Electronic Supplementary Information

A highly selective fluorescent probe for hypochlorite and its endogenous imaging in living cells

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Instruments and experimental procedures

General information

All reagents were purchased from Aldrich and were used without further purification. HPLC grade Acetonitrile was used in UV-vis and fluorescence studies. UV-vis spectra were recorded on a SHIMADZU UV-2450 spectrophotometer, with a quartz cuvette (path length 1 cm). The cell holder was thermostatted at 25 °C. The fluorescence spectra were recorded with a SHIMADZU 5301 PC spectrofluorimeter. Elemental analysis was done using a Flash EA 1112 CHNS/O analyzer from Thermo Electron Corporation. ^{1}H spectra were recorded on a JEOL-FT NMR-AL 300 MHz and Bruker Avance 3D 500 MHz spectrophotometer using $CDCl_3$ as solvent and tetramethylsilane as the internal standard. Data are reported as follows: chemical shift in ppm (*d*), multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet, br = broad singlet), coupling constants *J* (Hz), integration and interpretation. Fluorescence quantum yields[1a] were determined by using optically matching solution of perlyene orange (Φ_{fr} = 0.99 in chloroform)[1b] as standard at an excitation wavelength of 450 nm and quantum yield is calculated using the equation:

$$\Phi_{fs} = \Phi_{fr} \times \frac{1-10^{-ArLr}}{1-10^{-AsLs}} \times \frac{N_s^2}{N_r^2} \times \frac{D_s}{D_r}$$

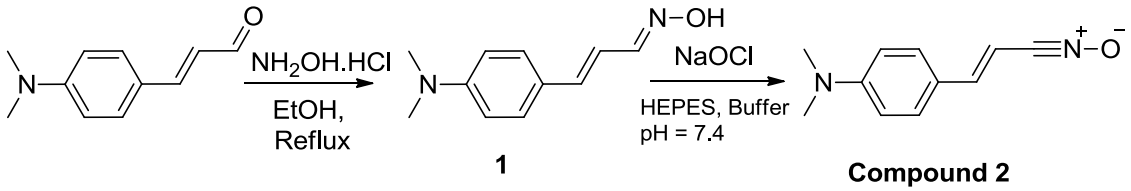
Φ_{fs} and Φ_{fr} are the radiative quantum yields of sample and the reference respectively, A_s and A_r are the absorbance of the sample and the reference respectively, D_s and D_s the respective areas of emission for sample and reference. L_s and L_r are the lengths of the absorption cells of sample and reference respectively. N_s and N_r are the refractive indices of the sample and reference solutions (pure solvents were assumed respectively).

[1a] Deams, J. N.; Grosby, G. A. *J. Phys. Chem.*, 1971, **75**, 991; [1b] W. E. Ford, P. V. Kamat, *J. Phys. Chem.*, 1987, **91**, 6373.

Procedure for sensing

UV-vis and fluorescence titrations were performed on 5.0 and 1.0 μM solution of ligand in H_2O/CH_3CN (99.5:0.5, v/v; buffered with HEPES, pH = 7.4; at 25 °C) mixture. Typically, aliquots of freshly prepared $M(ClO_4)_n$ (M = Hg^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+}, Cd^{2+}, Pb^{2+}, Fe^{3+}, Fe^{2+}, Ag^{+}, Mg^{2+} and Ca^{2+}; n = 1 or 2 or 3) and anions ($H_2PO_4^-$, CN^-, F^-, Br^-, I^-, AcO^-, SCN^- and SO_4^{2-} as tetrabutylammonium salt) standard solutions (10^{-1} M to 10^{-3} M) were added to record the UV-vis and fluorescence spectra. Hydrogen peroxide (H_2O_2), *tert*-butyl hydroperoxide (TBHP), and hypochlorite (OCl^-) were delivered from 30%, 70%, and 5% aqueous solutions, respectively. Hydroxyl radical (•OH) and tert-butoxy radical (•OtBu) were generated by reaction of 1 mM Fe^{2+} with 100 μM H_2O_2 or 100 μM TBHP, respectively. In titration experiments, each time a 3 ml solution of ligand was filled in a quartz cuvette (path length, 1 cm) and spectra were recorded after the addition of appropriate analyte.

Synthetic routes and characteristic data



Synthesis of probe 1:

A mixture of compound *N*,*N*-dimethylaminocinnamaldehyde (50 mg, 0.2857 mmol) and hydroxylamine hydrochloride (30 mg, 0.4285 mmol) in absolute ethanol (10 ml) was refluxed for 24 hrs. The solvent was dried in vacuo and the crude product was subjected to column chromatography to afford the desired product as a yellow solid (78% yield); ^{1}HNMR ($CDCl_3$, 500 MHz): d = 3.01 (s, 6 H, $N(CH_3)_2$), 6.70 (d, *J* = 10 Hz, 2 H, Ar-H), 7.22 (t, *J* = 10 Hz, 1 H, CH), 7.37 (d, *J* = 10 Hz, 2 H, CH), 7.43 (d, *J* = 5 Hz, 1 H, Ar-H), 7.92 (d, *J* = 10 Hz, 1 H, Ar-H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 40.12, 111.95, 116.88, 128.40, 128.98, 139.16, 140.84, 152.63 ppm. ESI-MS: m/z = 191.1138 $[M + H]^+$; Anal. Calcd for $C_{11}H_{14}N_2O$: C, 68.96; H, 7.09; N, 13.96; Found: C, 69.11; H, 6.87; N, 14.33.

Synthesis of compound 2:

A mixture of compound **1** and NaOCl in ACN/H_2O buffered with HEPES, 50 mM; was stirred for 1 hr. After the completion of the reaction the product was purified by column chromatography give compound **2** as brown solid in 68% yield; ^{1}HNMR (DMSO-d_6, 300 MHz): d = 2.91 (s, 6 H, N(CH_3)$_2$), 6.57 (d, *J* = 9 Hz, 1 H, CH), 7.06 (d, *J* = 6 Hz, 2 H, Ar-H), 7.26 (d, *J* = 9 Hz, 2 H, Ar-H), 7.76 (d, *J* = 9 Hz, 1 H, CH). ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 99.90, 111.63, 117.83, 123.46, 128.10, 138.11, 150.47 ppm. ESI-MS: m/z = 189.1030 $[M + H]^+$.

Fluorescence spectra of probe 1 in the presence of different metal ions and anions

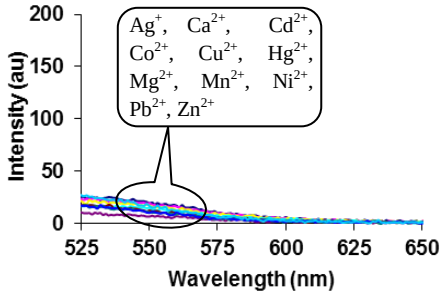


Fig. S7A: Fluorescence spectra of probe **1** (1.0 μM) in H_2O/CH_3CN (99.5: 0.5, v/v) buffered with HEPES, 50 mM, pH = 7.4 in presence of different metal ions (25 μM), λ_{ex} = 450 nm.

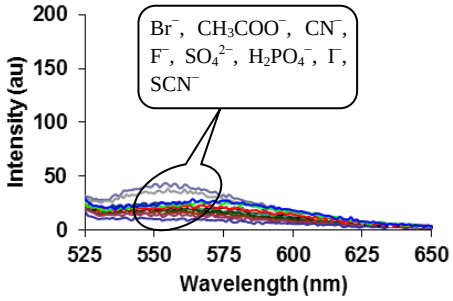


Fig. S7B: Fluorescence spectra of probe **1** (1.0 μM) in H_2O/CH_3CN (99.5: 0.5, v/v) buffered with HEPES, 50 mM, pH = 7.4 in presence of different anions (25 μM), λ_{ex} = 450 nm.

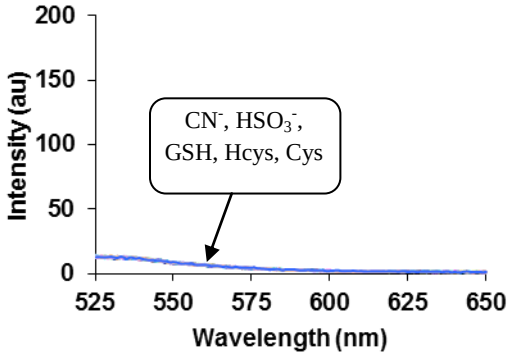


Fig. S7C: Fluorescence spectra of probe **1** (1.0 μM) on addition of CN^- (25 μM), HSO_3^-(25 μM), GSH (10 mM), Hcys (5 mM), Cys (5 mM) in H_2O/CH_3CN (99.5:0.5, v/v) buffered with HEPES, pH = 7.4; λ_{ex}= 450 nm.

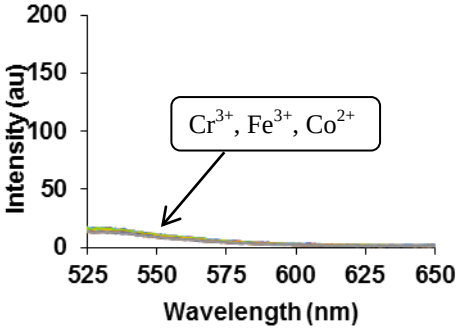


Fig. S7D: Fluorescence spectra of probe **1** (1.0 μM) on addition of Cr^{3+}, Fe^{3+} and Co^{2+} (25 μM each) in H_2O/CH_3CN (99.5:0.5, v/v) buffered with HEPES, pH = 7.4; λ_{ex}= 450 nm.

UV-vis spectra of probe 1 in the presence of different analytes

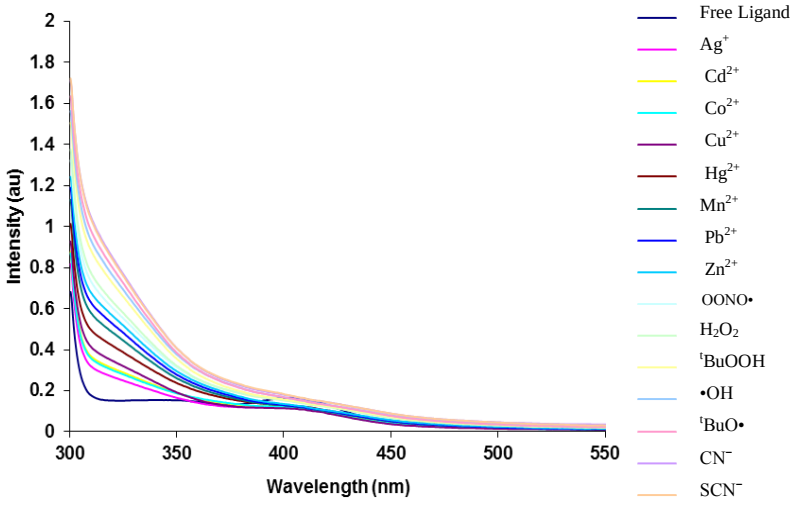
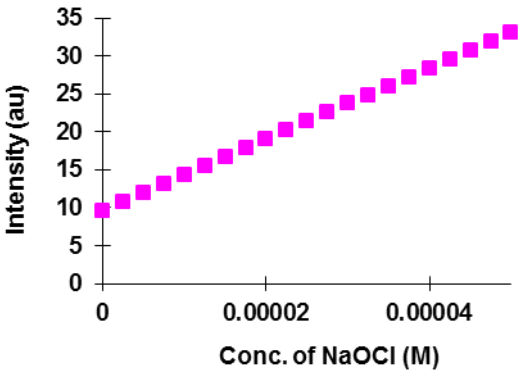


Fig. S8: UV-vis spectra of probe **1** (5.0 μM) in H_2O/CH_3CN (99.5: 0.5, v/v) buffered with HEPES, 50 mM, pH = 7.4; in the presence of different analytes (25 μM) (H_2O_2, ^{t}BuOOH, •OH, ^{t}BuO•, OONO•, Zn^{2+}, Pb^{2+}, Mn^{2+}, Hg^{2+}, Cu^{2+}, Co^{2+}, Cd^{2+}, Ag^+, CN^-, SCN^-)

Detection limit:



Multiple R = 0.968969

R^2 = 0.9589

Observation = 10

Intercept = 9.5918

Slope = 469734

The detection limit[2] was calculated based on the fluorescence titration. To determine the S/N ratio, the emission intensity of receptor **1** without NaOCl was measured by 10 times and the standard deviation of blank measurements was determined. The detection limit is then calculated with the following equation:

$DL = 3 \times SD/S$

Where SD is the standard deviation of the blank solution measured by 10 times; S is the slope of the calibration curve.

From the graph we get slope (S) = 469734, and SD value is 0.0256

Thus using the formula we get the Detection Limit (DL) = 1.63×10^{-7} M i.e. probe **1** can detect NaOCl in this minimum concentration through fluorescence method.

[2] S. Goswami, S. Das, K. Aich, D. Sarkar, T. K. Mondal, C. K. Quah, H.-K. Fun, *Dalton Trans.*, 2013, **42**, 15113.

Bar diagrams showing competitive selectivity of receptor 1

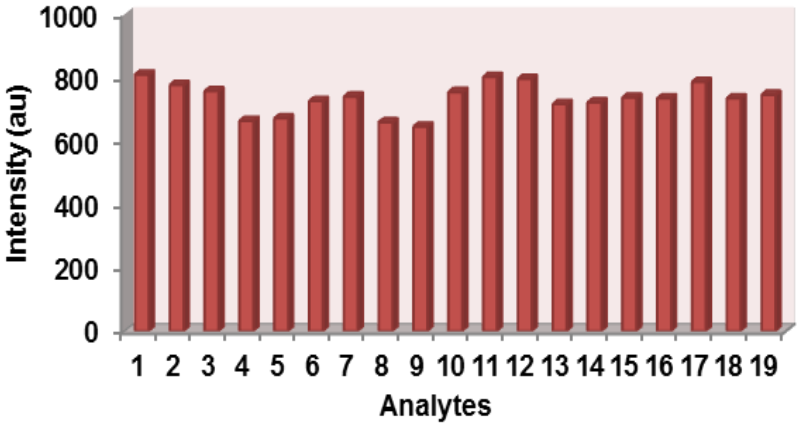


Fig. S10A: Competitive fluorescence selectivity of probe **1** (1.0 μM) towards addition of NaOCl (25 μM) in H_2O:CH_3CN (99.5:0.5, v/v) buffered with HEPES, 50 mM; pH = 7.4; λ_{ex} = 450 nm in the presence of other analyte (25 μM each). Bars represent the fluorescence emission intensity at 556 nm after the addition of other analyte. 1. H_2O_2; 2. $^{\bullet}OONO$; 3. TBHP; 4. Zn^{2+}; 5. Fe^{2+}; 6. Fe^{3+}; 7. $^{\bullet}O^{t}Bu$; 8, Cu^{2+}; 9. Ca^{2+}; 10. Hg^{2+}; 11. AcO^{-}; 12. SCN^{-}; 13. $^{\bullet}OH$; 14. F^{-}; 15. Mg^{2+}; 16. Cr^{3+}; 17. $H_2PO_4^{-}$; 18. Co^{2+}; 19. SO_4^{2-}. Data were given after the incubation period of 35 minutes with appropriate analytes.

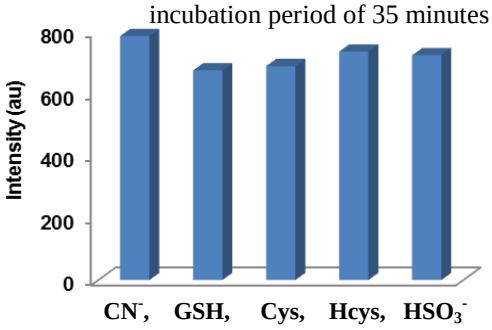


Fig. S10B: Competitive fluorescence bar diagram of probe **1** (1.0 μM) towards NaOCl (25 μM) in H_2O:CH_3CN (99.5:0.5, v/v) buffered with HEPES, 50 mM; pH = 7.4; λ_{ex} = 450 nm in the presence of CN^{-} (25 μM), GSH (10 mM), Cys (5 mM), Hcys (5 mM), HSO_3^{-} (25 μM).

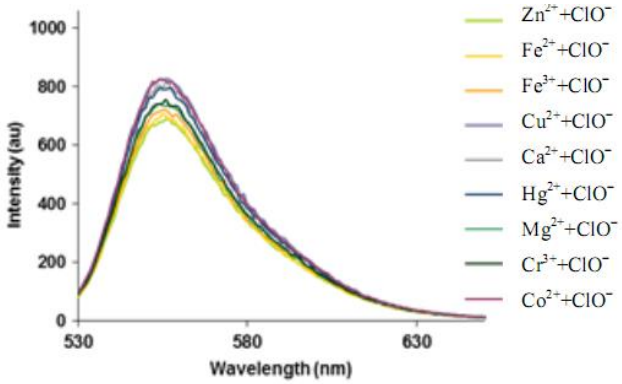


Fig. S10C: Competitive fluorescence spectra of probe 1 (1.0 μM) towards NaOCl in the presence of different metal ions.

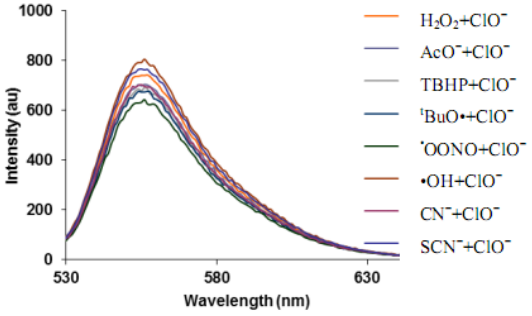


Fig. S10D: Competitive fluorescence spectra of probe **1** (1.0 μM) towards NaOCl in the presence of ROS and anions.

Table: Quantum yield and molar absorptivity in different solvent system of probe 1 towards addition of hypochlorite

Solvent	Addition of Hypochlorite	Quantum yield (ϕ)	ε (M^{-1} cm^{-1}) × 10^4 at 391 nm
H_2O/CH_3CN (99.5:0.5; v/v)	25 μM	0.51	4.64
H_2O/DMF (99.5:0.5, v/v)	25 μM	0.43	4.23
H_2O/DMSO (99.5:0.5; v/v)	25 μM	0.41	3.45
H_2O/EtOH (99.5:0.5; v/v)	25 μM	0.28	2.67
H_2O/MeOH (99.5:0.5; v/v)	25 μM	0.26	3.12
H_2O/THF (99.5:0.5; v/v)	25 μM	0.46	3.51

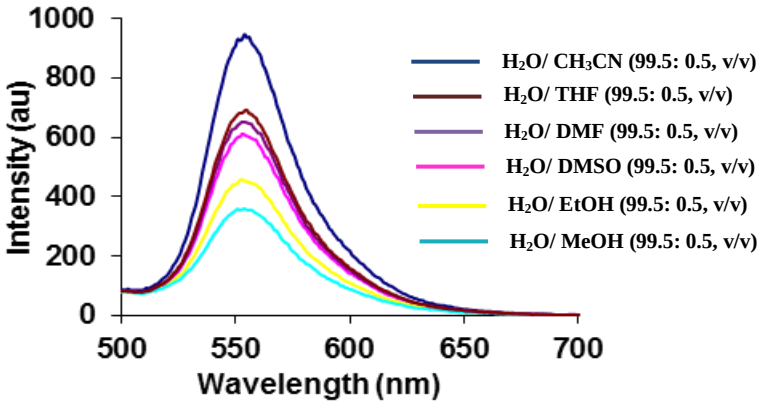
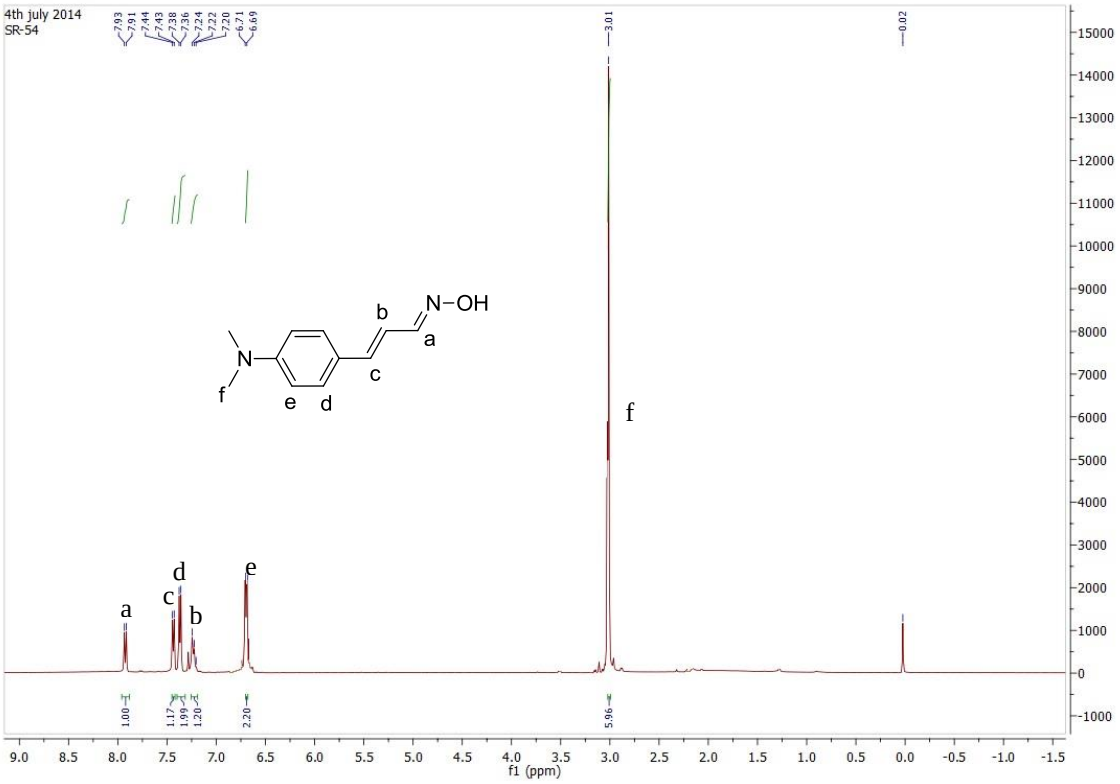
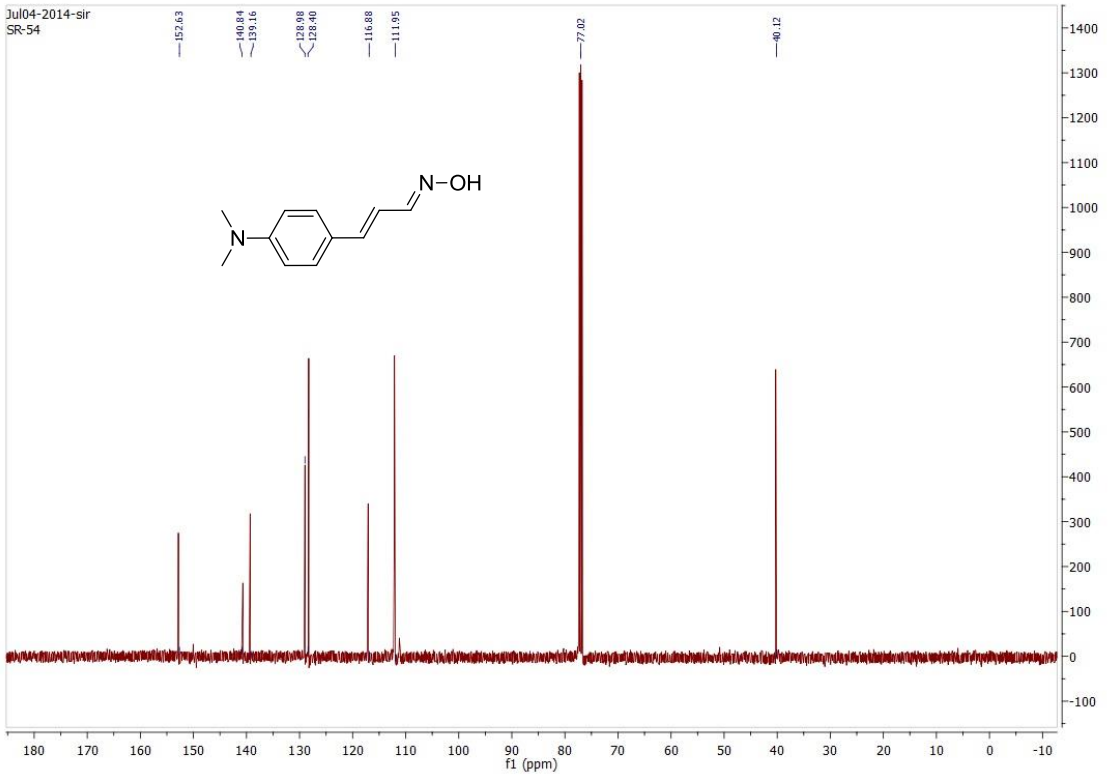


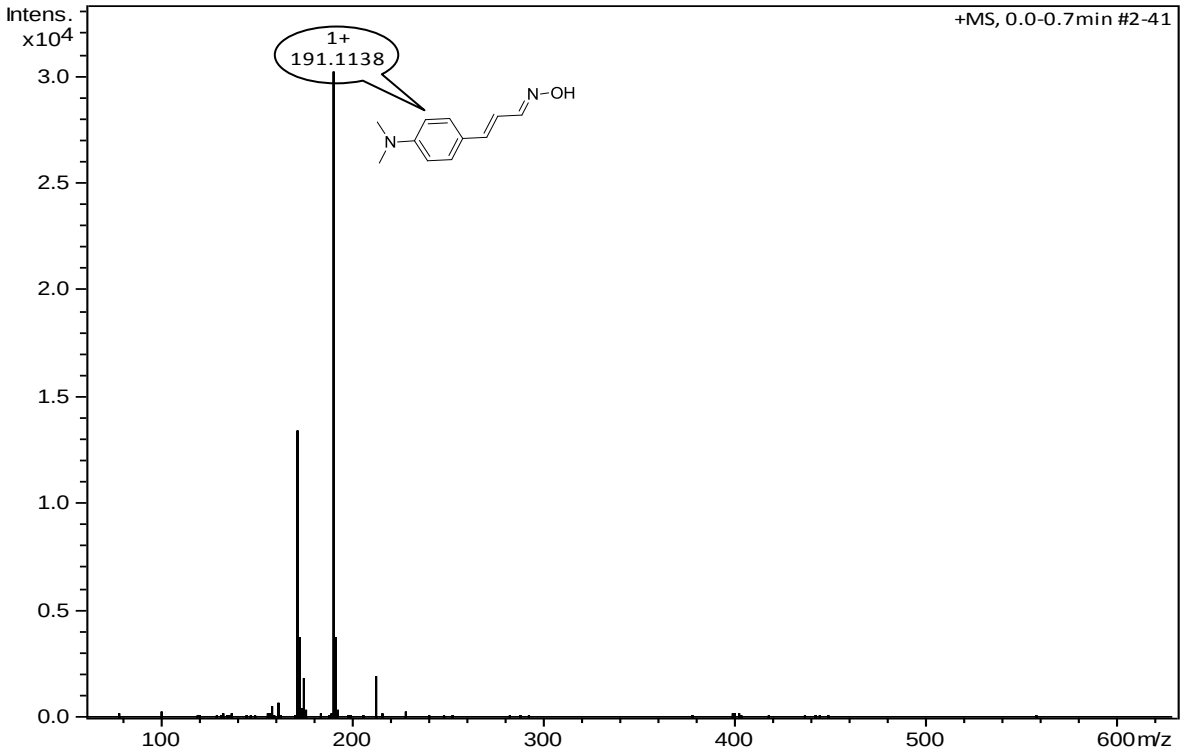
Fig. S11: Fluorescence spectra of probe **1** (1.0 μM) upon the addition of 25.0 μM hypochlorite in different solvent system, buffered with HEPES (50 mM), pH = 7.4; (λ_{ex} = 450 nm).

^{1}H and ^{13}C NMR spectra of probe 1 in $CDCl_3$ (500 and 125 MHz)

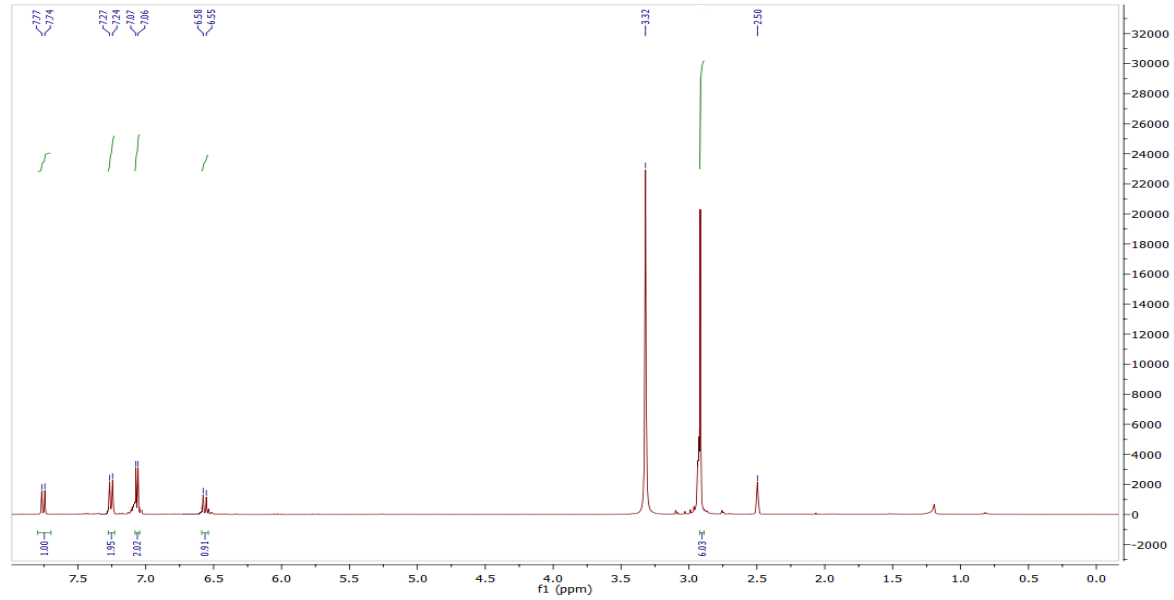


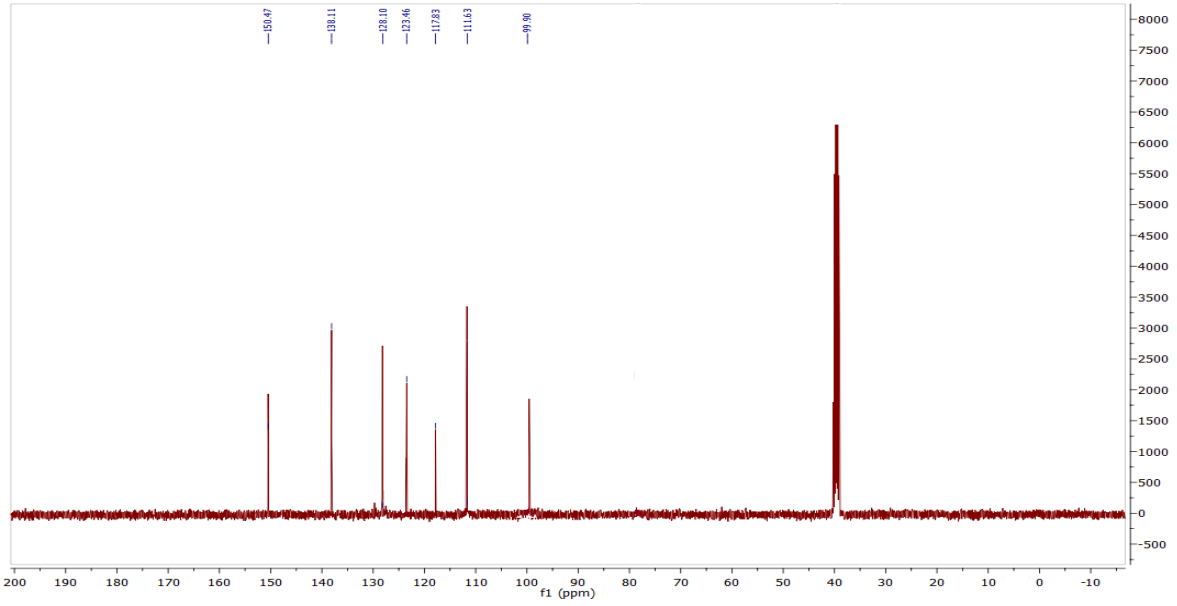


Mass spectrum of probe 1

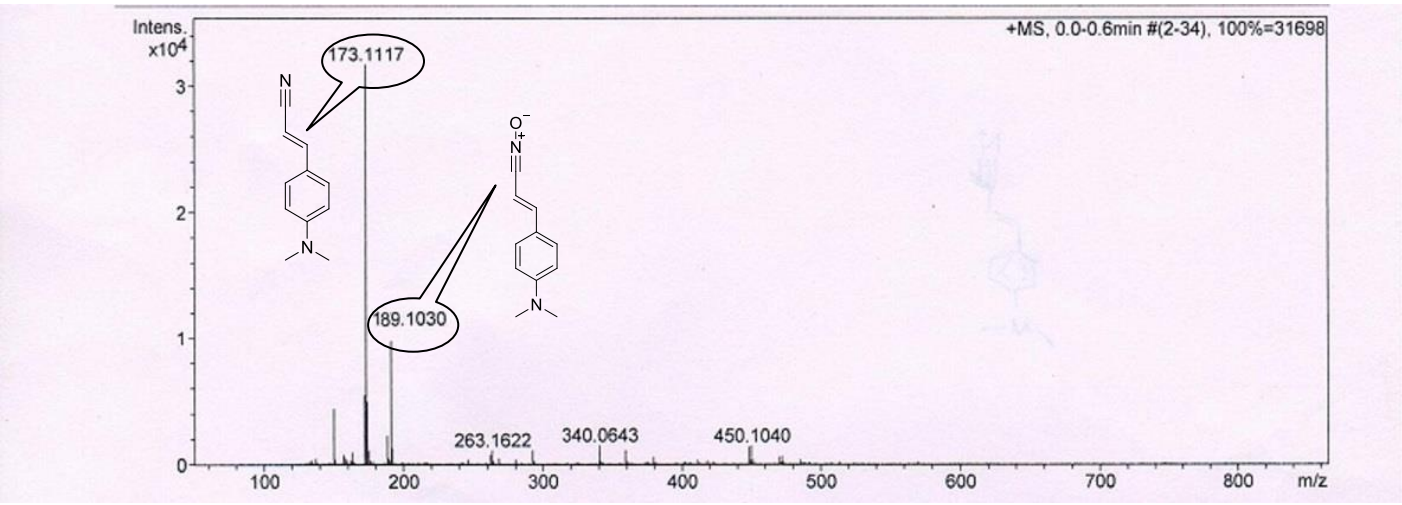


^{1}H and ^{13}C NMR spectrum of compound 2 in DMSO-d_6 (300 and 75 MHz)





Mass spectrum of compound 2



Mass spectrum of isoxazoline 3 (impure not isolated)

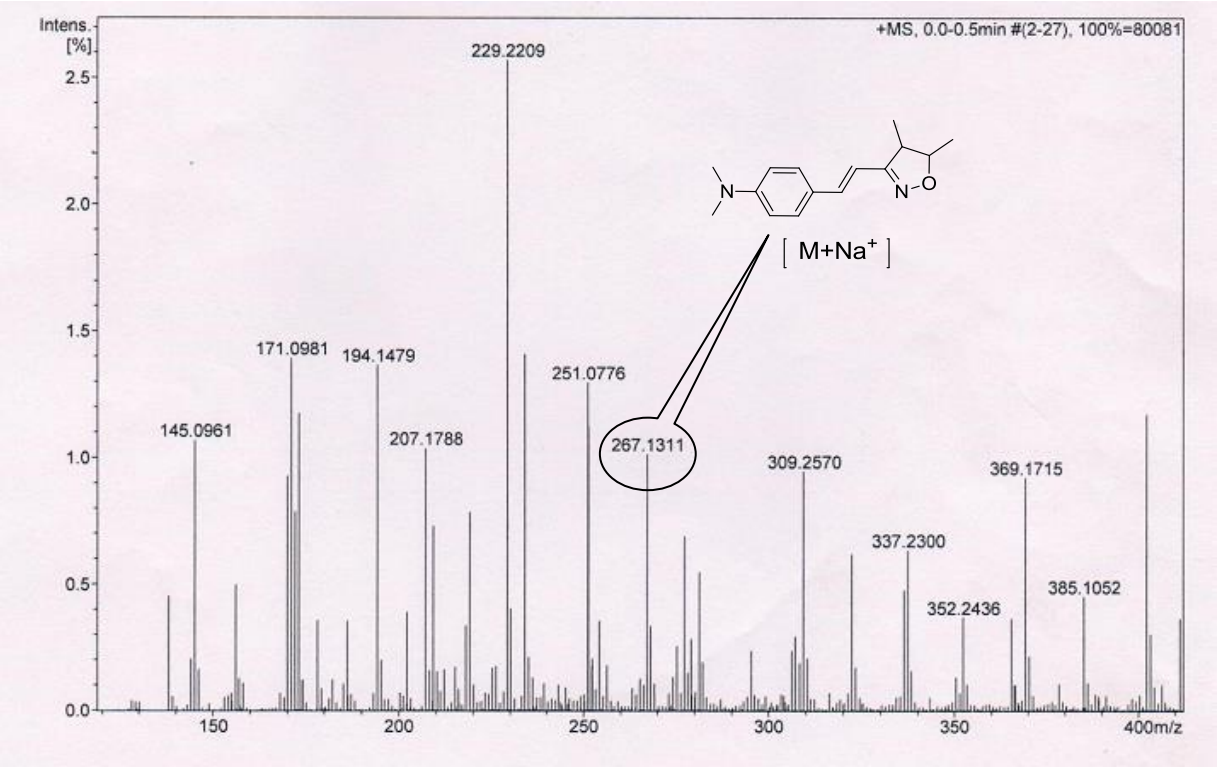


TABLE: Comparison of probe 1 with previous reported hypochlorite sensors in the literature

Journal	Fluorescence response	Enzymatic generated NaOCl detection	Detection limit	Fast detection	Low molecular weight	Experimental media	Endogenous NaOCl detection	Flow cytometric examination
Present manuscript	**Turn-On**	**Yes**	1.63×10^{-7} M	**Yes (within minutes)**	**Yes**	**H_2O/CH_3CN (99.5:0.5, v/v) HEPES buffered, pH = 7.4**	**Yes**	**Yes**
Anal. Chem., 2014, **86**, *671*	Turn-Off	Yes	0.13 μM	Yes	Yes	50 mM PBS/EtOH (9:1)	Yes	No
Inorg. Chem., 2013, **52**, 12863	Turn-On	No	----	Yes	No	DMF/phthalate buffer (50 mM, pH 5) (1:9,v/v)	No	No
Analyst, 2013, **138**, 6091	Turn-On	No	0.52 μM	Yes	Yes	PBS buffer (pH 7.4, 10 mM) and EtOH (1/9, v/v)	Yes	No
Analyst, 2011, **136**, 2277	Turn-On	No	-----	Yes	No	DMF-HEPES (50 mM, pH 7.2, v/v, 4 : 1) solutions	No	No
Chem. Eur. J. 2009, **15**, 2305	Turn-On	No	μM	Yes	Yes	0.1m potassium phosphate buffer, pH 9.0/DMF (v/v, 1:4)	No	No
Anal. Chem. 2010, **82**, 9775	Turn-Off	Yes	----	Yes	Yes	-----	Yes	No
Chem. Commun., 2013, **49**, 7836.	Turn-On	No	0.5 μM	Yes	Yes	pH 7.2 buffer/DMF (v/v, 4:1)	No	No
Chem. Commun., 2011, **47**, 11978	Turn-On	No	40 mM	Yes	Yes	DMSO–H_2O (1 : 9, v/v, 10 mM HEPES, pH 7.05)	No	No

Intensity analysis

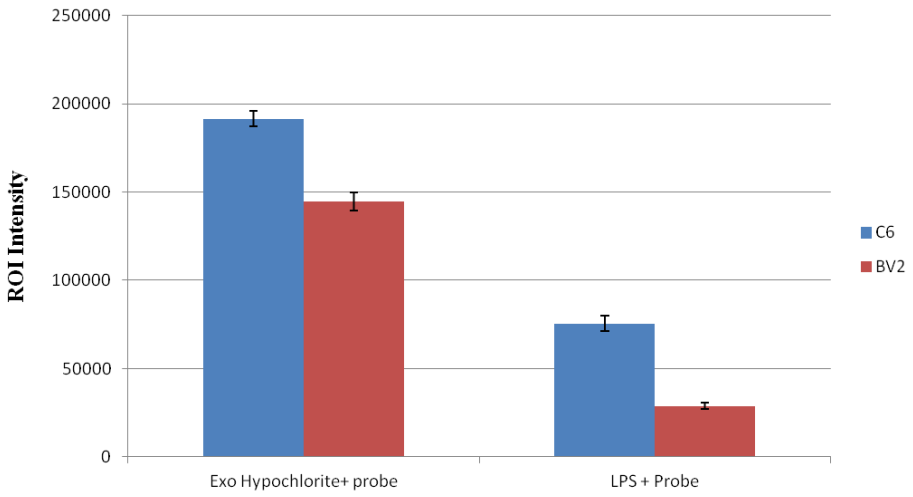


Fig. S19: Histogram depicts the relative fluorescence intensity expression of the probe **1** with addition of exogenous hypochlorite and LPS stimulated cell lines.

Cell culture and treatments

C6 glioma and BV2 microglial (Resident macrophage of brain) cell lines were obtained from NCCS, Pune and NBRC, Manesar, respectively. These Cell lines were maintained in DMEM medium supplemented with 10% FBS, 1X PSN antibiotic solution at 37°C in humid environment containing 5% CO_2 .Cells were seeded in 12 well plates and 90mm petri dishes at a cell density of 10,000cells/ml for fluorescence imaging and 30,000cells/ml for apoptosis assay. Four groups were studied as follows:

(I) Control group,

(II) Unstimulated cells exposed to probe (5.0 μM) for 30 min,

(III) Unstimulated cells treated with NaOCl (1.0 μM) for 30 min followed by washing with 1X and exposed to probe (5.0 μM) for 30 min,

(IV) For the detection of endogenous ClO^- ions, LPS activated C6 glial cells (2μg/ml for 24 hrs) and BV-2 microglial cells (100ng/ml for 48 hrs) were exposed to probe (5.0 μM) for 30 min.

Fluorescence Imaging

Control and treated cells were washed with chilled PBS and then fixed with 4% Paraformaldehyde (15 mins). After fixing and three washings with PBS of 5 mins each, cells were mounted on microscopic slides with antifading agent (fluoromount) and observed. Images were taken with A1R Nikon Laser Scanning Confocal microscope.

Annexin V/ FITC apoptosis assay

To test whether the probe has any cytotoxic effects on cells, Annexin V/ FITC apoptosis assay was carried out. Annexin V is a cellular protein of annexin group which possesses high affinity for Phosphotidylserine which is present in the inner leaflet of normal cells and is exposed on the surface of apoptotic cells. Propidium Iodide is a DNA intercalating agent which labels late apoptotic and necrotic cells. Control and treated cells were harvested by trypsinization and washed with PBS. Cells were pelleted down and resuspended in 1ml of 1X Annexin V binding buffer followed by addition of 10μl Annexin V/FITC. After Incubation for15 min in dark at

room temperature, cells were washed with 1X Annexin V binding buffer and centrifuged at 300g for 10 mins. Pellet was resuspended in 500µl Annexin V binding buffer and added 5µl of PI (Propidium Iodide) immediately before detection by Flow Cytometry (Accuri C6 Flow Cytometer). Four populations of cells viable (Annexin V-, PI-), early apoptotic (Annexin V+, PI-), late apoptotic (Annexin V+, PI+) and necrotic (Annexin V-, PI+) cells were detected and analyzed by FCS Express 4 flow research edition software (De novo software).