Electronic Supporting Information

Bay Functionalized Perylenediimide as deaggregation based intracellular fluorescent probe for perchlorate

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1. Experimental Section

(a) Details of Materials and methods

Chemicals and solvents were reagent grade and used without further purification unless otherwise stated. All reactions were performed under N_2 atmosphere. N-Methylpyrrolidine (NMP), DMSO and DMF solvents were of HPLC grade and dried over 4 Å molecular sieves. Chromatographic purification was done with silica gel 60-120 mesh. TLC was performed on aluminium sheets coated with silica gel 60 F254 (Merck, Darmstadt). NMR spectra were recorded on Bruker and JEOL (operating at (500, 400) and 300 for ^{1}H; (125, 100) and 75 MHz for ^{13}C, respectively). The peak values were obtained as ppm (δ), and referenced to the TMS as reference in ^{1}H NMR and deutrated solvent in ^{13}C NMR spectra. Abbreviations used for splitting patterns are s = singlet, bs = broad singlet, t = triplet, q = quartet, m = multiplet. Fourier transform infrared (FT-IR) spectra were recorded on Perkin Elmer 92035. HRMS spectra were recorded on BrukerMicroToff/QII.

Photophysical studies: Spectroscopic grade DMSO solvent and MilliQ water were used for measurement. The absorption spectra were recorded on Shimadzu-2450 spectrophotometer from Shimadzu equipped with Peltier system as temperature controller. Quartz cells of appropriate length were used for sample measurement. The spectral bandwidth and the scan rate were fixed at 2 nm and 140 nm min^{-1}, respectively. The fluorescence titrations were performed on Varian Carey Eclipse fluorescence spectrophotometer using slit width (excitation = 10 nm, emission = 2.5 nm) with excitation at 500 nm, unless otherwise stated. The stock solutions for various measurements of PDI **1-2** were prepared and dilutions of these stock solutions were used for the photophysical measurements. All absorption and fluorescence scans were saved as ACS II files and further processed in Excel™ to produce all graphs shown. The spectral data were analyzed through curve fitting procedures by using non-linear regression analysis SPECFIT 3.0.36 to determine the stability constants and the distribution of various species.

WXRD: WXRDs were recorded using a Rigakudiffractometer with CuKα (λ = 1.54 Å) emission and spectra were recorded in the (2) range of 5-50^{o}C. The radiation used was with a Ni filter and a data collection was carried out using flat holder in Bragg Brentano geometry.

SEM: SEM measurements were performed on a ZEISS SUPRA^{TM}55 operating at an acceleration voltage of 10 KV with tungsten filament as electron source. Samples of PDI **1** were dissolved in DMSO:H_2O (1:9 v/v). 5 μL aliquot of fresh solution of PDI **1** was deposited on glass surface using drop cast method. After drying the glass surface was imaged.

DLS:DLS measurements were performed at 298.15 K using a light scattering apparatus (Zetasizer Nano ZS Malvern Instrument Ltd., UK). The samples were thermally equilibrated for 10 minutes before each measurement and an average of 10 measurement runs were considered as data. The temperature was controlled with an accuracy of ±0.1 K using an inbuilt peltier device.

NMR titration studies:^{1}H NMR titration of PDI **1** against ClO_4^- was performed in DMSO(d_6)-H_2O (9:1 v/v) on Bruker 400 MHz spectrometer.Addition of higher amounts of water leads to precipitation of the compound. All the data were then processed in Top Spin software to draw the stacking spectra of PDI **1** and PDI **1**+ClO_4^- complex at different concentrations.

Threotical calculations: All the calculations were carried out using density functional theory (DFT) at B3LYP/6-31G* basis set.

(b) Details of Sample preparations

Preparation of TLC Strips:TLC strips were made by dipping into acetonitrile solution of PDI **1**followed by drying under vacuum at room temperature. In the TLC strip method, the different concentrations of ClO_4^- were prepared in aqueous solution followed by addition of aliquot of 6 μL of each solution on the TLC strips previously coated with PDI **1** (10 μM). For control experiment, drop of water alone was also added on the TLC strip coated with PDI**1**. The TLC strips were then visualized under 365 nm UV light.

Preparation of thin films: Thin films of PDI **1** on glass surface were fabricated using drop cast technique. In this technique the solution of PDI 1 (10 μM) was prepared in DMSO:H_2O (1:9 v/v)andvarious concentration (5, 10, 20 μL)of this solution were deposited on glass surface.

Similarly, the solution of PDI **1**+ClO_4^- complex were deposited. The glass surfaces were then dried under incubator at constant temperature and then imaged under different microscopic techniques.

Sample solution from flash powder:300 mg powder from firework was dissolved in about 25ml water pre warmed at about 60°C in a conical flask. The flask was closed and left overnight for complete dissolving of any soluble substances. Vigorous shaking is required to free all the powder so that is comes in contact with water. After leaving the mixture for overnight, the solution was filtered from a filter paper for 2 times to obtain the dissolved material in solution as the filtrate. After washing with water, the un-dissolved material was dried carefully and weighed. The test was performed in triplicates.

Weight of un-dissolved material = 240 mg
Weight of material actually dissolved in water = 300 mg – 240 mg = 60 mg

Live cell Imaging: Cell imaging studies were performed with glial cells of the rat brain (C6 glioma cells). C6 glioma cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 µg/ml each of streptomycin and gentamycin. The cells were grown and maintained in a humidified environment in an incubator at 37 ^oC with 5% CO_2. One day before treatment, a total of $2x10^5$ cells were seeded on glass coverslips (11 mm) into each well of a 24-well plate, and C6 cells were grown for 24 hours (until 60-70% confluence). Treatment was carried out in FBS and antibiotics free DMEM. C6 cells were incubated with PDI **1** (10 µM) (in three replicates) at 37 ^oC with 5% CO_2 for 30 min followed by 2 times wash with 1X phosphate buffered saline (PBS) (pH = 7.4) and the addition of ClO_4^- (40 and 80 µM in triplicates) for another 30 min by incubating the cells at same conditions. The C6 cells were then washed three times with 1X PBS supplemented with 5.0**%** DMSO, fixed in ice cold 4% paraformaldehyde, washed again thrice with 1X PBS supplemented with 5.0**%** DMSO and mounted on glass slides.

To test the cytotoxicity of PDI **1**, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay with C6 glioma cell lines was performed so as to determine the effect of PDI **1** on cell proliferation.

Confocal microscopy imaging was performed on NIKON AIR machine using laser with excitation at 488 nm. Imaging was done with 60X objective lens with oil-emersion. The fluorescent signals were visible in the cytoplasmic region of the C6 cells sparing the nuclei. Brightfield imaging after treatment with PDI **1** and ClO_4^- indicated that cells were viable throughout the experiment. C6 glioma cells were observed to be permeable to both the PDI **1** and ClO_4^-ions.

(c) Details of Synthesis

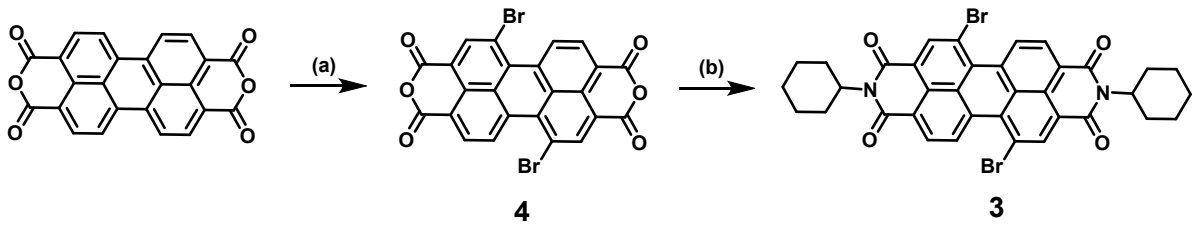
(i) Synthesis of 1,7-dibromoperylene-3,4,9,10-tetracarboxylicdianhydride (4)

Following the published procedure [1], perylenedianhydride (PTCDA) (5 g, 12.7 mmol) was taken in round bottomed flask and to this concentrated sulfuric acid was added and reaction mixture was stirred at 55^oC for 24 h. Iodine (0.12 g, 0.469 mmol) was then added to the reaction mixture and reaction mixture continuously stirred for another 5 h at 55^oC. Bromine (1.41 ml, 27.94 mmol) was slowly added dropwise in the reaction mixture with dropping funnel during time interval of 2 h. After complete addition of bromine, reaction mixture further stirred for additional 24 h at 85^oC. After this time interval, reaction mixture was cooled to room temperature and then water was added to the cooled mixture. The precipitate was collected by filtration, washed with 86% H_2SO_4 followed by water to afford 5.5 g of crude product as red solid, yield 85%, which was further used without purification.

(ii) Synthesis of N, N'-dicyclohexyl-1,7-dibromoperylene-3,4,9,10-tetracarboxylic acid diimide (3)

A mixture of **4** (500 mg, 0.909 mmol), glacial acetic acid (0.250 ml, 4.38 mmol) and cyclohexylamine (0.307 ml, 2.67 mmol) in N-methyl-2-pyrrolidone was stirred at 85^oC under N_2 atmosphere for 6 hours. After this time interval reaction mixture was poured onto ice and the resulting precipitate was collected by filtration and then dried to give 745 mg of PDI **5** which was further purified by column chromatography (SiO_2, chloroform/hexane) to isolate pure compound as red solid, yield 500 mg (0.704 mmol, 67.5%); R_f = 0.6 (chloroform/hexane 70:30);^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ1.31-1.90 (m, 16H, cyclohexyl), 2.49-2.59 (m, 4H,

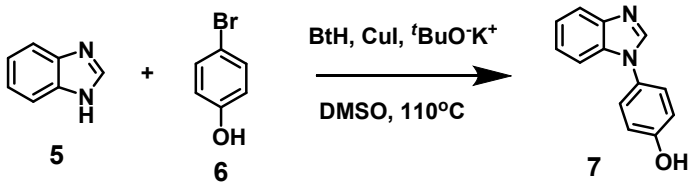
cyclohexyl), 4.99-5.05 (m, 2H, cyclohexyl), 8.66 (d, *J* = 8.2 Hz, 2H, perylene-ArH), 8.87 (s, 2H, perylene-ArH), 9.47 (d, *J* = 8.2 Hz, 2H, perylene-ArH) ppm;IR (ATR): ν = 3066.48, 2851.12, 2921.09, 1697.40, 1655.47, 1591.91, 1508.29, 1331.25, 1261.95, 1139.03, 1035.66, 741.52 cm^{-1}. We have also taken the ^1H NMR spectrum at 500 MHz and we have seen the formation of 1,6-regioisomer less than 5%.



Scheme S-1: Reagents and conditions: (a) H_2SO_4, I_2, reflux, Br_2, 24 h (b) NMP, cyclohexylamine, reflux

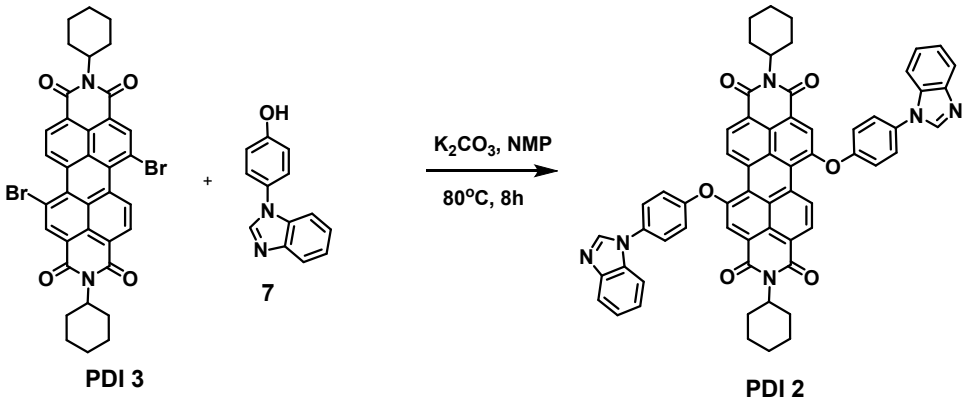
(iii) Synthesis of 4-(1H-benzo[d]imidazol-1-yl)phenol (7):

In a 100 ml two-neck RBF purged with nitrogen, CuI(0.96 gm, 10 mol%) and benzotriazole(1.21 gm, 20 mol%) was dissolved in DMSO (10 mL) and stirred at RT under N_2 atm.To this solution, 4-bromophenol (**6**) (8.79 gm, 51.11mmol), benzimidazole (**5**) (5.0 gm, 42.37 mmol) and potassium *tert*-butoxide(5.69 gm, 61.01 mmol)were subsequently added at RT under N_2atm and then resulting solution was stirred at 110^o C for 24 h. After completion of the reaction the mixture was treated with aqueous solution of EDTA and extracted with ethyl acetate. After evaporating the organic layer, the crude mixture was column chromatographed using gradient of 40% ethyl acetate:hexane to isolate pure compound **7**, as solid, 75% yield.^1H NMR ($CDCl_3$+DMSO-d_6, 300MHz): δ 7.03 (d, 2 H, *J* = 9.3Hz, ArH), 7.28-7.33 (m, 4H, ArH), 7.45-7.48 (m, 1H, ArH), 7.80-7.83 (m, 1H, ArH), 8.07 (s,1H, OH), 9.50 (s, 1H, Bim-C2H); IR (ATR): ν = 3436, 2587, 1615, 1519, 1375, 1231, 924 cm^{-1}



(iv) Synthesis of N, N'-bis(cyclohexyl)-1,7-bis(4-(1H-benzo[d]imidazol-1-yl)phenoxyperylene-3,4,9,10-tetracarboxylic acid diimide PDI 2

The mixture of 4-(1H-benzo[d]imidazol-1-yl)phenol (**7**) (37 mg, 0.18 mmol) and K_2CO_3 (36 mg, 0.26 mmol) was stirred in N-methyl-2-pyrrolidine (NMP) at room temperature. Then compound **3** (50 mg, 0.070 mmol) was added under N_2. The reaction mixture was stirred at 80 ^oC for 8 h. After cooling to RT, the reaction mixture was poured into 1 N HCl and the precipitates were filtered, washed with water and then dried under vacuum to give 50 mg of PDI which was further purified by column chromatography (SiO_2, chloroform/ethyl acetate) to isolate pure PDI **2** as red color solid, yield 33 mg (0.034 mmol, 48.5%);R_f = 0.5 (chloroform/ethyl acetate 96:4).^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ 1.74-1.89 (m, 16H, cyclohexyl), 2.47-2.58 (m, 4H, cyclohexyl), 4.97-5.05 (m, 2H, cyclohexyl), 7.35-7.38 (m, 8H, ArH), 7.56-7.62 (m, 6H, ArH), 7.89 (d, 2H, ArH), 8.15 (s, 2H, perylene-ArH), 8.41 (s, 2H, BIm), 8.66 (d, *J* = 8.4 Hz, 2H, perylene-ArH), 9.58(d, *J* = 8.4 Hz, 2H, perylene-ArH) ppm; ^{13}C NMR (75 MHz, TFA:$CDCl_3$ (1:9), 25 °C): δ 25.2, 26.3, 29.1, 29.8, 56.0, 113.2, 115.8, 117.9, 120.4, 122.9, 124.6, 125.8, 125.9, 126.1, 126.9, 128.0, 128.5, 128.8, 129.0, 129.5, 130.1, 130.6, 131.9, 132.3, 133.9, 139.3, 154.4, 157.4, 162.2, 164.8; IR (ATR): ν = 2924, 2852, 1697, 1655, 1593, 1455, 1329, 1507, 741 cm^{-1}.

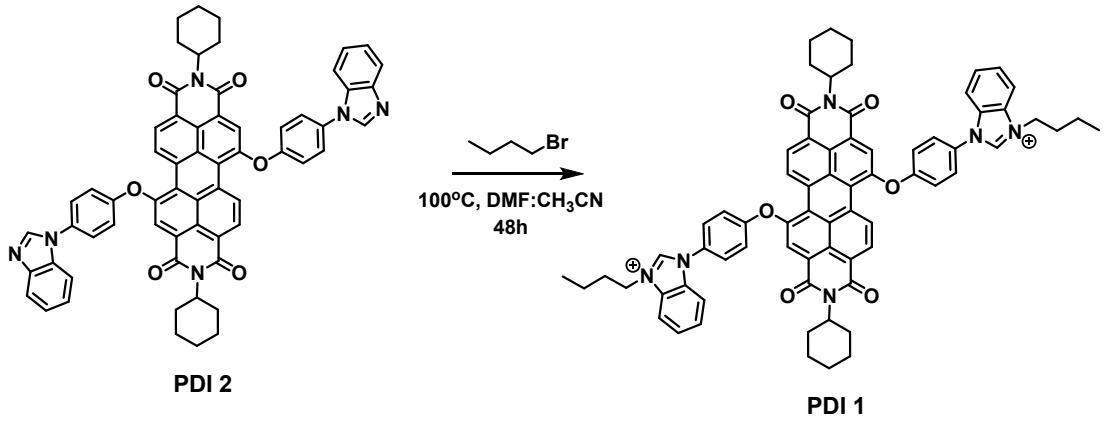


Scheme S-2: Synthetic schemes showing synthesis of PDI **2**.

(v) Synthesis of PDI 1

To a stirred solution of PDI **2** (200 mg, 0.26 mmol) in DMF:CH_3CN (2:8), was added n-butylbromide (76.7 mg, 0.55 mmol) at room temperature. The mixture was stirred for an

additional 48 h at 100 °C. After the completion of reaction (TLC), the reaction mixture was concentrated under reduced pressure. The crude product thus obtained was washed twice with diethyl ether to isolate pure PDI **1** as red color solid, yield 240 mg (0.192 mmol, 75.7%); ^{1}H NMR (400 MHz, DMSO-d_6, 25 °C): δ 1.16 (t, J = 7.2 Hz, 6H, $N^+CH_2CH_2CH_2\underline{\boldsymbol{CH_3}}$), 1.26-1.31 (m, 4H, cyclohexyl), 1.40 (quintuplet, J = 7.2 Hz, 4H, $N^+CH_2CH_2\underline{\boldsymbol{CH_2}}CH_3$), 1.69-1.71 (m, 8H, cyclohexyl), 1.82-1.85 (m, 4H, cyclohexyl), 1.97 (quintuplet, J = 7.2 Hz, 4H, $N^+CH_2\underline{\boldsymbol{CH_2}}CH_2CH_3$), 2.33-2.36 (m, 4H, cyclohexyl), 4.56 (t, J = 7.2 Hz, 6H, $N^+\underline{\boldsymbol{CH_2}}CH_2CH_2CH_3$), 4.76-4.83 (m, 4H, cyclohexyl), 7.57 (d, J = 8.4 Hz, 4H, Phenyl ArH), 7.71-7.80 (m, 4H, BImArH), 7.81 (d, J = 7.6 Hz, 2H, BImArH), 7.91 (s, 2H, perylene-ArH), 7.95 (d, J = 8.4 Hz, 4H, Phenyl ArH), 8.17 (d, J = 8.0 Hz, 2H, BImArH), 8.54 (d, J = 7.6 Hz, 2H, perylene-ArH), 9.36 (d, J = 6.8 Hz, 2H, perylene-ArH), 10.10 (s, 2H, Bim C2-H); ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ 13.7, 20.0, 25.4, 26.5, 29.1, 31.5, 54.2, 58.0, 113.7, 113.8, 120.4, 120.6, 123.3, 124.7, 125.0, 125.1, 125.4, 125.9, 127.7, 127.9, 128.2, 128.9, 129.0, 130.8, 131.4, 131.5, 132.5, 142.0, 153.1, 157.2, 162.9, 163.3 ppm; IR (ATR): ν = 2928, 2855, 1702, 1660, 1595, 1454, 1333, 842 cm^{-1}.



Scheme S-3: Synthetic schemes showing synthesis of PDI **1**.

2. Solvent Dependent Emission Spectra

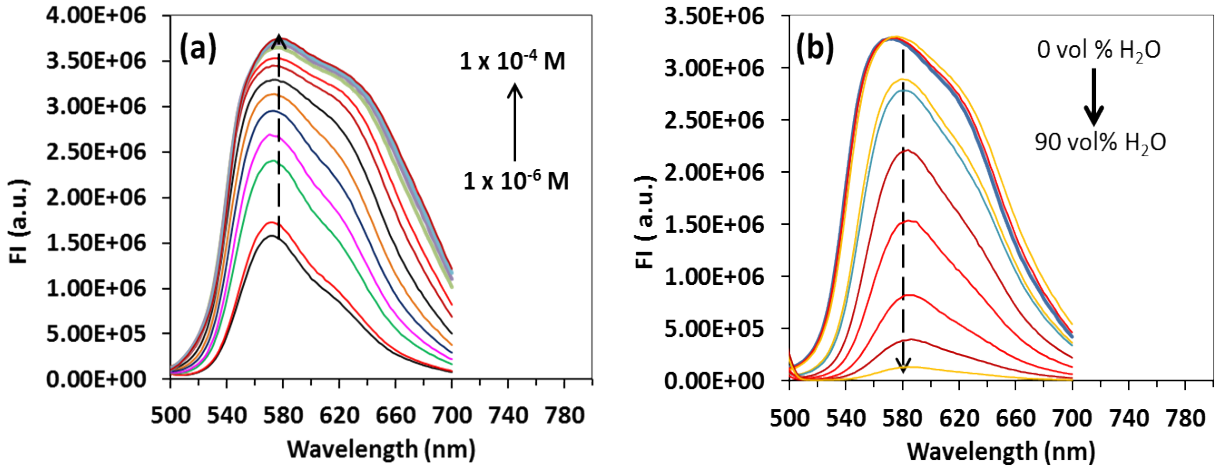


Figure S1: (a) Concentration-dependent emission spectra of PDI **1** (10^{-6} to 10^{-4} M) in pure DMSO; (b) Solvent-dependent emission spectra of PDI **1** after incremental addition of 10 vol% of H_2O in DMSO at a concentration of 10 μM.

For each solvent composition we have calculated the aggregation degree α_{agg}, aggregation constant K and Gibbs energy ΔG^o using the following equations [2].

$$\alpha_{agg} = (\varepsilon_A^{0\text{-}0})_{max} - (\varepsilon_A^{0\text{-}0}) / (\varepsilon_A^{0\text{-}0})_{max} - (\varepsilon_A^{0\text{-}0})_{min}$$

$$K = \alpha_{agg} / 2(1 - \alpha_{agg})^2 C_T$$

$$\Delta G^o = -RT\ln K$$

3. pHtitration of PDI 1

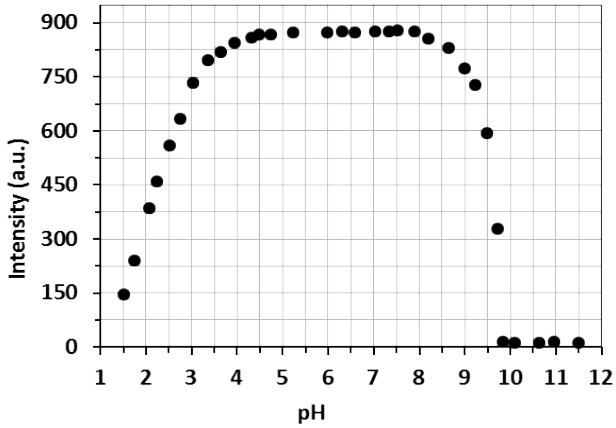


Figure S2: The plot of gradual change in fluorescence intensity of PDI **1** (10 μM) in water (10% DMSO) taken at 577 nm vs. change in pH of the solution.

4. Absorbance titration data of PDI 1 and its ClO_4^- complex

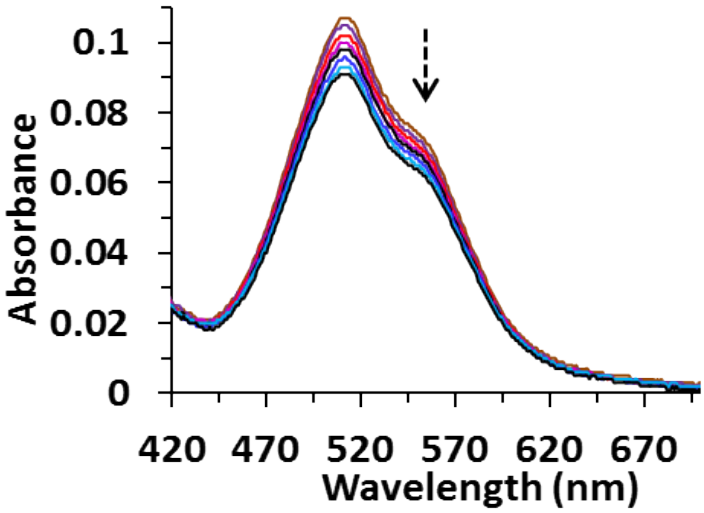


Figure S3a: Absorbance intensity changes of PDI **1** (10 μM) in the presence of ClO_4^- recorded in HEPES buffer (0.01 M, pH 7.4)- 10% DMSO (v/v).

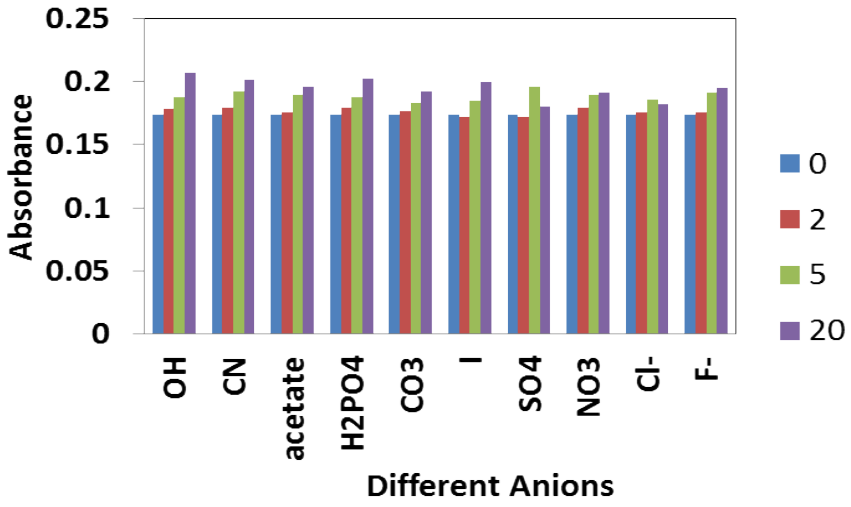


Figure S3b: Absorbance intensity changes of PDI **1** (10 μM) in the presence of different anions (except ClO_4^-) recorded in HEPES buffer (0.01 M, pH 7.4)- 10% DMSO (v/v).

5. Fluorescence titration data of PDI 1 and its ClO_4^- complex

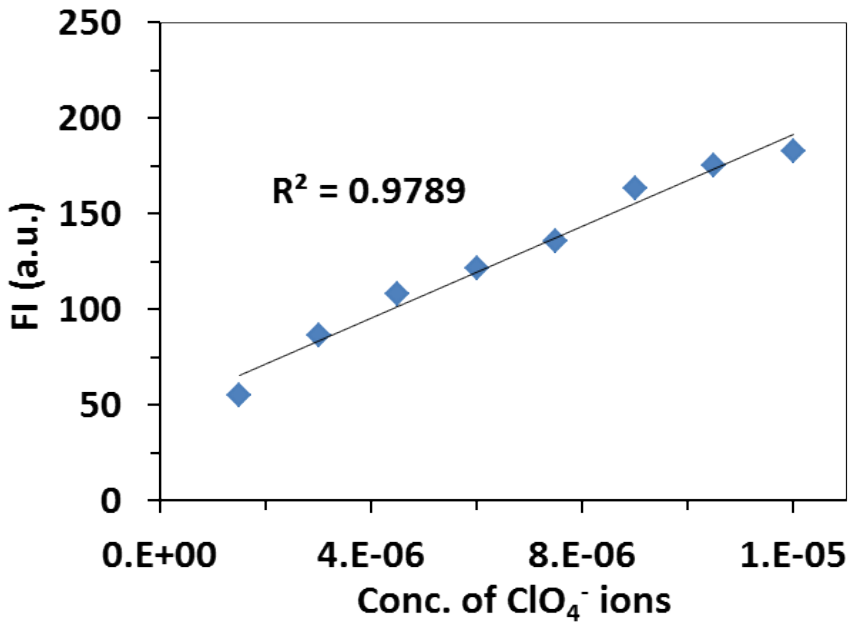


Figure S4a. The plot of PDI **1** in the presence of increasing concentrations of ClO_4^- ions to calculate the lowest limit of detection.

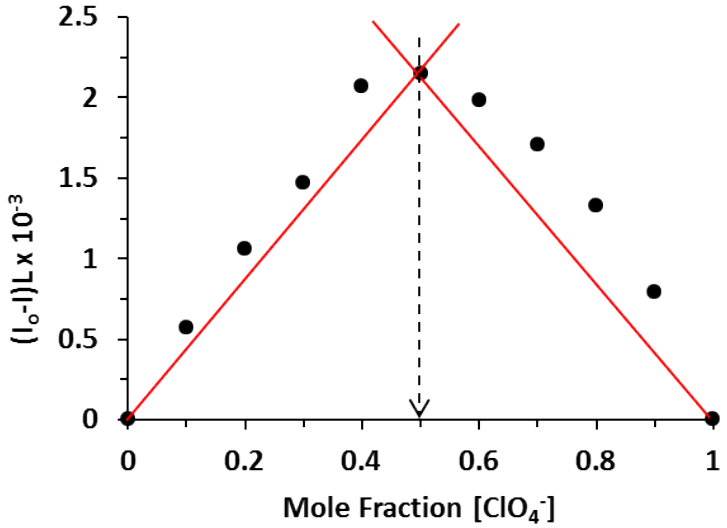


Figure S4b. Job's plot (fluorometrically) showing PDI **1**:ClO_4^- (1:1) complex recorded in pH 7.4 HEPES buffered aqueous solution containing 10% DMSO (v/v).

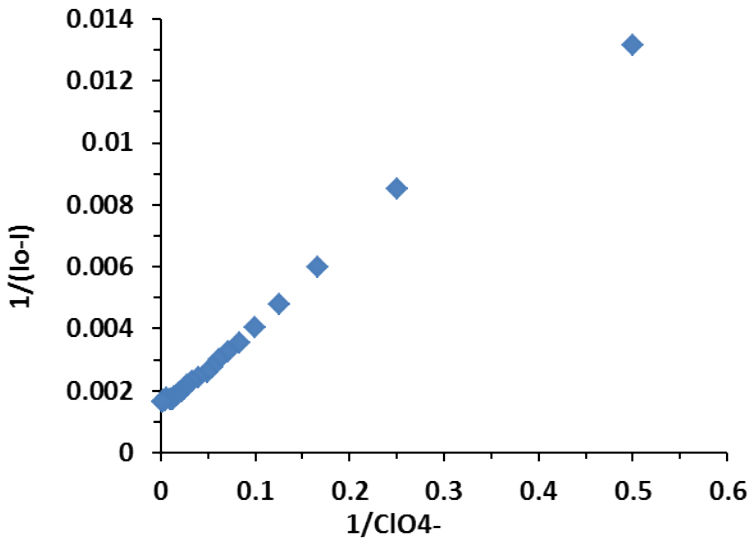


Figure S4c. Benesi-Hildebrand plot of PDI **1** in the presence of increasing concentrations of ClO_4^- ions

PDI **1** PDI **1**+ClO_4^-

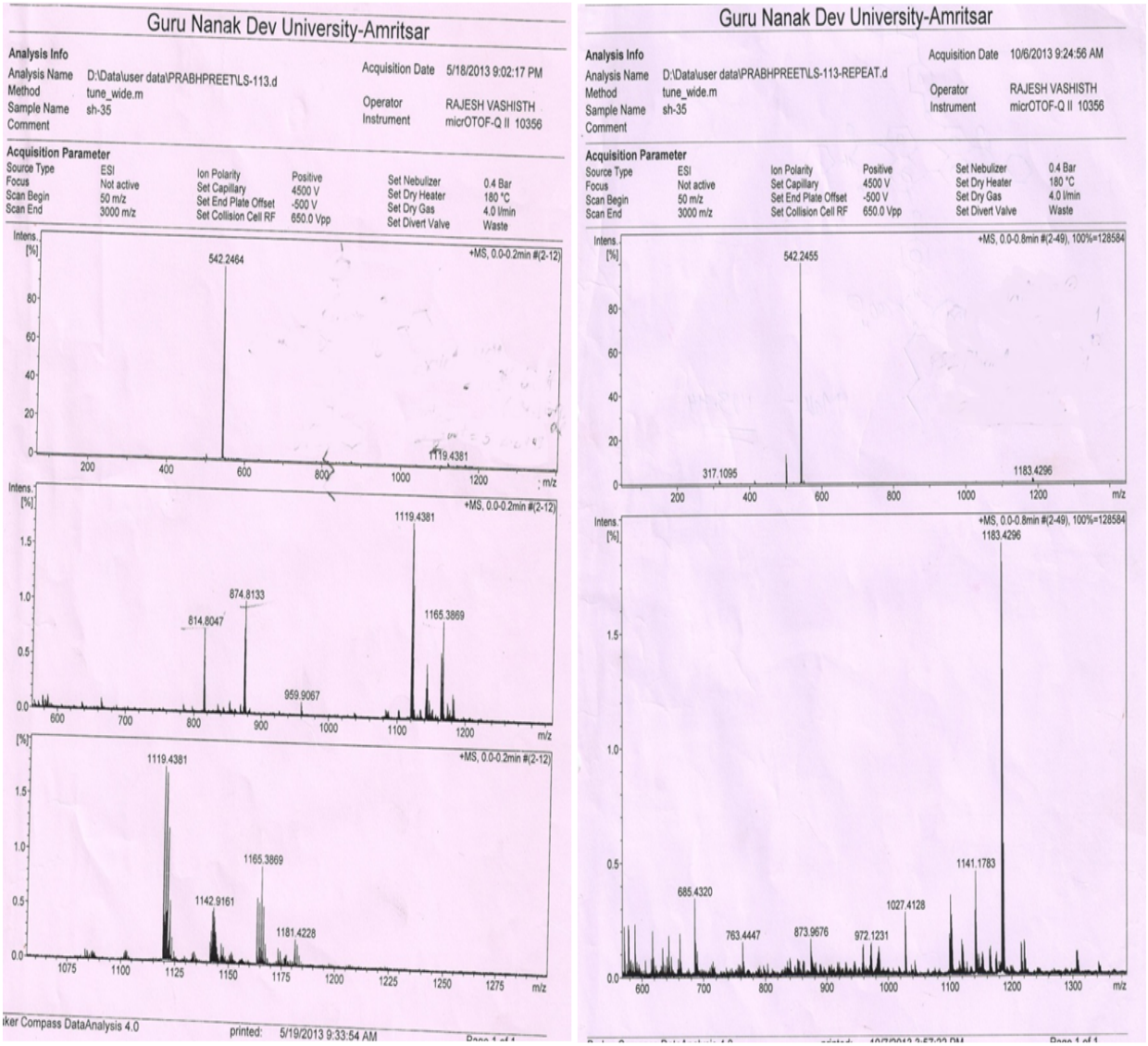


Figure S4d: HRMS analysis of PDI **1** andPDI **1**+ClO_4^- complex

6. Competition experiment of PDI 1 with interfering anions

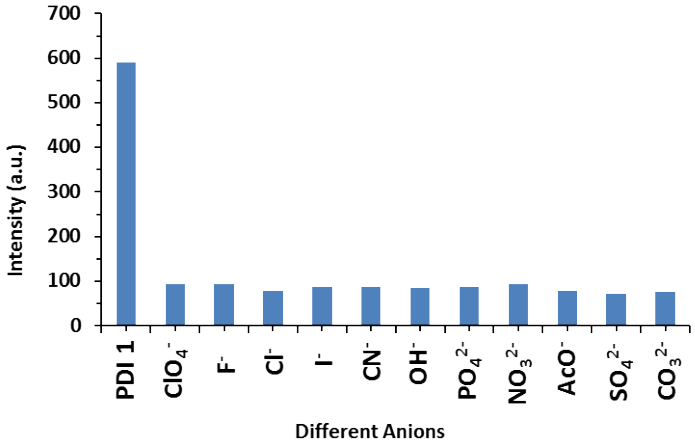


Figure S5. Effect of different anions on the fluorescence quenching of PDI **1**(10 μM) by ClO_4^- alone (100 μM) and other competitive anions (500 μM).

7. EDX spectra of PDI 1 and its ClO_4^- complex

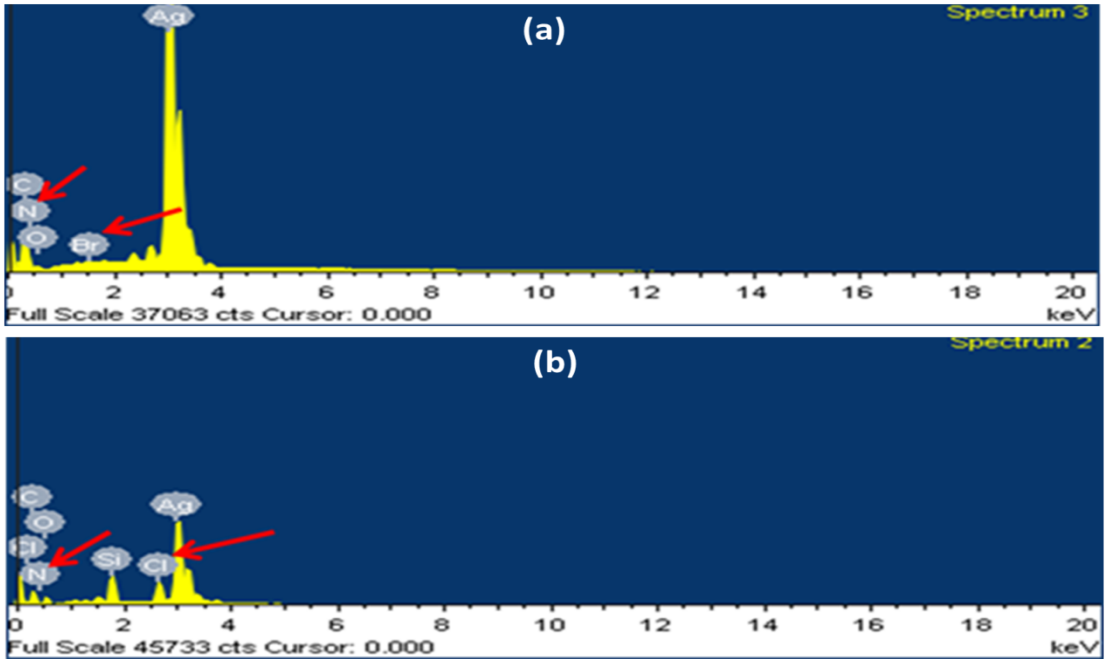


Figure S6: EDX spectrum of (a) PDI **1** alone and (b) PDI **1**with ClO_4^- ions recorded on EDX coupled SEM machine.

8. Fluorescence titration of PDI 1 in HEPES Buffer:DMSO (1:1) with ClO_4^-

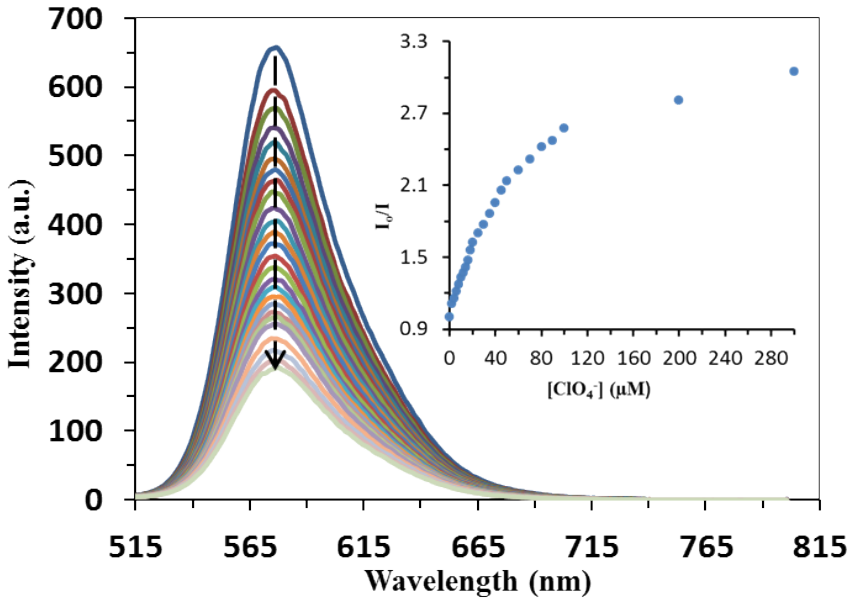


Figure S7: Fluorescence spectra of PDI **1** (10 μM) recorded in pH 7.4 HEPES buffered (0.01 M) aqueous solution containing 50% DMSO (v/v) on addition of the ClO_4^-solution; Excitation at 500 nm.

9. NMR titration Studies

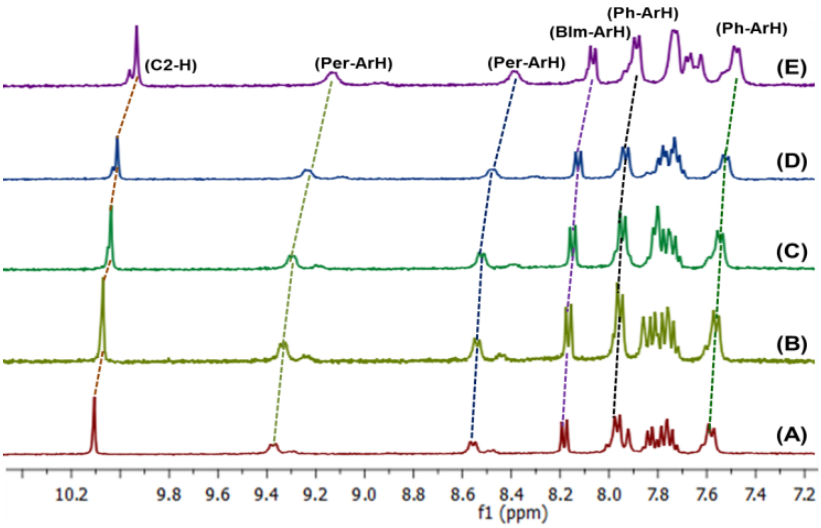


Figure S8a Partial ^{1}H NMR titration spectra (aromatic range) of PDI **1** (5 mM) with perchlorate (ClO_4^-) in DMSO-d_6 + H_2O (9:1). (A) PDI **1** alone; (B) 0.5 equivalent ClO_4^-; (C) 1.0 equivalent ClO_4^-; (D) 2.0 equivalent ClO_4^-; (E) 3.0 equivalent ClO_4^-. The dotted line follows the resonance shifts of the protons.

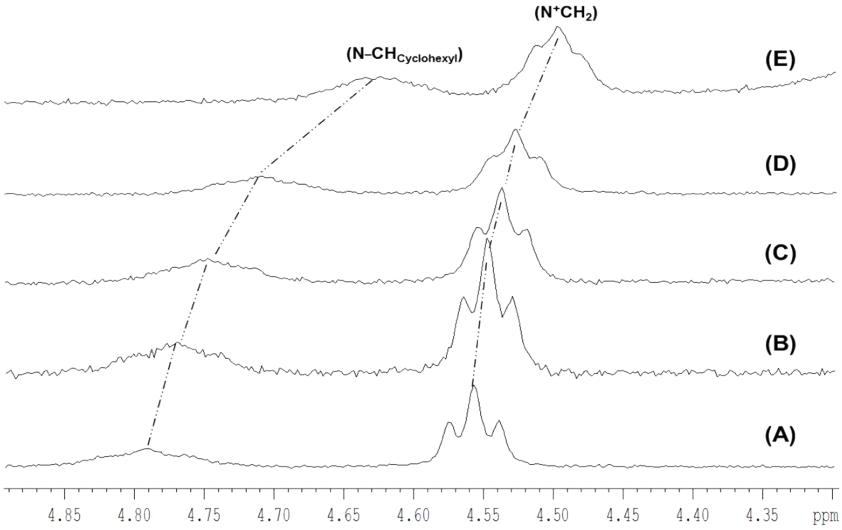


Figure S8b. Partial ^{1}H NMR titration spectra of PDI **1** (5 mM) with perchlorate (ClO_4^-) in DMSO-d_6 + H_2O (9:1). The dotted line follows the resonance shifts of the protons. (A) PDI alone; (B) 0.5 equivalent ClO_4^-; (C) 1.0 equivalent ClO_4^-; (D) 2.0 equivalent ClO_4^-; (E) 3.0 equivalent ClO_4^-.

10. Table SI-1: Application of PDI 1 in determination of ClO_4^- in tap water

Sr. No.	Conc. Added (μM)	Conc Determined ± SD[a] (μM)	Relative Error (%)
1	10	10 ± 0.2	1.4
2	20	20 ± 0.7	2.7
3	30	30 ± 0.6	1.4
4	40	40 ± 0.7	1.4

[a]Average ± standard deviation of three determinations

11. Detection of perchlorate in fireworks

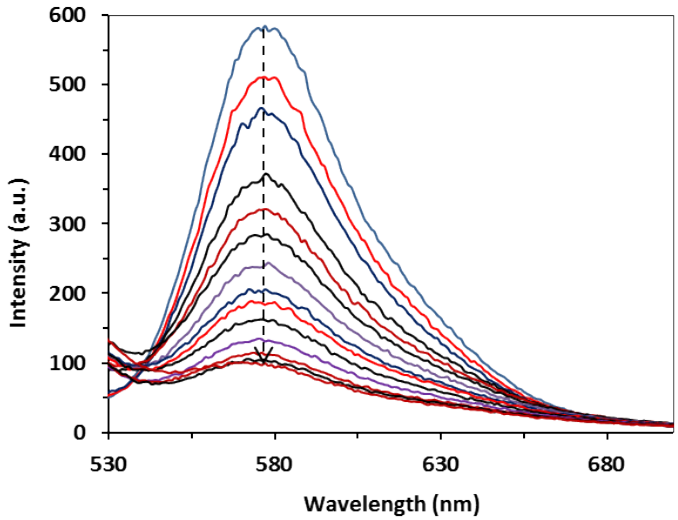


Figure S9: Fluorescence spectra of PDI **1** (10 μM) recorded in pH 7.4 HEPES buffered (0.01 M) aqueous solution containing 10% DMSO (v/v) on addition of the solution obtained after dissolution of powder from firework; Excitation at 500 nm.

We established that the concentration of the perchlorate ion in sample solution is 3.7 x 10^{-4} M from our standard calibration curve however exact quantification of ClO_4^- could not be established due to presence of other organic and inorganic constituents in flash powder which remained in sample matrix in dissolved form and are not removed by filtration.

12. MTT Assay

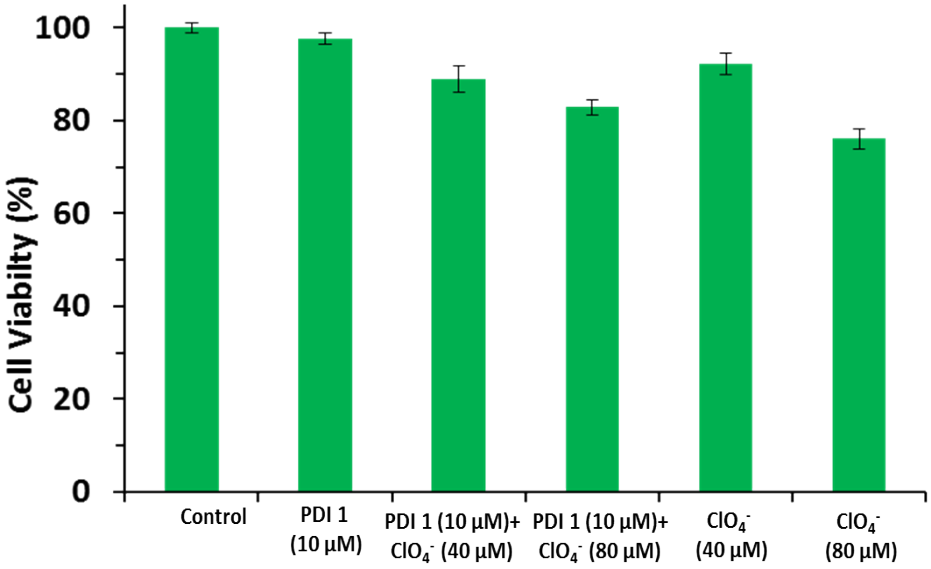


Figure S10. Cell viability values (%) tested by an MTT assay using C6 glioma cells.

References

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