285, found 370.1552.

General methods for UV-Visible and Fluorescence Spectroscopy

All chemicals and solvents used for the spectroscopic study, were received from a commercial source and used without further purification. Absorption spectra were recorded on a UV-1800 spectrophotometer (Shimadzu, Japan) and fluorescence spectra were recorded using a RF-6000 fluorescence spectrofluorometer (Shimadzu) with a 3000 μL volume of 1 cm standard quartz cell. The stock solutions of **P_1CS** and **P_2CS** were prepared in phosphate buffered saline (0.1% DMSO). The fluorescence emission spectra were recorded at an excitation wavelength of 450 nm and emission at 540 nm with slit widths set at 3 nm. The solutions of biologically relevant species were prepared from LiCl, NaCl, KCl, AgCl, $MgCl_2$, $CaCl_2$, $BaSO_4$, $FeCl_2$, $CuCl_2$, $ZnCl_2$, $FeCl_3$, $AlCl_3$, KBr, $NaHCO_3$, $NaNO_2$, $NaNO_3$, NaOAc, Na_2SO_4, and H_2O_2.

Determination of Fluorescence quantum yield

The quantum yields of probe **P_1CS** and **P_2CS** at pH 4.0 and pH 8.5 were determined according to the literature.[2]

Fluorescence quantum yields for **P_1CS** and **P_2CS** at pH 4.0 and pH 8.5 were determined by using RhodamineB ($\Phi F = 0.89$ in ethanol) as a standard.

The quantum yield was calculated using the following equation:

$$\Phi_S = \Phi_X (A_X/A_S) (F_S/F_X) (n_S/n_X)^2$$

Where, Φ_X = The fluorescence quantum yield of the reference,

A_X = The absorbance of the reference,

A_S = The absorbance of the sample,

F_X = Relative integrated fluorescence intensities at the same excitation wavelength for reference,

F_S = Relative integrated fluorescence intensities at the same excitation wavelength for the sample,

and n = the refractive index of the solvents used.

Quantum yields were determined using fluorescein as a standard. The quantum yield was calculated according to the equation: $\Phi s = \Phi_X (A_S F_S / A_X F_X)$;

The quantum yield of probe **P_1CS** at pH 4.0 and pH 8.5 is 0.625 and 0.129, respectively, upon excitation at 450 nm. The quantum yield of probe **P_2CS** at pH 4.0 and pH 8.5 is 0.712 and 0.135, respectively, upon excitation at 450 nm.

Solvatochromic study: For solvatochromic study we dissolved **P_1CS** (5 μM) in different solvents for eg. EtOAc, DMF, DMSO, EtOH and THF and checked fluorescence emission (λ_{ex}= 450 nm).

Viscosity study: For viscosity based fluorescent emission study we used **P_1CS** (5 μM) in water: glycerol solvent mixture from 100:0 to 0.01:99.9 and collected fluorescence emission (λ_{ex}= 450 nm).

Cell culture and maintenance

Human cancer cell lines HeLa (cervical cancer), and MDA-MB-231 (breast cancer) were obtained from American Type Culture Collection (Manassas, USA). Human lung fibroblast cell line WI-38 was kindly gifted form IICB, Kolkata, India. Spheroids of HeLa cells were developed with the transfer of cells into ultra-low attachment cell culture flasks. Cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) and 5% CO_2 at 37 °C, while the resistant cell lines were maintained in the media with the desired drug concentration.

Cytotoxicity assay

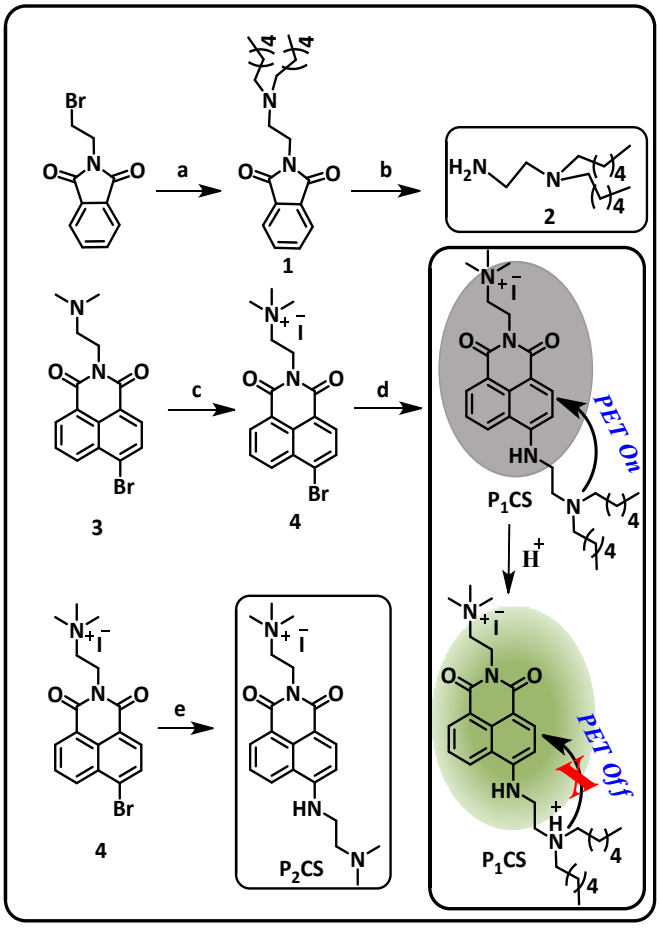
The cytotoxicity of HeLa, and WI-38 cells was evaluated over a wide range of concentrations (1 μM to 10 mM) of **P_1CS** for a period of 12 h. The cell growth inhibitory potential was measured using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay as described before.[3] Finally, absorbance was recorded at 570 nm (BioTek, PowerWave XS, USA).

Fluorescence imaging

For fluorescence imaging experiments, the adherent cells obtained after removing the culture medium were subjected to washing with phosphate-buffered saline (PBS pH 7.4) two times. Cells were treated with the fluorescent probes; then cells were washed for three times with PBS to remove the excess dye molecules from the culture medium. Initially both **P_1CS** and **P_2CS** (1 μM) were treated with HeLa cells over 10 to 40 min to check the optimal imaging conditions. After optimizing the imaging conditions (5 μM for 15 min), the cells were co-stained with Hoechst (1 μM). In order to determine the pH-dependent behavior of **P_1CS**, studies were performed on HeLa cells. The cells were incubated with high K^+ buffers (30 mM NaCl, 120 mM KCl, 1 mM $CaCl_2$,

0.5 mM $MgSO_4$, 1 mM NaH_2PO_4, 5 mM glucose, 20 mM HEPES, 20 mM NaOAc) of different pH such as 6.5, 7.4 and 8.5 for 30 min. Further, the effect of glucose was studied by maintaining the cells in medium with different glucose concentrations. The cells were maintained in DMEM media supplemented with 25 mM glucose (12 h) or 100 mM glucose (12 h) or with 10 mM 2-Deoxy-D glucose (4 h). In an attempt to estimate cell membrane targeting ability of **P_1CS**, a co-staining approach was followed with Trypan Blue where nucleus was stained with SYTO™ 17 as previously described.[4] Trypan Blue is a membrane impermeable dye which can quench the fluorescence emission of fluorophores in the range of 500-600 nm. Finally, to access whether **P_1CS** can distinguish cells with varying metastatic potential, imaging studies were performed on MDA-MB-231, HeLa, W1-38 and spheroids of HeLa cells. For all the imaging studies, cells were observed under appropriate filter of an inverted fluorescence microscope (Olympus 1X51, Singapore).

Reaction scheme:



Scheme S1 Synthesis of probes (P_1CS, P_2CS)

Reagents and reaction conditions: (a) Dihexylamine, K_2CO_3, ACN, 70 °C, 12 h. (b)

NH_2NH_2, H_2O, ACN, rt, 12 h. (c) Methyl Iodide, 70 °C, 2 h. (d) comp-2, ethylene glycol, 150 °C, 12 h. (e) DMEN, ethylene glycol, 150 °C, 12 h.

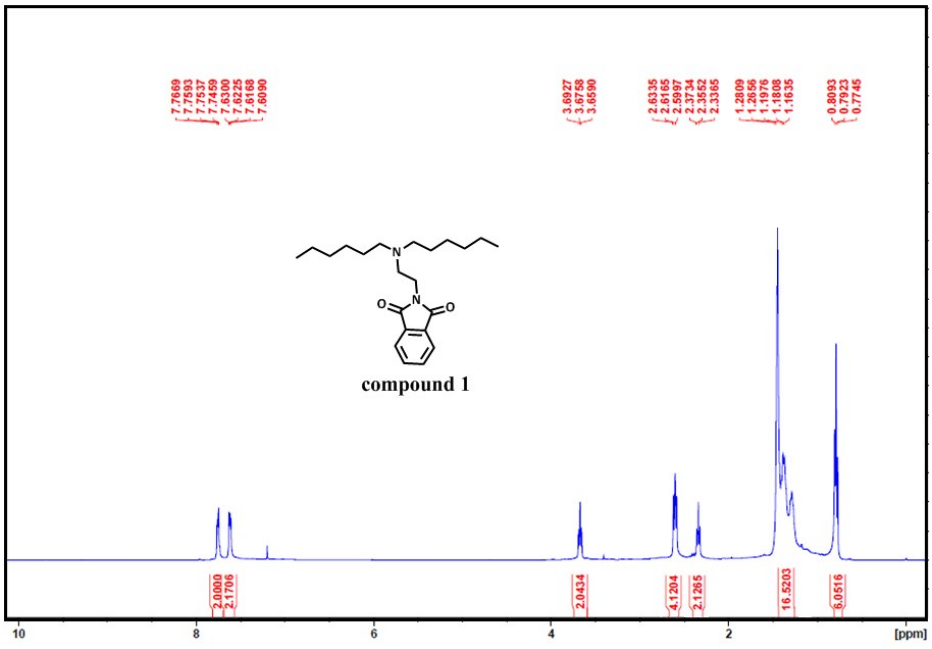


Fig. S1 ^{1}H NMR of compound **1** in $CDCl_3$.

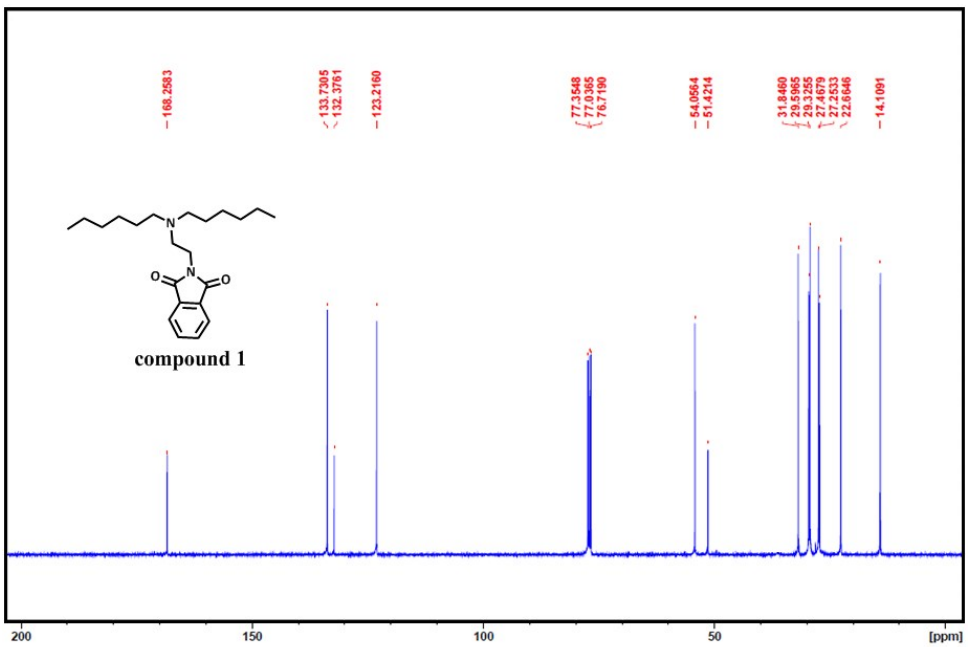


Fig. S2 ^{13}C-NMR of compound **1** in $CDCl_3$.

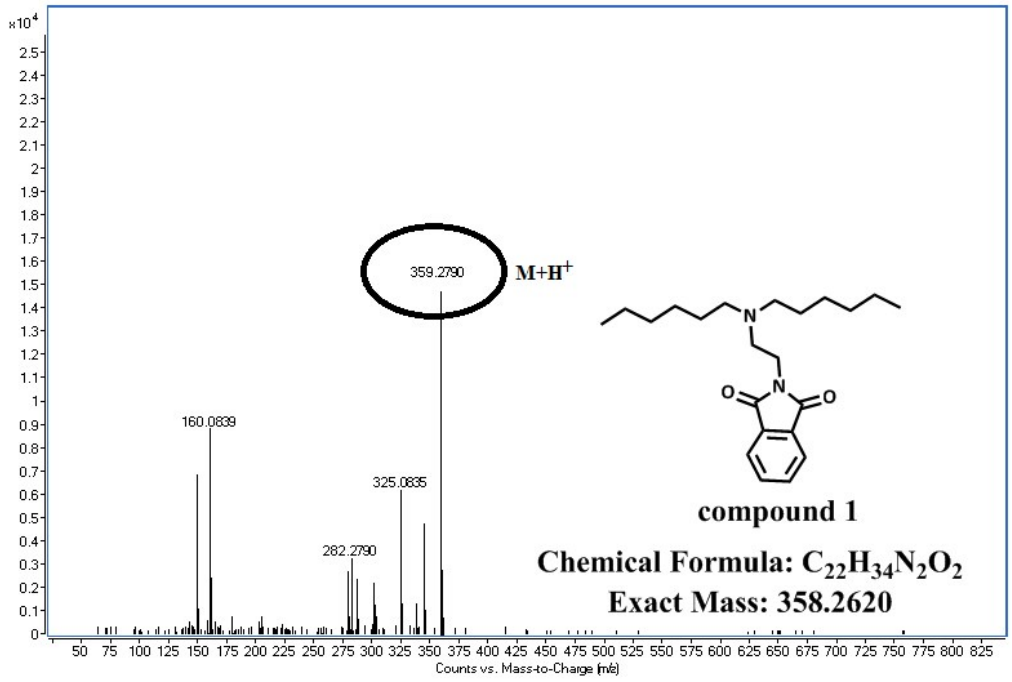


Fig. S3 ESI-HRMS of compound **1**.

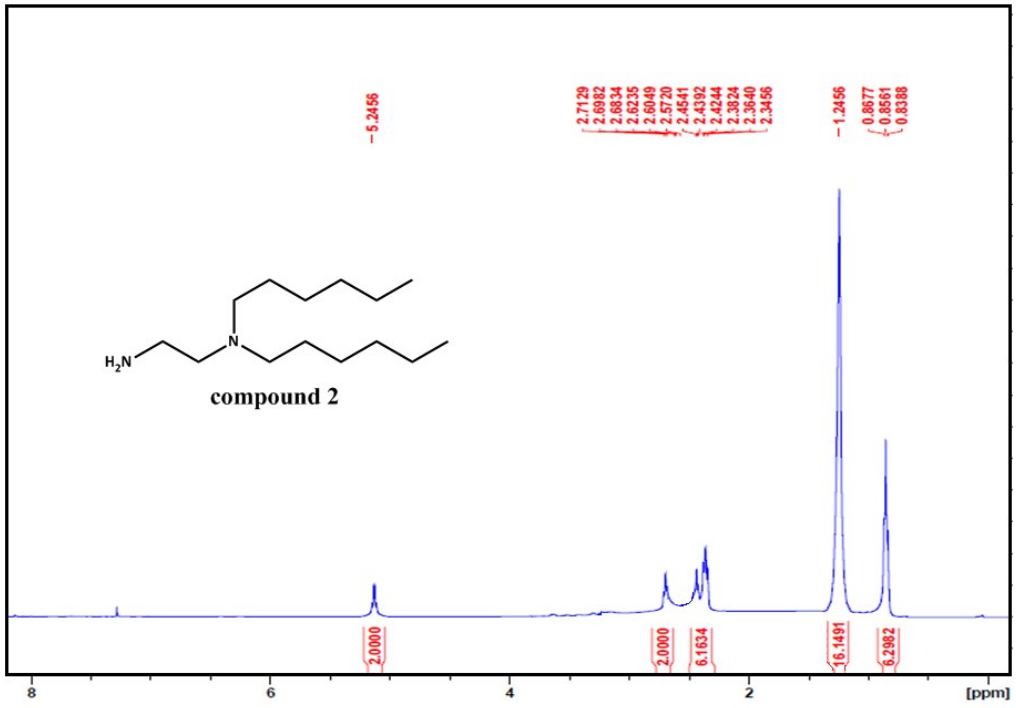


Fig. S4 ^{1}H NMR of compound **2** in $CDCl_3$.

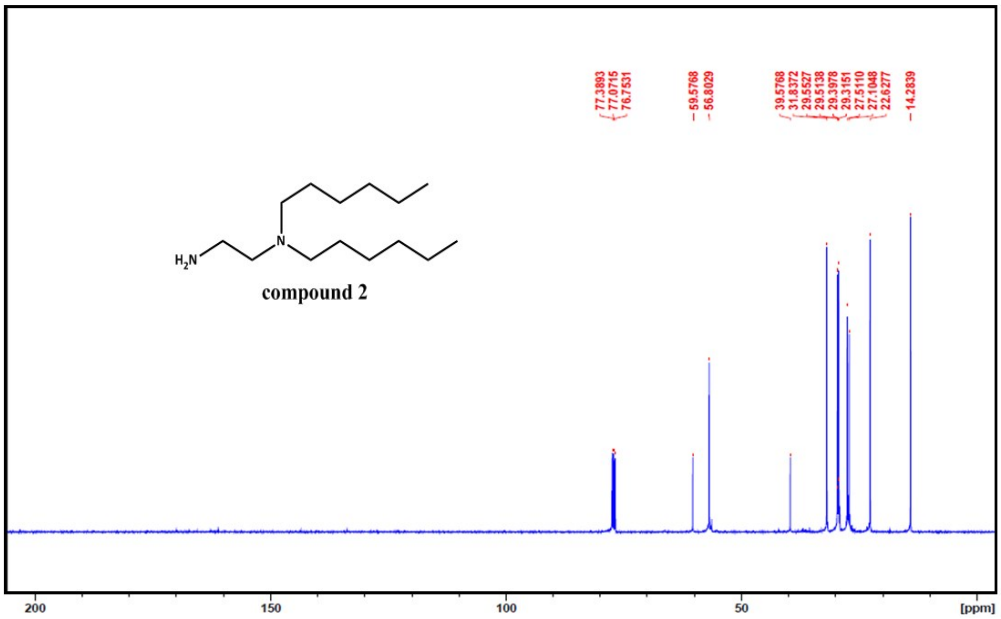


Fig. S5 ^{13}C-NMR of compound **2** in $CDCl_3$.

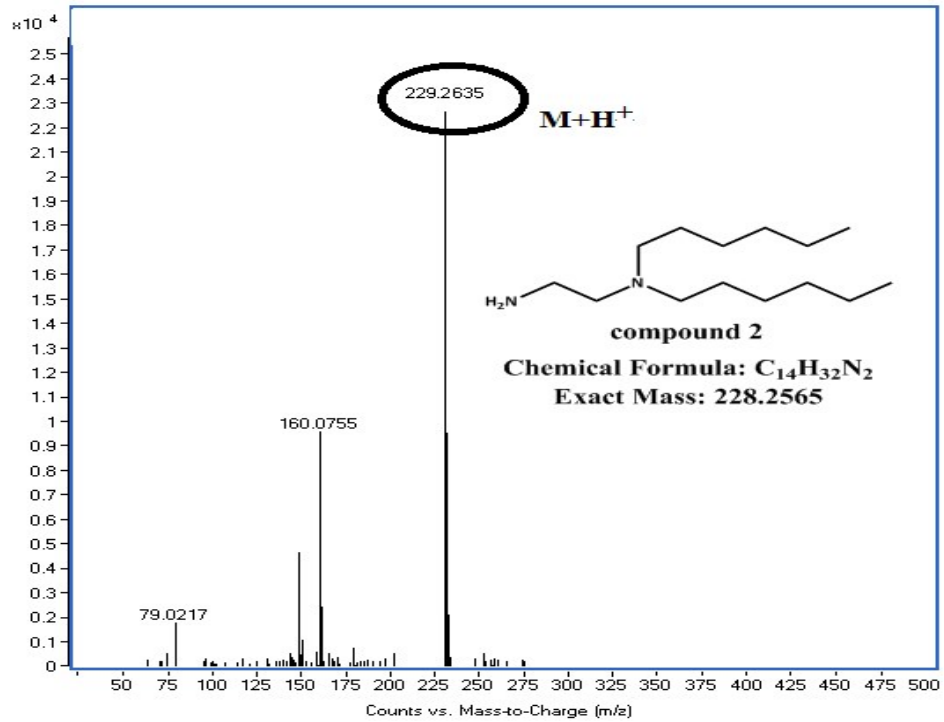


Fig. S6 ESI-HRMS of compound **2**.

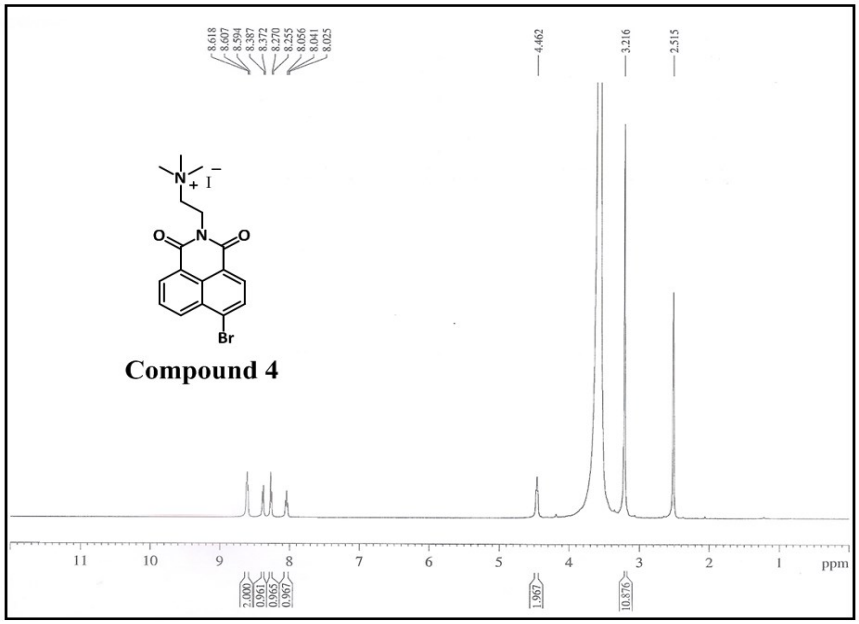


Fig. S7 ^{1}H NMR of compound **4** in DMSO-d6.

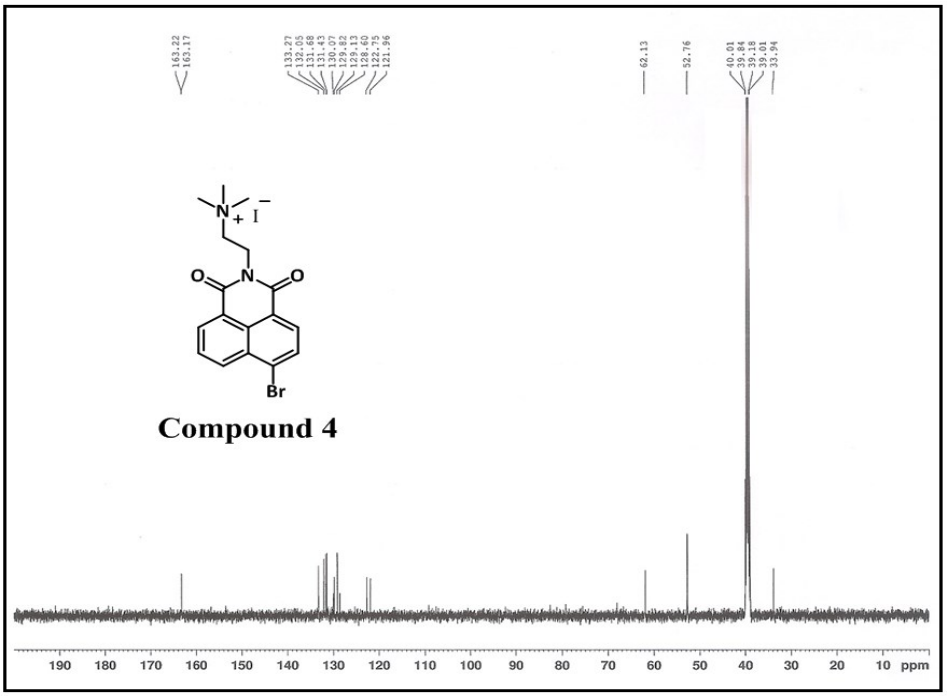


Fig. S8 ^{13}C-NMR of compound **4** in DMSO-d6.

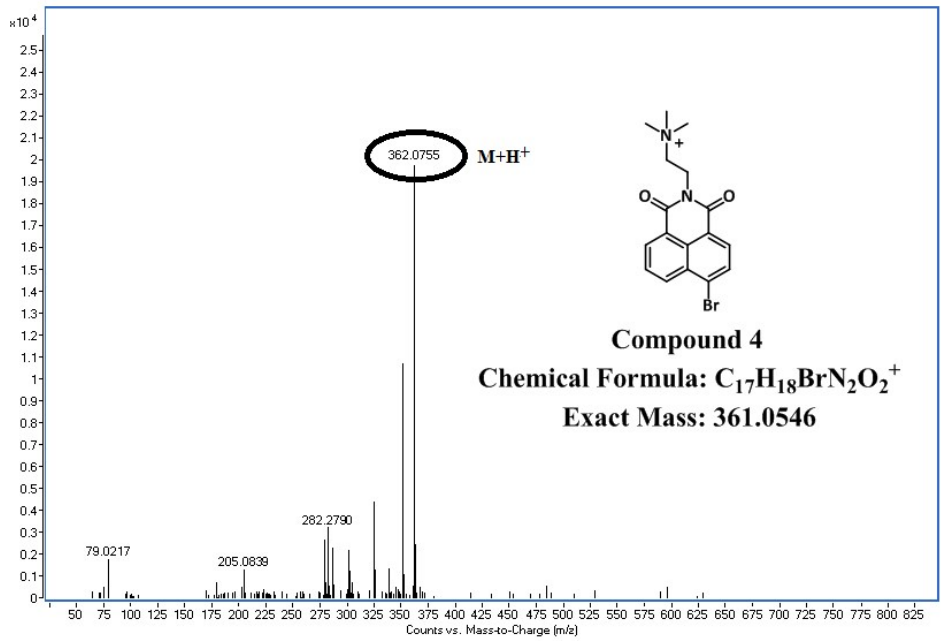


Fig. S9 ESI-HRMS of compound **4**.

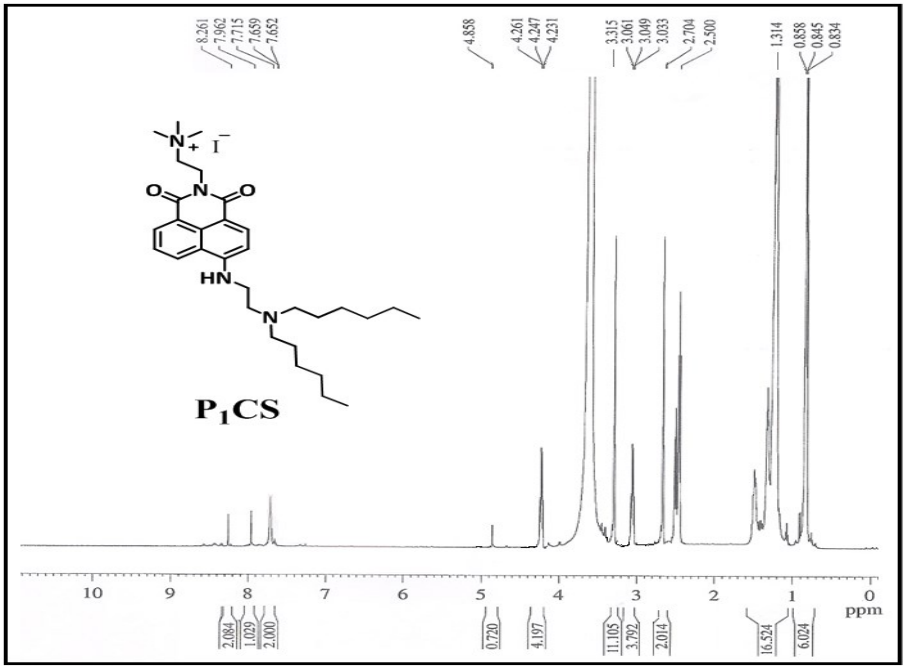


Fig. S10 ^{1}H NMR of compound **P_1CS** in DMSO-d6.

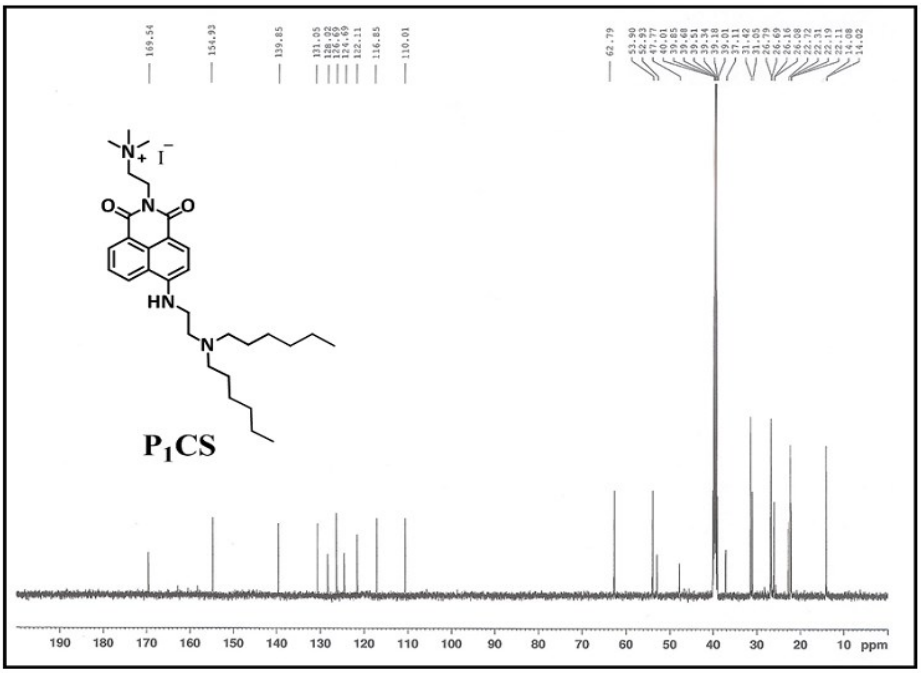


Fig. S11 ^{13}C-NMR of compound **P_1CS** in DMSO-d6.

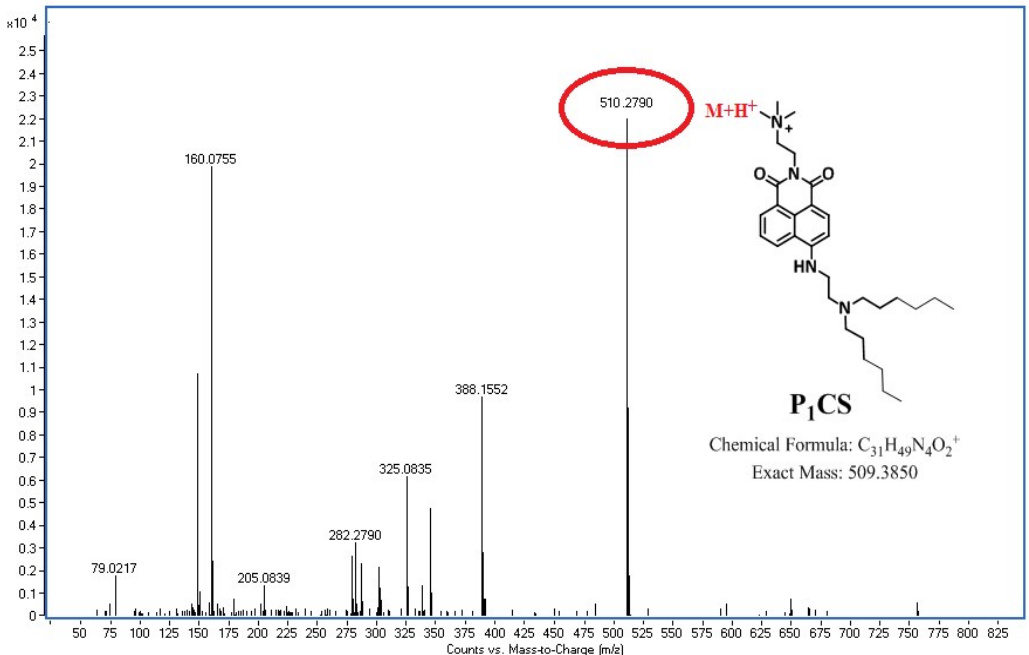


Fig. S12 ESI-HRMS of **P_1CS**.

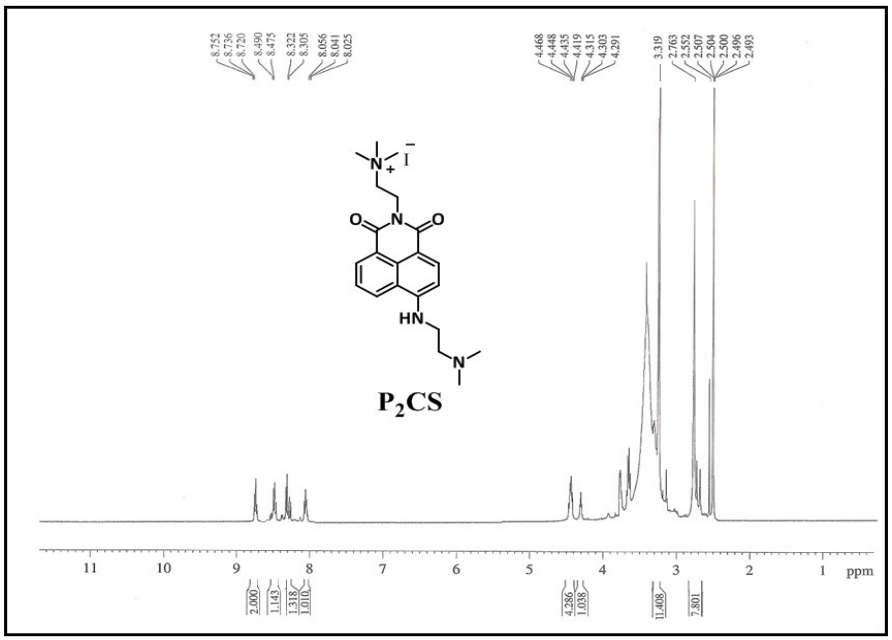


Fig. S13 ^{1}H NMR of **P_2CS** in DMSO-d6.

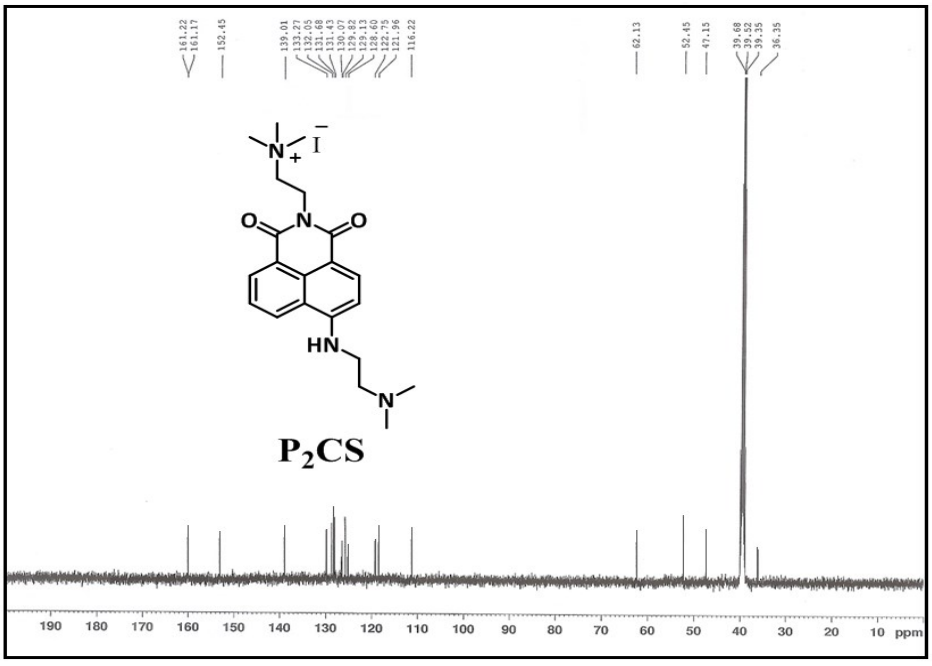


Fig. S14 ^{13}C-NMR of compound **P_2CS** in DMSO-d6.

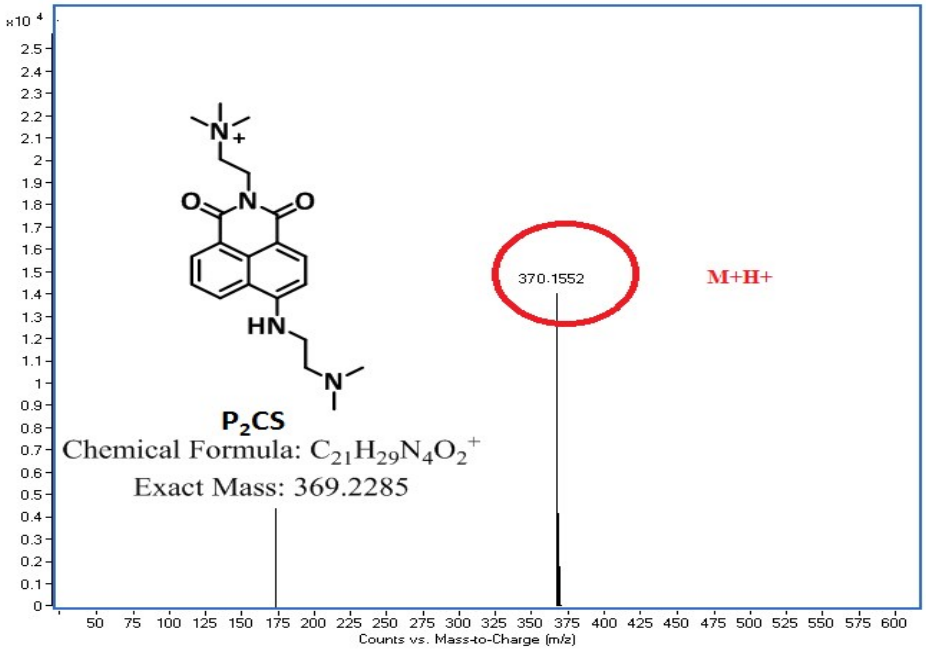


Fig. S15 ESI-HRMS of **P_2CS**.

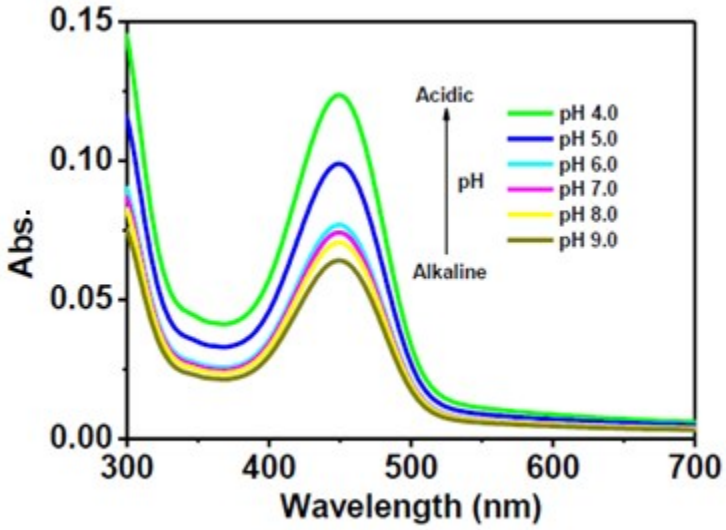


Fig. S16 Absorption changes of **P_1CS** (5 μM) at variable pH (4.0 - 9.0) in PBS buffer solution (0.1% DMSO). **P_1CS** was incubated with various pH solution for 30 min at 310 K.

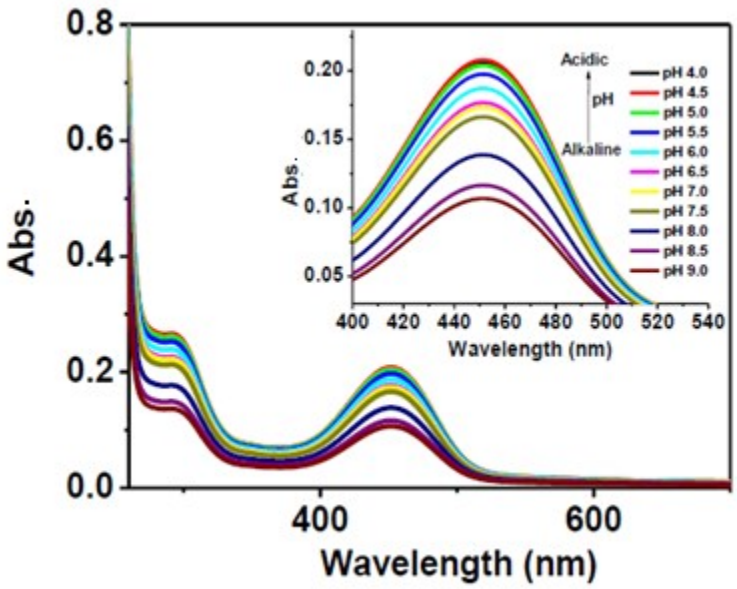


Fig. S17 Absorption changes of **P_2CS** (5 μM) at variable pH (4.0 - 9.0) in PBS buffer solution (0.1% DMSO). **P_2CS** was incubated with various pH solution for 30 min at 310 K.

Quantum yield (ϕ)	pH 4.0	pH 8.5
P_1CS (5 μM)	0.625	0.129
P_2CS (5 μM)	0.712	0.135

Table S1 Quantum yield of the pH probes at variable pH.

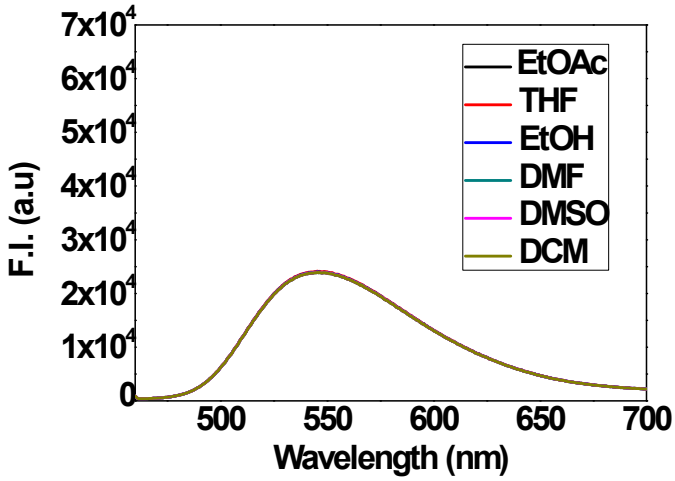


Fig. S18 Solvatochromic test was done in different solvents a) EtOAc, b) THF, c) EtOH, d) DMF, e) DMSO, f) DCM with using **P_1CS** (5 μm) (λ_{ex}= 450 nm).

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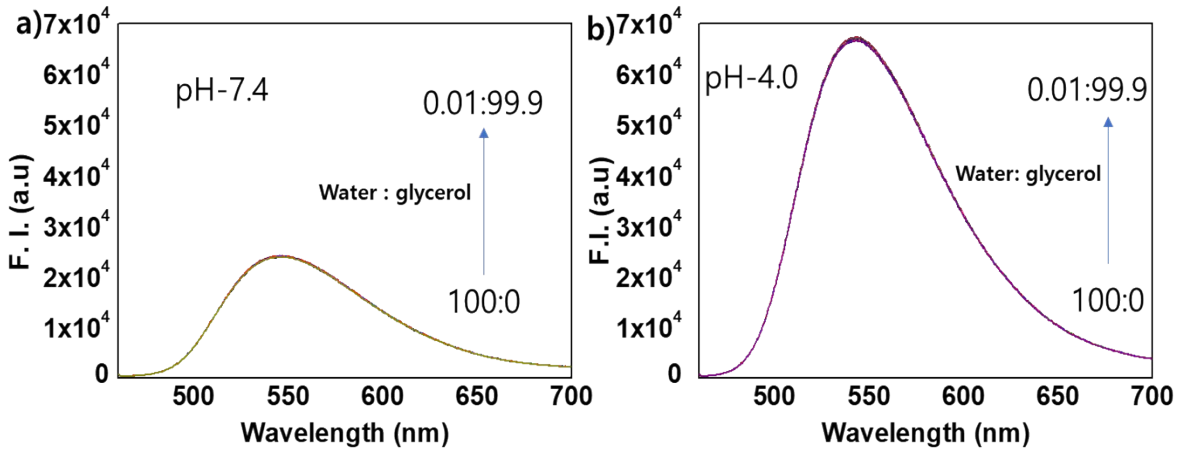

Fig. S19 The fluorescence spectral changes of the probe **P_1CS** (5.0 μM) in different ratios of water:glycerol mixtures (λ_{ex} = 450 nm) at pH 4.0 and 7.4 respectively.

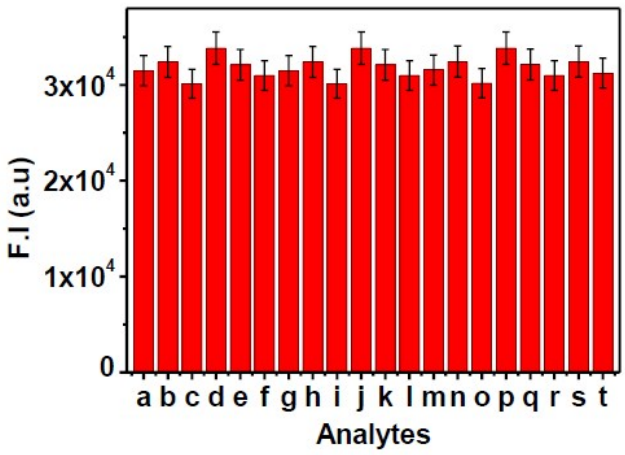

Fig. S20 Fluorescent emission (FI_{em} at 540 nm) of **P_1CS** (5 μM) in the presence of 500 μM of various analytes, in PBS buffer solution (pH 6.0) [a) Li^+, b) Na^+, c) K^+, d) Ag^+, e) Mg^{2+}, f) Ca^{2+}, g) Ba^{2+}, h) Fe^{2+}, i) Cu^{2+}, j) Zn^{2+}, k) Fe^{3+}, l) Al^{3+}, m) Cl^-, n) Br^-, o) HCO_3^-, p) NO_2^-, q) NO_3^-, r) AcO^-, s) SO_4^{2-}, t) H_2O_2].

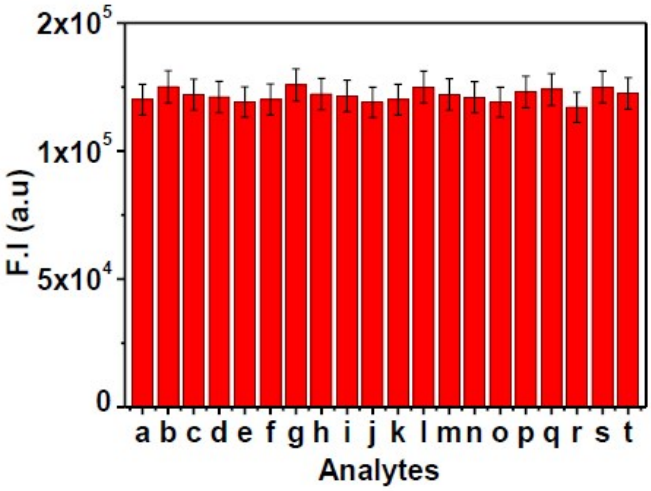

Fig. S21 Fluorescent emission (FI_{em} at 540 nm) of **P_2CS** (5 μM) in the presence of 500 μM of various analytes, in PBS buffer solution (pH 6.0) [a) Li^+, b) Na^+, c) K^+, d) Ag^+, e) Mg^{2+}, f) Ca^{2+}, g) Ba^{2+}, h) Fe^{2+}, i) Cu^{2+}, j) Zn^{2+}, k) Fe^{3+}, l) Al^{3+}, m) Cl^-, n) Br^-, o) HCO_3^-, p) NO_2^-, q) NO_3^-, r) AcO^-, s) SO_4^{2-}, t) H_2O_2].

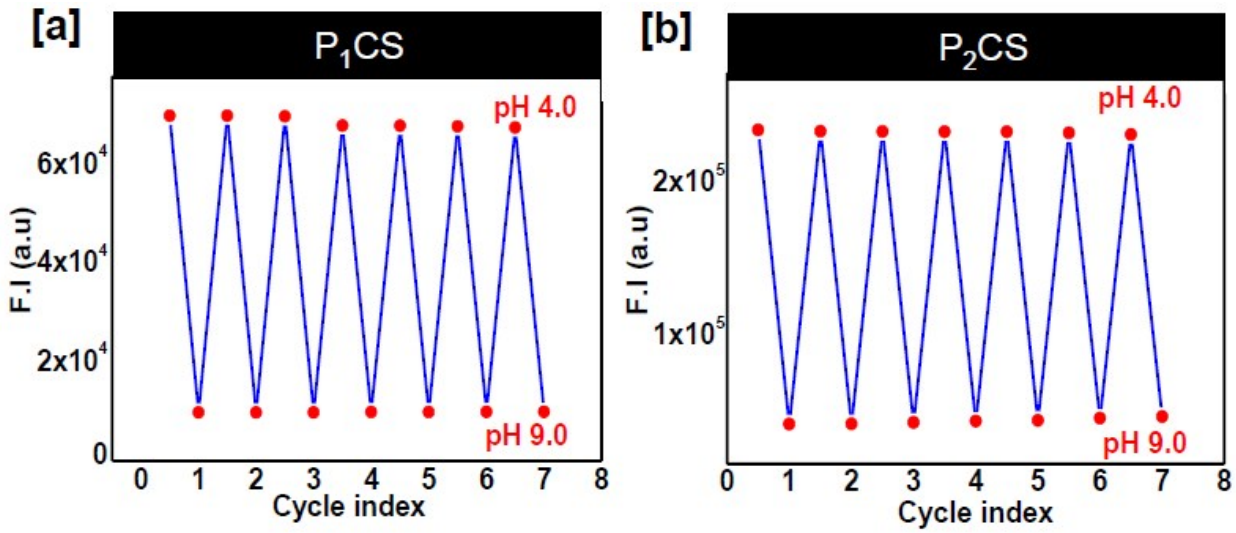

Fig.S22 [a] and [b] are the pH reversibility study of **P_1CS** (5.0 μM) and **P_2CS** (5.0 μM) between pH 4.0 and 9.0 respectively.

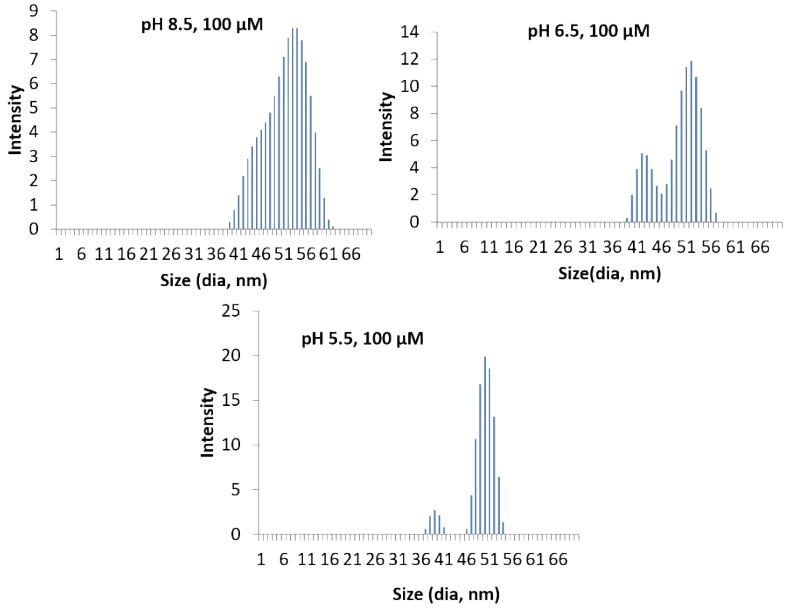

Fig. S23 DLS study of probe **P_1CS** (100 μM) at variable pH.

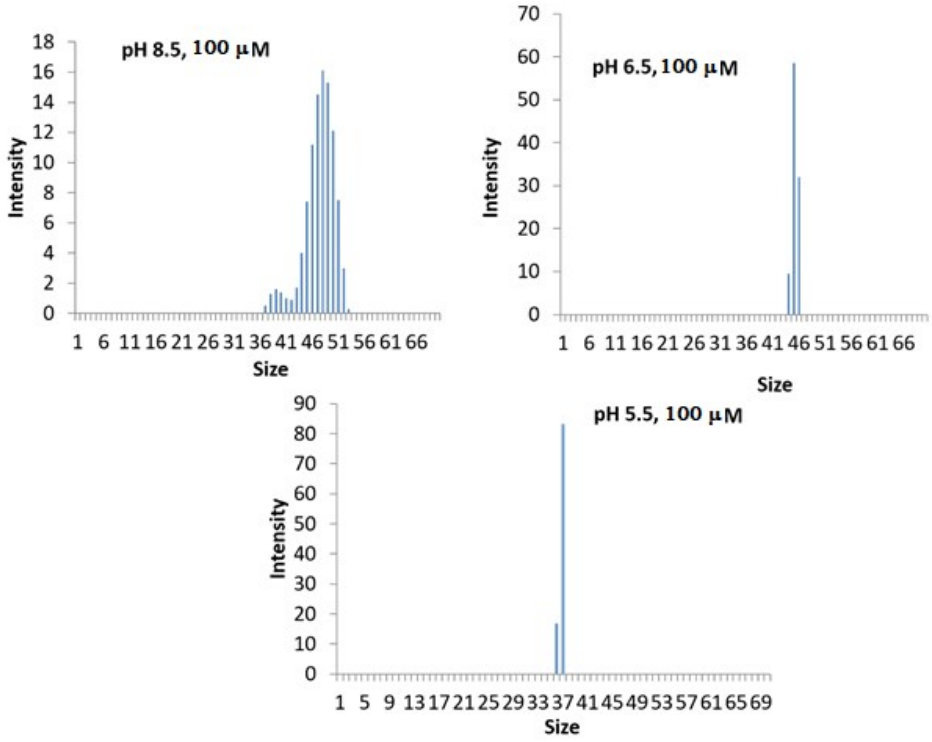

Fig. S24 DLS study of probe **P_2CS** (100 µM) at variable pH.

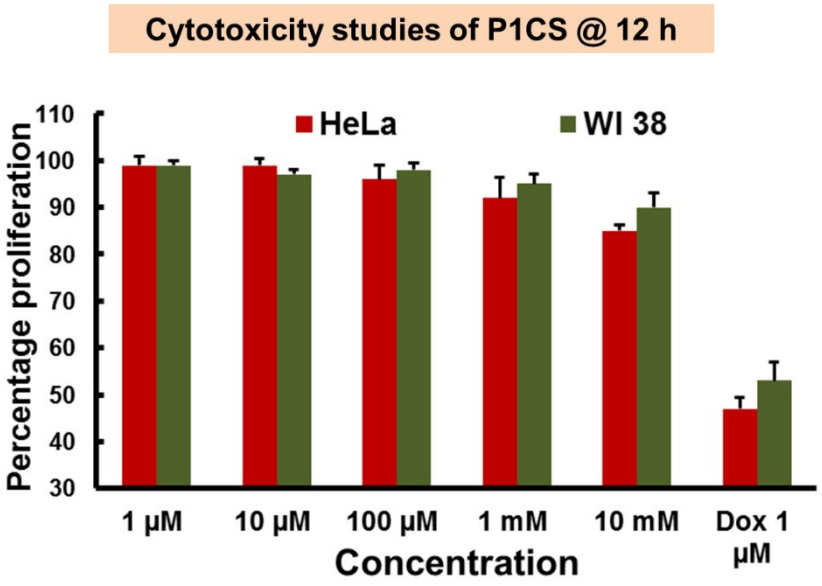

Fig. S25 Evaluation of the cytotoxicity of **P_1CS** by MTT assay on HeLa and W1-38 cells after 12 h incubation. Doxorubicin (1 μM) was used as a positive control. Data are the mean ± SD of three independent experiments.

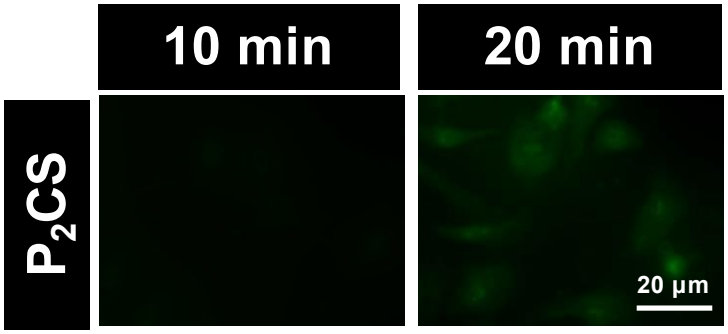

Fig. S26 Time depending imaging for **P_2CS** in HeLa cells. Cells were incubated with 1 μM of **P_2CS** for a different time period (5-20 min). The images were captured at 500 nm - 600 nm, excitation wavelength 450 nm. Scale bar 20 μm

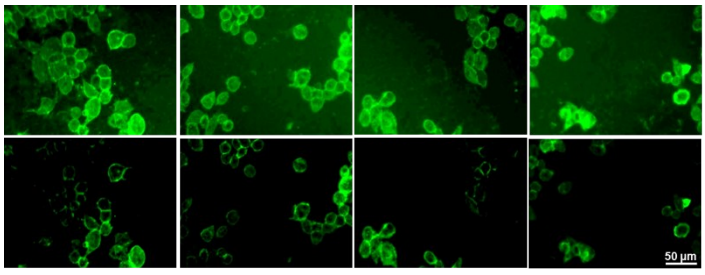

Fig. S27 Evaluation of the fluorescence emission on HeLa cells maintained in DMEM media supplemented with 10 % foetal bovine serum for 24 h. The cells were later incubated with P1CS (5μM) and imaged after 15 min using an inverted fluorescence microscope. Upper panel represents cells without washing and the lower panel represents the respective images after washing. Cells were washed with phosphate buffered saline (PBS, pH 7.4) thrice for the cells represented in the lower panel. The images were captured at 500 nm - 600 nm, excitation wavelength 450 nm. Scale bar 50 μm

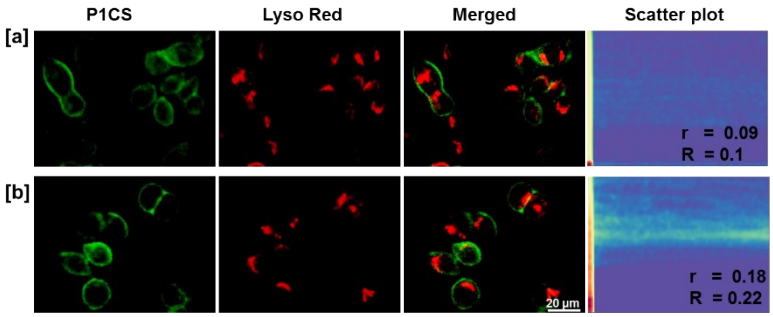

Fig. S28 Evaluation of the fluorescence emission in a time-dependent manner on HeLa cells with **P_1CS** (5μM) using fluorescence microscopy. Co-localization studies of **P_1CS** (5μM) after [a] 15 min and [b] 30 min incubation on HeLa cells and staining with Lysotracker Red (5μL). Red channel images were collected within range of 640-660 nm. Green channel images were collected within range of 530-600 nm. The co-localization was calculated using Pearson's correlation coefficient (r) and Mander's overlap coefficients (R).

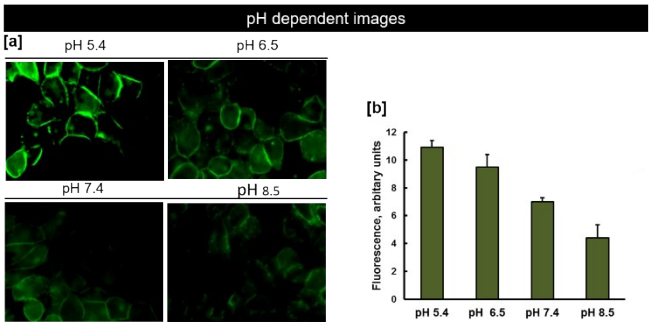

Fig. S29 [a] pH-dependent studies of **P_1CS** (5 μM) on HeLa cells and [b] quantification of the fluorescence intensity. Emission was collected at 500-600 nm, excitation set at 450 nm. The cells were incubated with high K^+ buffers (30 mM NaCl, 120 mM KCl, 1 mM CaCl2, 0.5 mM MgSO4, 1 mM NaH_2PO_4, 5 mM glucose, 20 mM HEPES, 20 mM NaOAc)

System	Excitation/Emission	Published Journal/Ref.
Synthetic **DNA nanomachine**	488 nm / 500-750 nm	*Nat. Commun.,* 2011, **2**, 340. (reference number 18)
Peptide based ratiometric fluorescent probe **SNARF-pHLIP**	531 nm/ 595 nm at **pH 5.9** 531 nm/ 645 nm at **pH 7.8**	*Proc. Natl. Acad. Sci. U.S.A.,* 2016, **113**, 8177. (reference number 19)
Lipid-DNA conjugated system	488 nm /520 nm for **FAM** dye and 543 nm/ 580 nm for **TAMRA** dye	*ACS Appl. Mater. Interfaces,* 2014, **6**, 15329. (reference number 20)
PEG-lipid conjugated fluorescein isothiocyanate system	458 nm / 525 nm 488 nm / 525 nm	*Sci. Rep.,* 2017, **7**, 17484. (reference number 21)
Bovine serum albumin protected gold nanoclusters (**BSA-AuNCs**), contained fluorescein isothiocyanate (**FITC**) labeled cationic peptides (**CPs**)	488 nm / 650 nm	*ACS Sens.,* 2018, **3**, 2278. (reference number 22)
P_1CS Amphiphilic **pH** system	450 nm / 540 nm	**This work**

Table S2: A tabular account of cell membrane specific probes.

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