Electronic Supporting Information (ESI)

Perylenemonoimide-based fluorescent probe: Ultrasensitive and selective tracing of endogenous peroxynitrite in living cells

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Experimental Section

Materials and Methods:

All solvents and reagents were purchased from commercial sources and used as received. Potassium acetate (KOAc), 1-bromobutane, and Bis(pinacolato)diboron were purchased from Alfa Aesar, Potassium Carbonate (K_2CO_3) was purchased from Otto, Bromine (Br_2) was purchased from Merck, Glacial acetic acid was obtained from SDFCL. [1,1'-Bis(diphenylphosphino)ferrocene]dichloropalladium(II), Perylene-3,4,9,10-tetracarboxylic dianhydride, 2,6-diisopropylaniline, *tert*-butyl alcohol, *tetra*-butylammonium hydroxide, Zinc Acetate and *N,N'*-dimethyl-1,3-propanediamine were purchased from Sigma. Imidazole, dichloromethane (DCM), methanol (MeOH), *N,N*-dimethylformamide (DMF) and toluene were purchased from SRL. Potassium hydroxide (KOH) was taken from CDH, 1-butanol and *p*-toluene sulphonic acid were taken from Spectrochem. Iodoethane was obtained from TCI. The water used was of Milli-Q grade. Thin layer chromatography (TLC) analysis was carried out using Merck TLC Silica gel 60 F_{254} and column chromatography was performed on silica gel (230-400 mesh size), purchased from Rankem.

Sample preparation

The stock solutions of 1 mM concentration for spectroscopy experiments of dye were prepared in fresh distilled tetrahydrofuran (THF) solvent. The concentration of dyes was kept 2 μM for all the measurements.

Preparation of reactive oxygen species (ROS) and reactive nitrogen species (RNS)

Hydrogen peroxide (H_2O_2), *tert*-butylhydroperoxide (TBHP), and sodium hypochlorite (NaOCl) were diluted from commercially available solutions. ROO· was generated from 2,2'-azobis (2-amidinopropane) (AAPH). Superoxide (O_2^-) was prepared from KO_2 in DMSO. Peroxynitrite solution was generated by following the reported procedure.[1] The stock solution

were prepared in Milli-Q water and the concentration was determined using molar extinction co-efficient of 1670 $cm^{-1}M^{-1}$ and absorbance at 302 nm obtained from UV- spectrophotometer.

Steady-state absorbance and fluorescence measurements

All the steady-state absorption measurements were carried out using Shimadzu 1800 UV-spectrophotometer using a quartz cuvette with a 1 cm path length. To avoid the inner filter effect, all measurements were taken with a dilute solution of the sample. The HORIBA Jobin Yvon Fluorimeter was used for all steady-state fluorescence measurements. The excitation and emission slits on a 1 cm path length quartz cuvette were used to record fluorescence spectra, and the integration time was varied accordingly. All the experiments were performed at ambient temperature (298 K) unless otherwise mentioned.

Nuclear Magnetic Resonance and Mass Spectroscopy

^{1}H and $^{13}C\{^{1}H\}$ NMR spectra were recorded on a Bruker spectrometer in $CDCl_3$ as a solvent. The chemical shifts were reported in ppm scale with respect to the residual solvent signal at □ = 7.26 ppm in $CDCl_3$ for ^{1}H NMR at 500 MHz and 400 MHz. The mass spectra for all the compounds were recorded using APCI and ESI with the solution of dyes in chloroform solvent.

Calculation of limit of detection (LOD)

The limit of detection was determined based on fluorescence titration. To get the slope, the decrease in fluorescence intensity at 578 nm was plotted with increasing concentration of $ONOO^-$. So, the detection limit (LOD) was calculated by using following equation

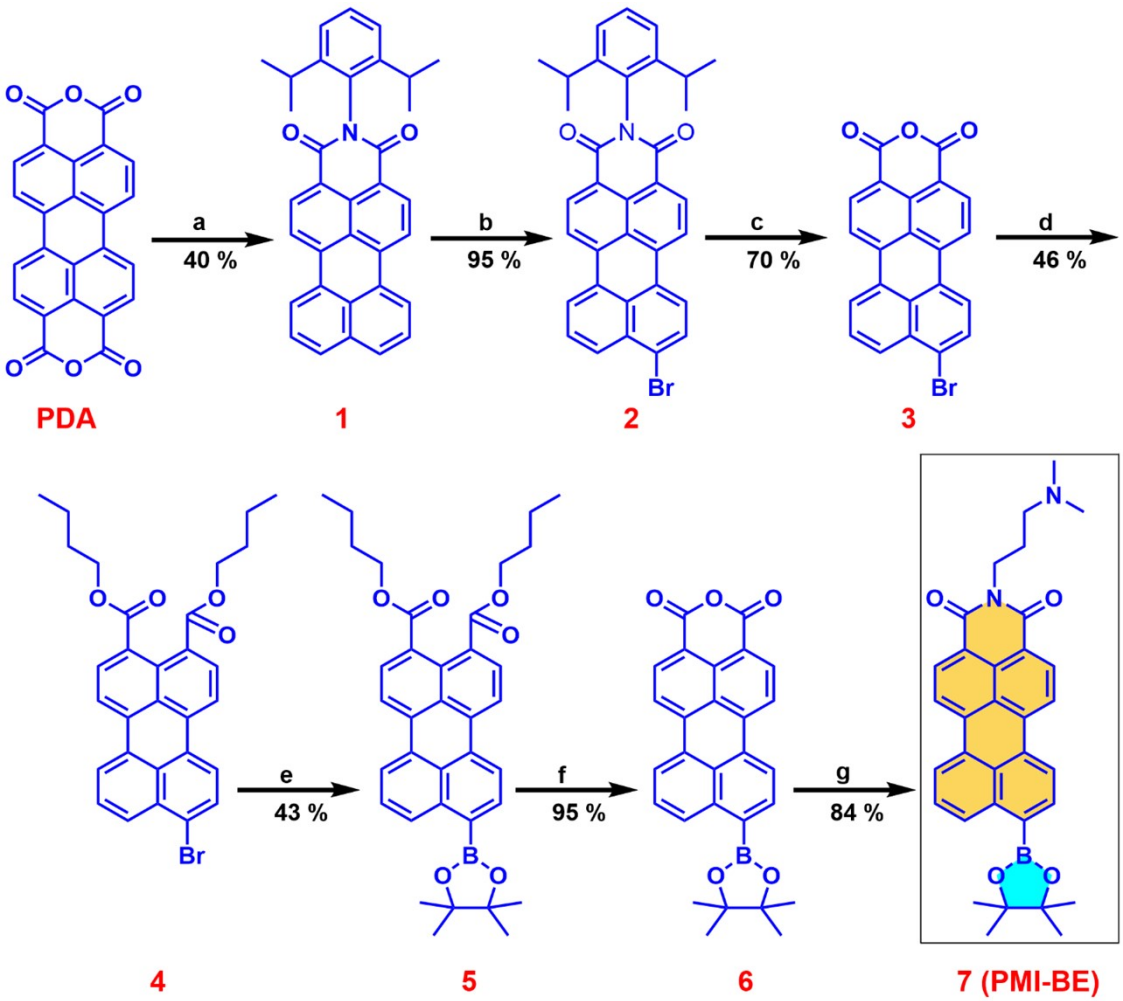
$$LOD = 3\sigma / k$$

Where, σ = Standard deviation of response

k = Slope of calibration curve

Cell culture

Dulbecco's Modified Eagle Medium (DMEM), Trypsin, Antibiotic cocktail, and Fetal Bovine Serum (FBS) were purchased from HiMedia (USA). Lyso-Tracker Red, ER Tracker Red, and MitoTracker Red were purchased from Thermo Fisher Scientific (USA). The 35 mm glass bottom imaging dishes were obtained from Ibidi (Germany). All the confocal microscopy imaging was performed with an Olympus FV3000 Confocal Laser Scanning Microscope (LSM). HeLa cells and RAW 264.7 cells were obtained from NCCS, Pune, India, and were grown in a 25 cm^2 cell culture flask (Corning, USA) using DMEM containing 10% (v/v) FBS and 1% (v/v) antibiotic cocktail in 5% CO_2 at 37 °C in a CO_2 incubator. For imaging purposes, cells were grown to 75% - 80% confluency in the 35 mm glass bottom imaging dishes (170 ± 5 μm) in DMEM with 10% FBS. For the colocalization experiment, the cells were co-incubated with indicated concentrations of **PMI-BE** and commercially available trackers for 10 minutes and washed with PBS (pH 7.4) twice before imaging. For the generation of cellular endogenous peroxynitrite, RAW 264.7 cells were incubated with 1 μg/mL lipopolysaccharide (LPS) and 50 ng/mL interferon-γ (IFN-γ) for 10 h at 37 °C and then stained with 1 μM **PMI-BE** for 10 min and imaged under the microscope.



(a) 2,6-diisopropylaniline, $Zn(OAc)_2.2H_2O$, Imidazole, H_2O 190 °C 18 h; (b) Br_2, DCM, reflux, 3 h; (c) KOH, ^tBuOH, 100 °C, 3 h; (d) 1-Butanol, 1-Bromobutane, *tetra*-butylammonium hydroxide, K_2CO_3, 120 °C, 3 h; (e) Bis(pinacolato)diboron, KOAc, [1,1'-Bis(diphenylphosphino)ferrocene]dichloropalladium(II), complex with dichloromethane, toluene, reflux, overnight; (f) *p*-Toluenesulfonicacid, toluene, 120 °C, 3 h; (g) N,N'-Dimethyl-1,3-propanediamine, DMF, 80 °C, 2.15 h

Scheme S1: Synthetic route for synthesis of the designed probe **PMI-BE**.

Synthesis of compound 6:

Compound **5** (50 mg, 0.0864 mmol) was dissolved in toluene (2.5 mL) and then *p*-toluenesulfonic acid (49.3 mg, 0.2592 mmol) added to it. The reaction mixture was kept in an oil bath at 120 °C for 3 h. After completion of reaction time, reaction mixture was allowed to cool at room temperature and then toluene was evaporated using rotatory evaporator. The precipitation was done using methanol and filtered out as desired brown-colored solid product (38 mg) in 95 % yield.

Synthesis of compound 7 (PMI-BE):

First, compound **6** (30 mg, 0.0669 mmol) was dissolved in dry DMF solvent (2 mL) and then *N,N*-Dimethyl-1,3-propanediamine (25 μL, 0.2007 mmol) added to it. The reaction mixture was kept in an oil bath at 80 ^{o}C for 2.15 h. After completion, reaction mixture was allowed to cool at room temperature and then water (4 mL) was added to get the precipitate The obtained precipitate was filtered out and dried under high vacuum as desired dark red-colored solid product (30 mg) in 84 % yield.

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.86 (d, J = 8.4 Hz, 1H), 8.55 (d, J = 8.0 Hz, 2H), 8.44 – 8.34 (m, 4H), 8.17 (d, J = 7.5 Hz, 1H), 7.65 (t, J = 8.0 Hz, 1H), 4.25 (t, 2H), 2.46 (t, J = 7.3 Hz, 2H), 2.27 (s, 6H), 1.99 – 1.90 (m, J = 14.8, 7.4 Hz, 2H), 1.47 (s, 12H).

^{13}C {^{1}H} NMR (400 MHz, $CDCl_3$): δ 163.92, 137.95, 137.50, 136.99, 136.21, 131.71, 131.49, 131.40, 131.32, 130.89, 129.59, 128.86, 127.56, 127.20, 126.99, 126.55, 123.68, 123.55, 122.58, 121.06, 120.68, 120.51, 120.16, 120.09, 96.13, 84.24, 57.08, 44.98, 38.54, 25.68, 25.02.

HRMS (APCI): calculated m/z of $[M+H]^+$ = 533.2612, obtained m/z = 533.2608

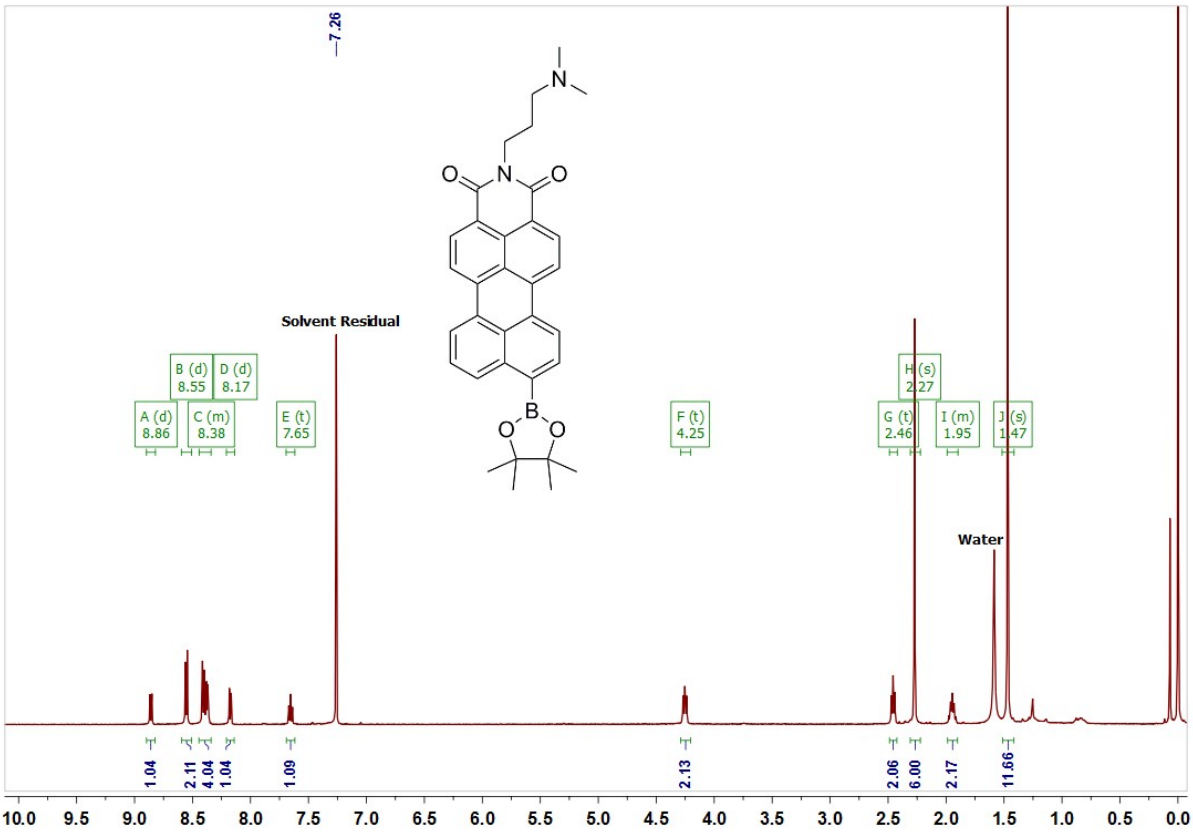


Fig. S1: ^{1}H NMR spectrum of **7 (PMI-BE)** recorded in $CDCl_3$ at 500 MHz

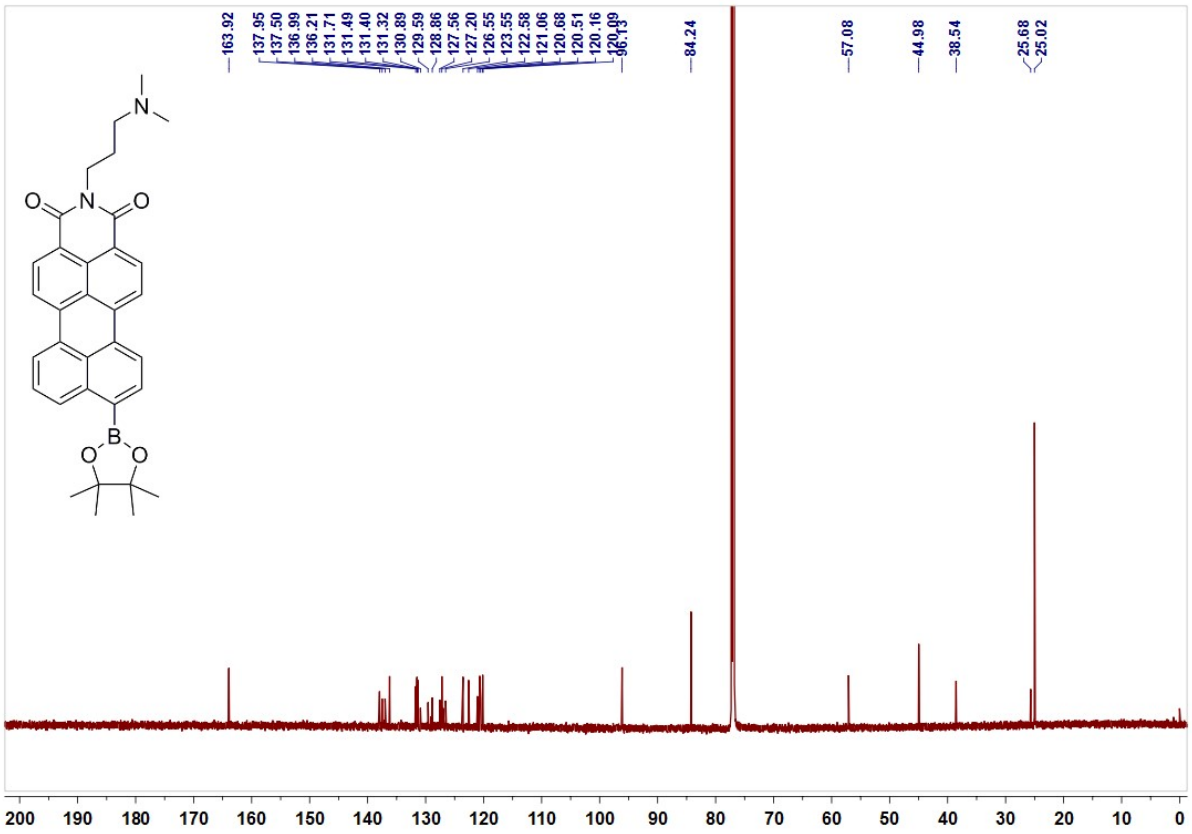


Fig. S2: ^{13}C NMR spectrum of **7 (PMI-BE)** recorded in $CDCl_3$ at 125 MHz

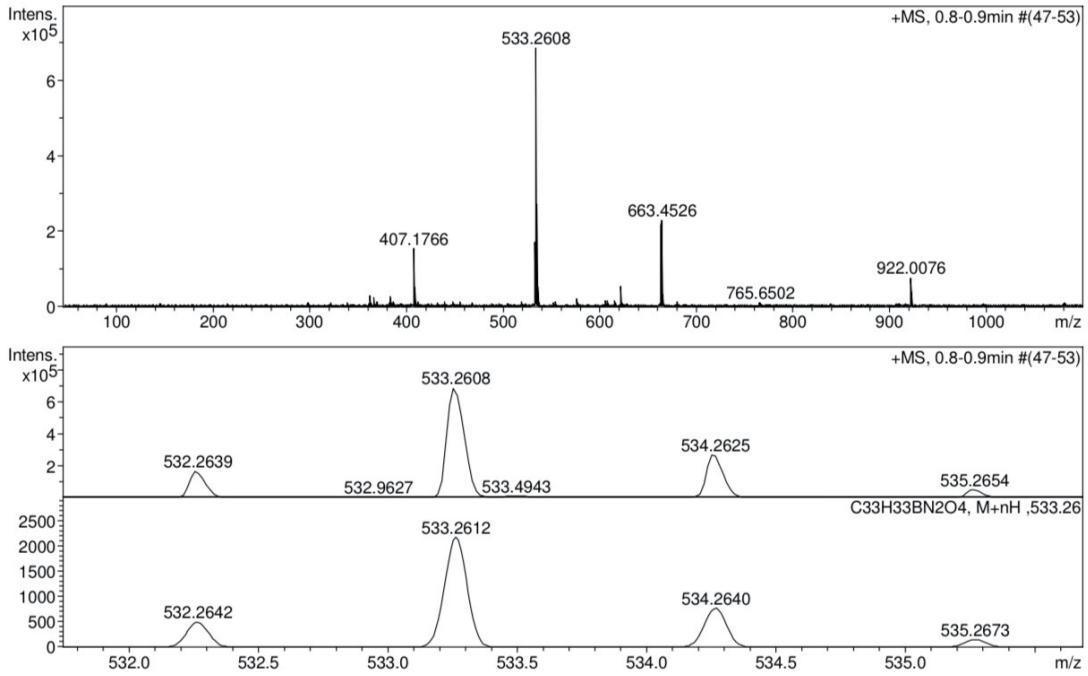


Fig. S3: APCI mass spectrum for compound **7 (PMI-BE)**, Calculated m/z of $[M+H]^+$ = 533.2612 and Obtained m/z = 533.2608

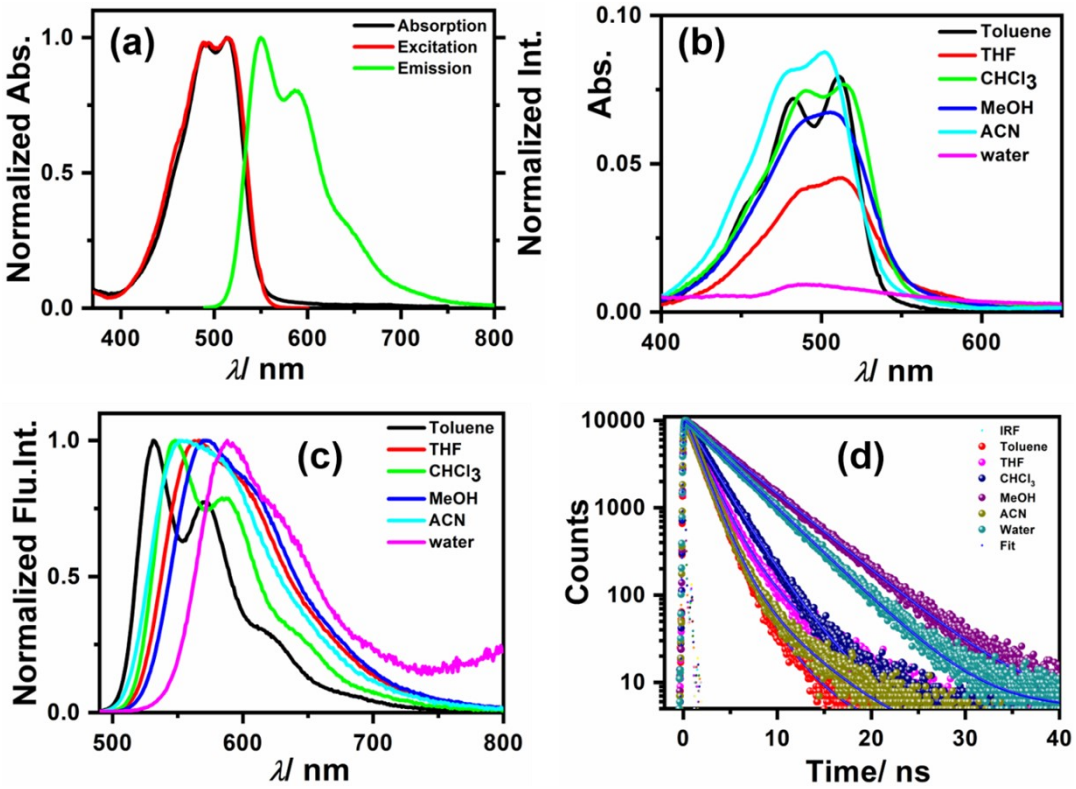


Fig. S4: (a) Optical absorption, emission, and excitation of **PMI-BE** (2 μM) in chloroform, solvent-dependent (b) UV-Vis. absorbance spectra (c) emission spectra, and (d) fluorescence lifetime decay.

Table S1: Summary of photophysical properties of **PMI-BE**

Solvents	$\lambda_{abs.}$/ **nm**	ε/ **M^{-1}cm^{-1}**	$\lambda_{em.}$/ **nm**	**Stokes Shift/ nm**	τ_f/ **ns**
Toluene	511	39,720	531	20	1.8
THF	513	22,630	565	52	2.8
$CHCl_3$	514	38,390	549	35	2.6
MeOH	508	33,575	572	64	5.0
ACN	502	43,855	554	52	2.2
Water	490	4,625	588	98	4.2

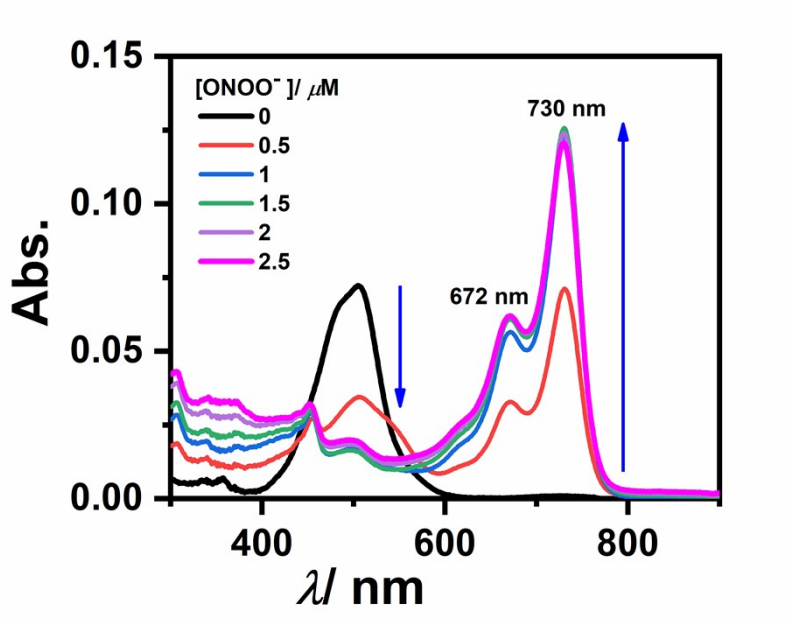


Fig. S5: Response of 2 μM **PMI-BE** towards peroxynitrite in acetonitrile (ACN) solvent

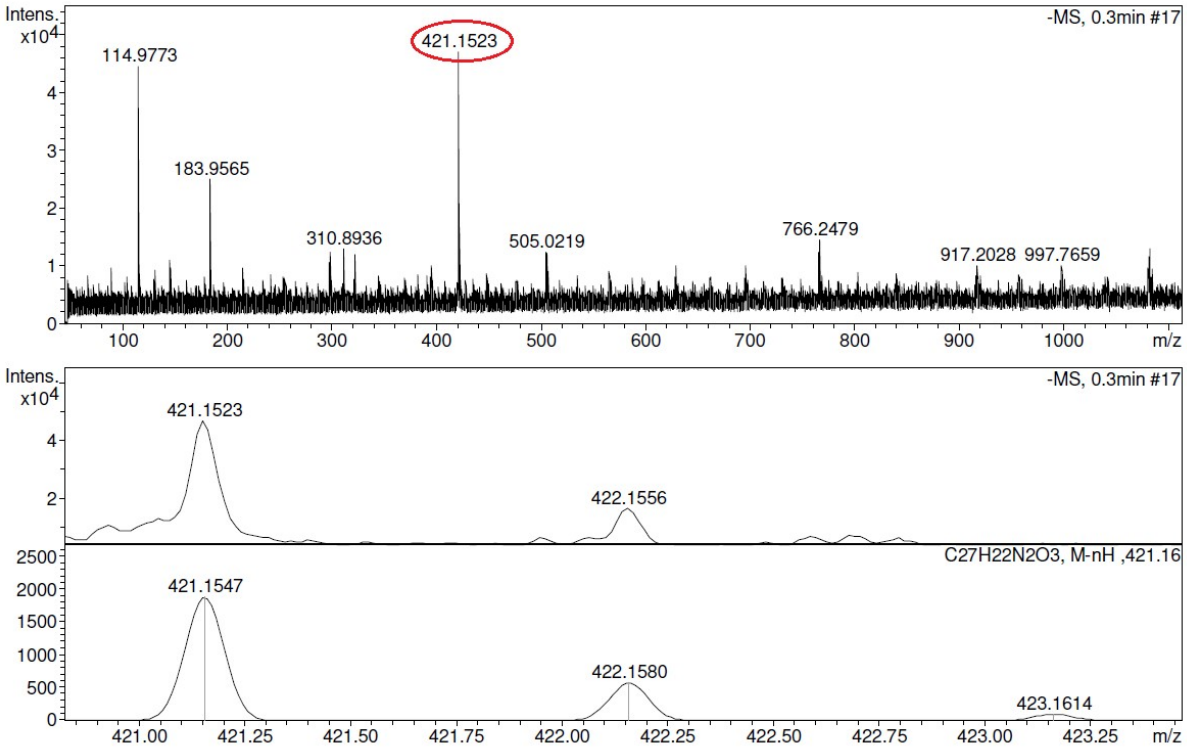
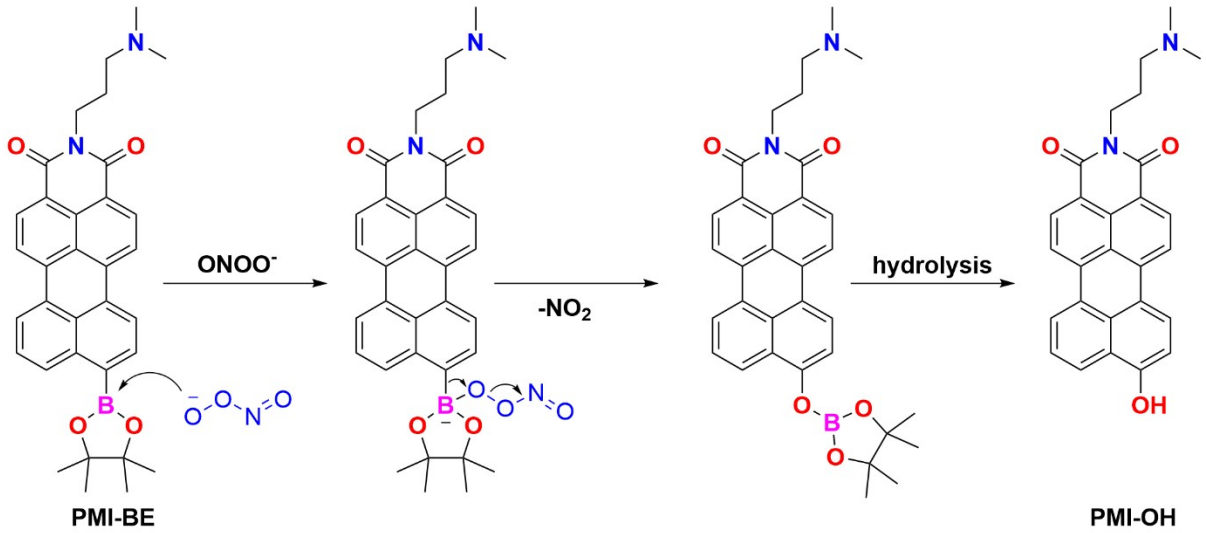


Fig. S6: ESI mass spectrum for reaction product between **PMI-BE** and $ONOO^-$ Calculated m/z of $[M-H]^+$ = 421.1547 and Obtained m/z = 421.1523



Scheme S2: Plausible mechanism of peroxynitrite detection

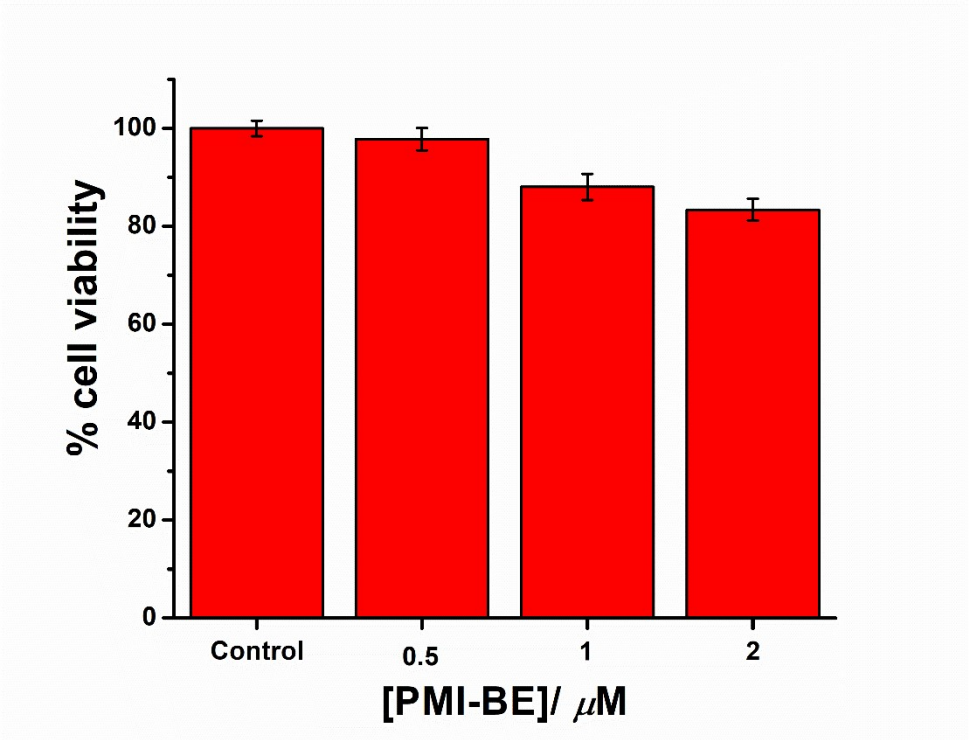
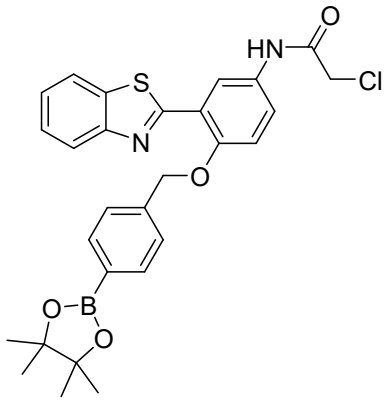
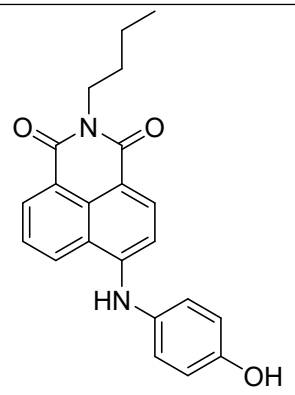
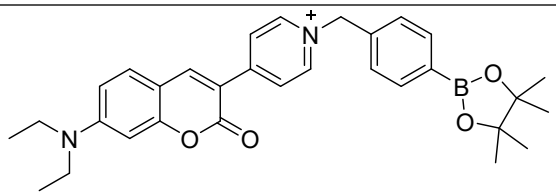
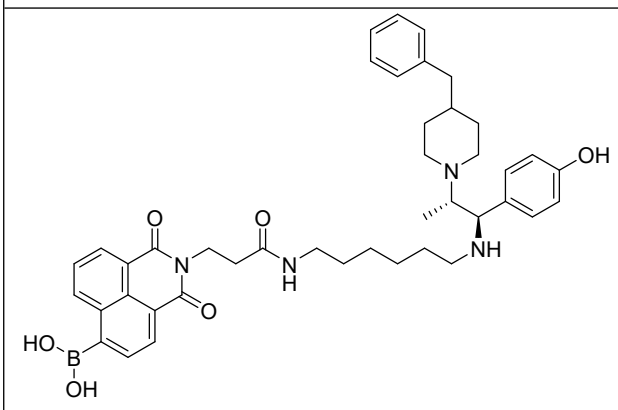
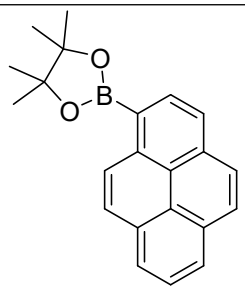
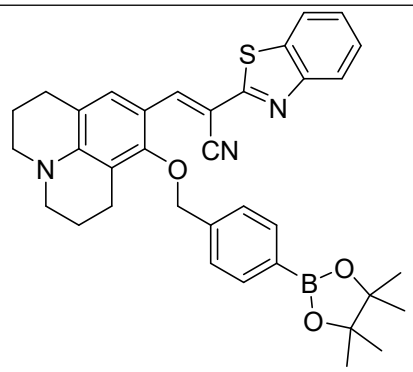
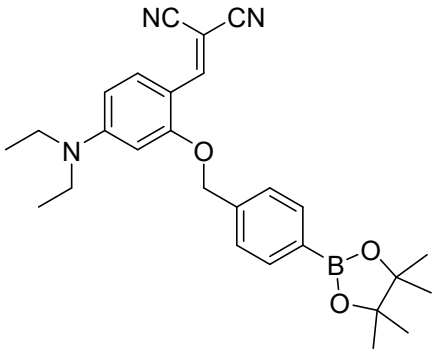
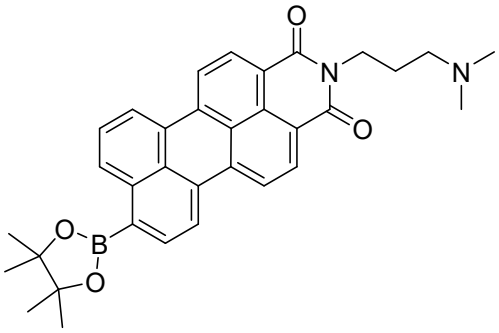


Fig. S7: MTT assay of **PMI-BE** in HeLa cell incubated with representative concentration for 24 h

Table S2: Table of comparison between reported $ONOO^-$ probes and **PMI-BE**

Probe	λ_{em}	Detection limit	Imaging	References
	405/ 481 nm	21.4 nM	Living cells	*Chem. Commun*., 2018, 54, 9953-9956
	548 nm	49.7 nM	Living cells and *C. elegans*	*RSC Adv.*, 2020, 10, 38281-38286
	500/ 565 nm	47 nM	Living cells	*Anal. Methods*, 2019, 11, 5699-5703
	450/ 550 nm	184 nM	Living cells and tissues	*Anal. Chem*. 2018, 90, 9347−9352

	347 nm/ 396 nm, 410 nm, 430 nm	100 nM	Living cells	*Analyst*, 2012, 137, 3740–3749
	540 nm	2.5 μM	Living cells	*Chem. Commun*., 2014, 50, 9353-9356
	480 nm/ 580 nm	35 nM	Living cells	*RSC Adv*., 2014, 4, 51589-51592
	507/ 743 nm and 578 nm	0.26 nM	Living cells	This work

References:

1. J. W. Reed, H. H. Ho and W. L. Jolly, *J. Am. Chem. Soc.*, 1974, **96**, 1248-1249.