Supporting information

Engaging highly fluorescent Conjugated Polymer Network for probing endogenous hypochlorite in macrophage Cells: Improved sensitivity via signal amplification

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# R.S.F and N.G have equal contribution towards the manuscript

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EXPERIMENTAL SECTION

Spectroscopic studies. The UV-vis spectroscopic studies were recorded on a JASCO (model V-650) UV-Vis spectrophotometer. The slit-width for the experiment was kept at 5 nm. Sensing was carried out by adding requisite amounts of sodium hypochlorite to water (1 % DMSO) solutions of probe **1** (1×10^{-6} M). On the other hand, fluorescence experiments were performed in FluoroLog-TM (Horiba Scientific). The slit-width for the fluorescence experiment was kept at 5 nm (excitation) and 5 nm (emission) and the excitation wavelength was set at 365 nm.

Fluorescence Titrations: Stock solutions of 1 mM of **1** was prepared in DMSO. A standard fluorescence cuvette (10 mm) was filled with 2 mL of the respective solvent, to which 10 uL of probe **1** (10 mM) was added. An initial PL spectrum was recorded using λ_{ex} = 365 nm. Subsequently, increasing concentrations of hypochlorite were added to the probe solution and the fluorescence spectra was recorded.

Fluorescence Decay Experiment

Fluorescence lifetime values were measured by using a time-correlated single photon counting fluorimeter (Horiba Delta flex Modular). The system was excited with 365 nm nano-LED of Horiba–Jobin Yvon with pulse duration of 1.2 ns (slit width of 5/5, monitored at 520 nm, 480 nm, 424 nm). Average fluorescence lifetimes (τ_{av}) for the exponential iterative fitting were calculated from the decay times (τ_i) and the relative amplitudes (a_i) using the equation.

$$\tau_{av} = (a_1\tau_1^2 + a_2\tau_2^2 + a_3\tau_3^2)/(a_1\tau_1 + a_2\tau_2 + a_3\tau_3) \quad \textbf{(1)}$$

where a_1, a_2, and a_3 are the relative amplitudes and τ_1, τ_2, and τ_3 are the lifetime values, respectively.

Detection limit determination. The method used for the calculation of the detection limit is known as the blank variability method. In this method, the calibration curve was prepared by recording fluorescence spectra of **1** in different amounts of hypochlorite.

From the equation obtained from the calibration plot, the added hypochlorite concentrations were calculated. Then another calibration curve was drawn between the C_{real} (added hypochlorite, μM) vs. $C_{calc.}$ (Calculated amount of hypochlorite, μM). This afforded a value of the slope (b).

The fluorescence spectra of **1** were taken as blank reading. A total 10 replicates of the blank were measured. The standard deviation from the blank readings was calculated by fitting the fluorescence reading into the equation obtained from the first calibration curve (titration spectra). Using this standard deviation value, we calculated decision limit by this following equation.

$$L_C = t_C \times s \times (1 + 1/N)1/2 \quad \text{..... ..... (1)}$$

where, N = the number of blank replicates taken; the value of t_c for 10 blank readings is 1.833; and s = the standard deviation value.

The detection limit (L_D) was calculated as the double of the decision limit obtained,

$L_D = 2\ L_c$ …… (2)

In concentration term, the detection limit appeared as,

$x_D = 2 \times C = 2\ L_C / b$ …… (3)

where, b = slope of the second calibration curve (C_{real} vs. C_{calc}.).

^{1}H NMR Studies. ^{1}H NMR titration studies of compound **1** (5 mM) were performed with sodium hypochlorite (6.0 equiv.) in DMSO-d_6. The spectra were recorded using identical parameters.

Determination of fluorescence intensity by confocal imaging

Treatment with the compound: Cells after reaching 80 % confluency, cultured onto the coverslips. The cells were incubated with the compound solutions (70 µM, 100 µM, and 500 µM in 200 µL DMEM media) for 2 h and 4 h to observe the internalization. Followed by incubation, the cells were washed three times with the sterile phosphate buffer saline (PBS), pH 7.4 solution (0.5 mL), to remove the excess compound present in the media, and later on incubated with 4 % (w/v) paraformaldehyde solution (0.5 mL) for 15 min for the fixation. The coverslips were mounted onto the glass slides and the images were captured using confocal microscope (Leica microsystems, Germany). The excitation of the compound was 350 nm, and the emission was observed from 430 nm-480 nm. The Ar laser was used at 20 % efficiency and magnified at 40X. The captured images were analyzed using *image J* software.

Using the same above-mentioned procedure, we have performed internalization study with HEK-293 cells with the optimised concentration and time period to evaluate probe's selectivity.

Effect of glucose induced and ABAH pre-treated RAW 264.7 cells followed by compound treatment: RAW 264.7 cells were seeded onto the coverslips on a 12 well plate at 80 % confluency. Glucose was exposed to the cells by treating them with lipopolysaccharides (LPS, 5 µg/well) for 16 h. Whereas, the production of hypochlorite was blocked inside the cells using 4-amino benzoic acid (ABAH) by incubating the cells with ABAH (200 µg/well). After the incubation with LPS/ABAH, the cells were treated with the compound of optimized concentration for 2 h and 4 h. After the timepoints, the cells were washed, fixed with 4 % paraformaldehyde, and images were captured using the confocal microscopy. The emission intensities were quantified as per the procedure mentioned in the above section.

Cytotoxicity by MTT assay and IC 50 determination: MTT assay procedure was followed to determine the in vitro cytotoxicity of the compound against a human macrophage RAW 264.7 cells. Cells were sub-cultured as per the ATCC protocol in their respective complete media and were subsequently seeded in a sterile 96-well plate with about 100 µL/well with a cell density of about 1x 10 4 cells per well and were incubated overnight. The desired concentration of the compound was prepared from the DMSO stock solution of the compound in the respective media. Cells were treated with 70 µM concentration with a control solution of 1% DMSO in the respective media and were incubated for 2 h, 4 h ,12 h and 24 h in the growth medium, respectively. Post-treatment period, the media was aspirated, and to this, 50 µL of 5 mg/mL of MTT solution in phenol red-free media was added and was incubated for about 3 h for the formation of formazan crystals. The MTT solution was removed, and to the wells, 150 µL of DMSO was added for the dissolution of the formed crystals. The absorbance was measured at 570 nm and 650 nm wavelengths. The % cell viability was calculated as follows:

$$\frac{Absorbance\ of\ treated\ cells}{Absorbance\ of\ untreated\ cells} \times 100$$

The % cell viability was determined, and the obtained results were depicted as the dose-response curve for the IC_{50} analysis using GraphPad Prism™ version 8.0.1.

Colocalization evaluation using Lyso-tracker Deep Red

RAW 264.7 cells were seeded onto coverslips in a 12-well plate once they reached 80% confluency in the cell culture flask. To evaluate the specific accumulation of the probe in phagolysosomes, we used the commercially available dye Lyso-tracker Deep Red for targeted lysosomal tracking. First, the cells were incubated with the probe (70 µM) for 2 h, followed by incubation with Lyso-tracker Deep Red (0.1 µM) for 1 h. After the incubation periods, the cells were washed three times with PBS to remove excess dye. The coverslips containing the fixed cells were then mounted onto glass slides using Fluoromount G mounting media. The images were captured using a Leica DMI8 fluorescence microscope at 40X magnification. The co-localization of the probe and the commercial Lyso-tracker dye within the cells was analyzed and quantified using Image J software to assess the extent of overlap between the two fluorescent signals.

Synthesis and characterization of monomers:

Synthesis of 3-(2-(2-ethoxyethoxy)ethoxy)thiophene : In a round bottom flask CuI (117 mg, 0.6 mmol), and NaH (245 mg, 6 mmol) were taken under N_2 atmosphere and then diethylene glycol monoethylether (2g, 15mmole) was added dropwise in ice cold condition. After the complete addition of diethylene glycol monoethylether, the reaction mixture was further stirred for 30 minutes at room temperature. Then 3-bromothiophene (500 mg, 3 mmol) was added in the reaction mixture and

heated 100 °C for 24 hours. The reaction mixture was allowed to cool to room temperature. The product was extracted with dichloromethane and washed with 10% aqueous ammonium chloride followed by brine. The solvent was then removed under reduced pressure. The crude product was then eluted over silica gel using a 80:20 solution of hexanes :ethyl acetate to give a yellow oil. The formation of the product was confirmed by NMR, with a yield of 70%. ^{1}H NMR ($CDCl_3$, 500MHz) δ=7.17(1H,d), 6.78(1H,d), 6.26(1H,s) 4.13(2H,t), 3.85(2H,t), 3.7(2H,t), 3.62(2H,t), 3.5 (2H,q), 1.22(3H,t)

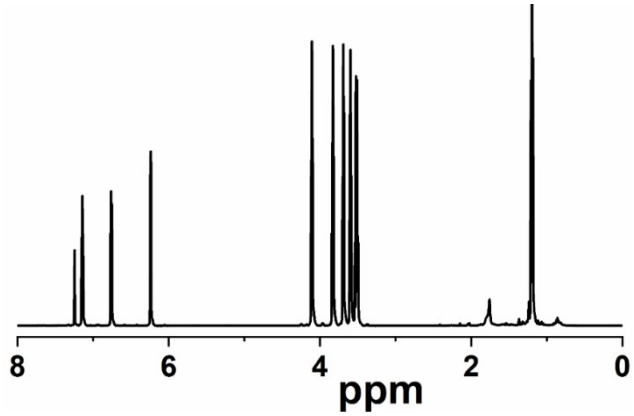


Fig. S1: ^{1}H-NMR spectra of 3-(2-(2-ethoxyethoxy)ethoxy)thiophene

Synthesis of 2,5-dibromo-3-(2-(2-ethoxyethoxy)ethoxy)thiophene:

3-[2-(2-ethoxyethoxy)ethoxy]thiophene (150 mg, 0.69 mmol) was dissolved in 10 mL of dry THF in a round bottom flask. The flask was put in ice bath and N-bromosuccinimide (271 mg, 1.52 mmol) was added and further stirred for 30 minutes before the flask was removed from the ice bath. Additionally, the reaction was stirred for 2 hours at room temperature. The product was extracted with dichloromethane, washed with water, brine followed by dried over anhydrous Na_2SO_4. The solvent was then removed under reduced pressure. The product was purified by eluting over silica gel using a 90:10 mixture of hexanes: ethyl acetate. The formation of the product was confirmed by NMR, with a yield of 80%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ = 6.82 (1H,s) 4.17(2H,t), 3.80(2H,t),3.7(2H,t), 3.60(2H,t), 3.54(2H,q), 1.22(3H,t).

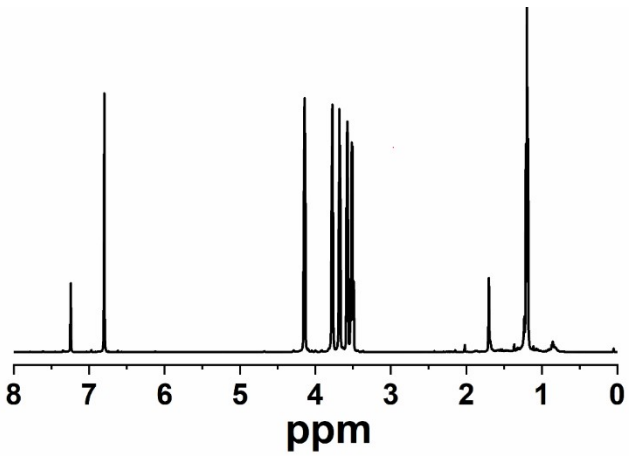


Fig. S2: ^{1}H-NMR spectra of 2,5-dibromo-3-(2-(2-ethoxyethoxy)ethoxy)thiophene

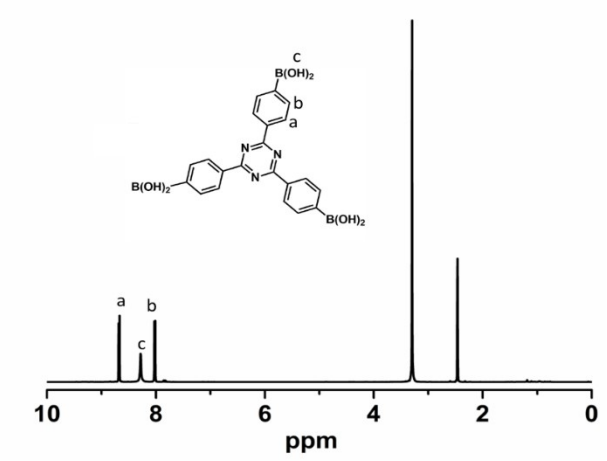


Fig. S3: ^{1}H NMR spectra of ((1,3,5-triazine-2,4,6-triyl) tris (benzene-4,1-diyl)) triboronic acid (TBBA) (in d_6 DMSO).

Synthesis of CPN: Initially, 4-cyanophenylboronic acid was trimerized by acid-catalyzed method to prepare monomer ((1,3,5-triazine-2,4,6-triyl)tris(benzene-4,1-diyl))triboronic acid (TBBA). TBBA (50 mg, 0.113 mmol), 2,5-dibromo-3-(2-(2-ethoxyethoxy)ethoxy)thiophene(84 mg, 0.227 mmol), K_2CO_3 (188 mg, 1.36 mmol), and $Pd(PPh_3)_4$ (3.9 mg, 0.003 mmol) were dissolved in toluene: ethanol: water (3:1:1). The mixture was degassed by a vacuum argon cycle several times. Then the reaction mixture was refluxed by 2 hr under argon atmosphere. After completion of the reaction the product was extracted by DCM, washed with water and the solvent was removed by evaporating to get green color solid residue. For purification of residue was further dissolved in $CHCl_3$ and precipitated in hexane three times. The formation of the polymer was confirmed by NMR data.

In ^{1}H NMR study, the proton signals at 8.69 from benzene ring of the pristine TBBA monomer (Fig. S3) broadens and shifts slightly down-field to 8.79 ppm (as indicated by "a" in Fig. S4), while the proton signal at 8.03 ppm also broadens and shows significant up-field chemical shift to 7.65 ppm (denoted by “b”, Fig. S4) in **CPN**. After attachment of donor 3-(2-(2-ethoxyethoxy)ethoxy)thiophene to benzene ring of triazine-benzene (TB), the ring current drops causing in shielding of nearby phenyl ring protons and their up-field chemical shift. The aromatic protons in thiophene moiety in **CPN** produce broad multiplet signals in the range 7.55 to 7.18, which could be attributable to the presence of repeating units in slightly different chemical environments in the polymeric **CPN**. Overall, the up-field chemical shift of thiophene ring proton in comparison to its pure monomer (Fig. S2) may be the result of its attachment with electron acceptor TB unit which further shield the aromatic thiophene ring proton.

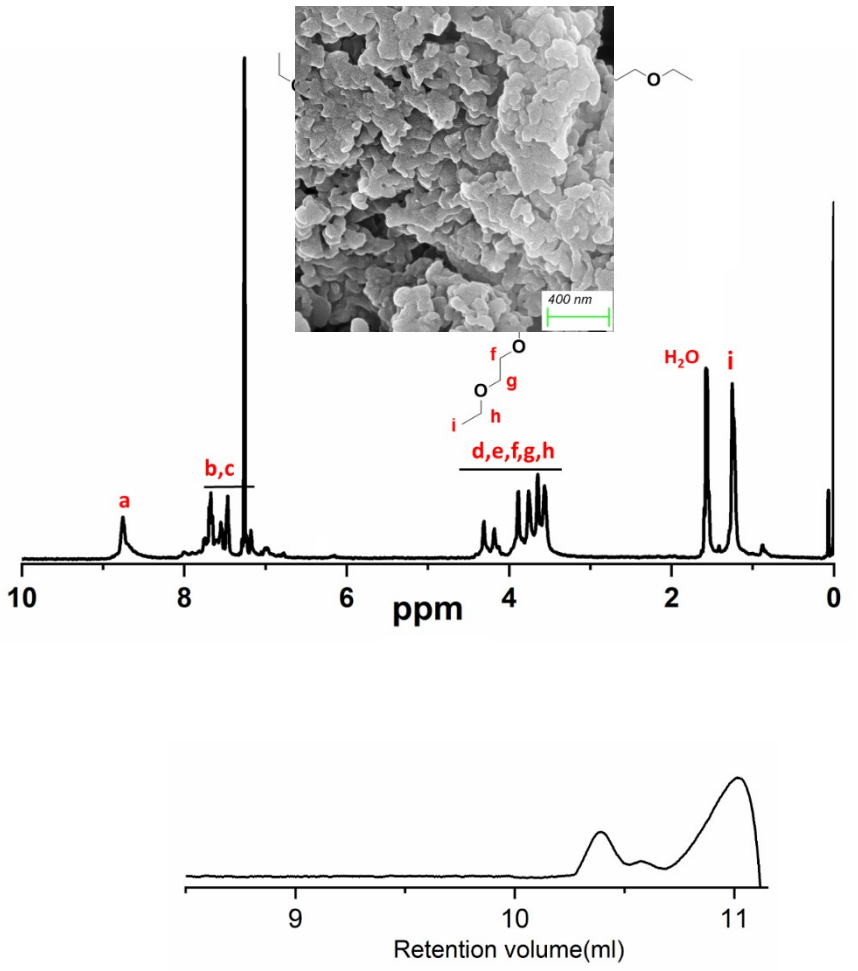


Fig. S4 : ^{1}H NMR spectra of polymer **CPN** with peak assigned.

Fig. S5: GPC traces of both **CPN** using DMF as eluent at 50 ^{o}C.

Fig. S6: High magnification FESEM image of **CPN**

BET adsorption summarized data of CPN

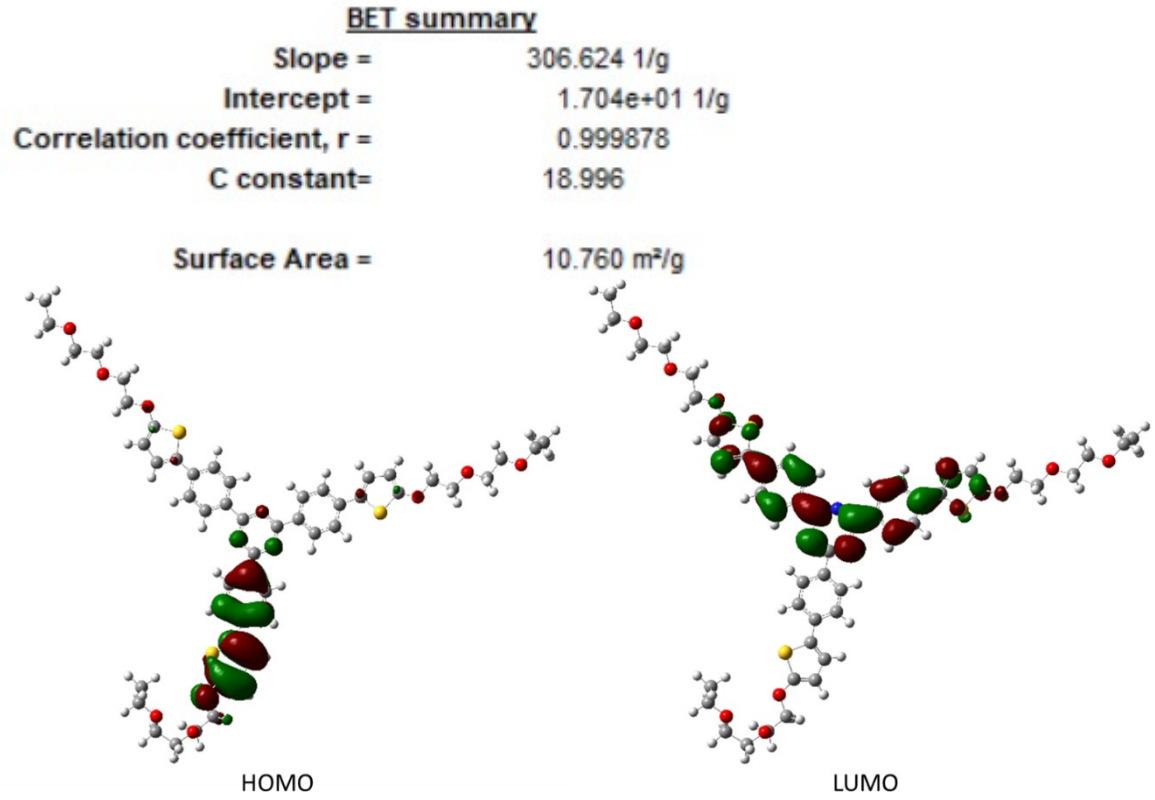
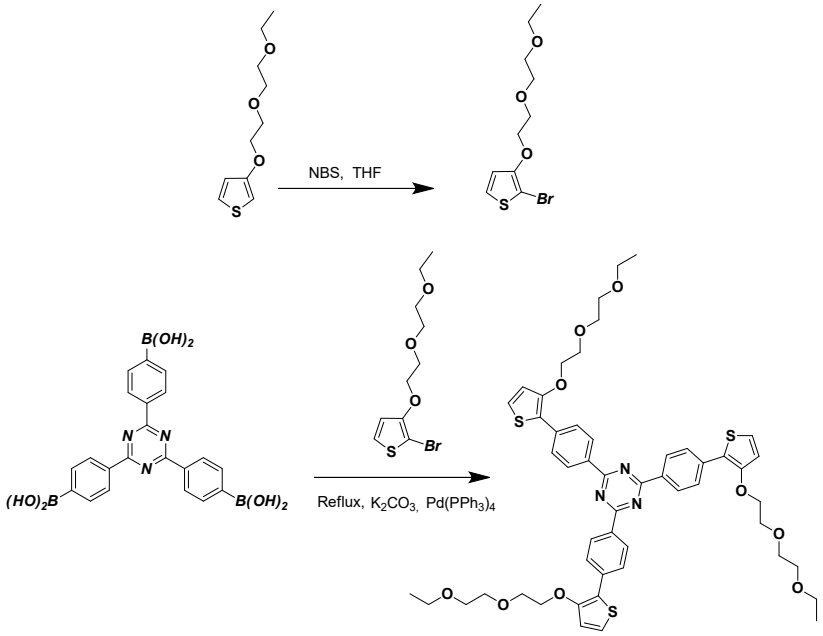


Fig. S7: Topographical representations of HOMO and LUMO molecular orbital of **CPN** (top view).

Synthesis of monomeric unit of CPN (control 1)



Scheme. **S1**: Total **s**cheme for the synthesis of control 1

Synthesis of 2-bromo-3-(2-(2-ethoxyethoxy)ethoxy)thiophene: In a round-bottom flask, 100 mg (0.46 mmol) of 3-[2-(2-ethoxyethoxy)ethoxy]thiophene was dissolved in 10 mL of dry THF. After

placing the flask in an ice bath, N-bromosuccinimide (91 mg, 0.51 mmol) was added, and it was further agitated for a further 30 minutes. Additionally, the reaction was stirred for 2 hours at room temperature. The product was extracted with dichloromethane, washed with water, brine followed by dried over anhydrous Na_2SO_4. The solvent was then removed under reduced pressure. The product was purified by eluting over silica gel using a 80:20 mixture of hexanes: ethyl acetate.

Synthesis of 2,4,6-tris(4-(3-(2-(2-ethoxyethoxy)ethoxy)thiophen-2-yl)phenyl)-1,3,5-triazine (control 1): In a round bottom flask TBBA (70 mg, 0.16mmol), 2-bromo-3-(2-(2-ethoxyethoxy)ethoxy)thiophene (150 mg, 0.51 mmol), K_2CO_3 (257 mg, 1.90 mmol), and $Pd(PPh_3)_4$ (5.5 mg, 0.0047 mmol) were dissolved in toluene: ethanol: water (3:1:1). The mixture was degassed by a vacuum argon cycle several times. Then the reaction mixture was refluxed by 18 hr under argon atmosphere. After completion of the reaction the product was extracted by DCM, washed with water , brine and the solvent was removed by evaporating to get brown color semi- solid residue. Further the product was purified by column chromatography using a 70:30 mixture of hexanes: ethyl acetate.

ADDITIONAL SPECTRAL DATA

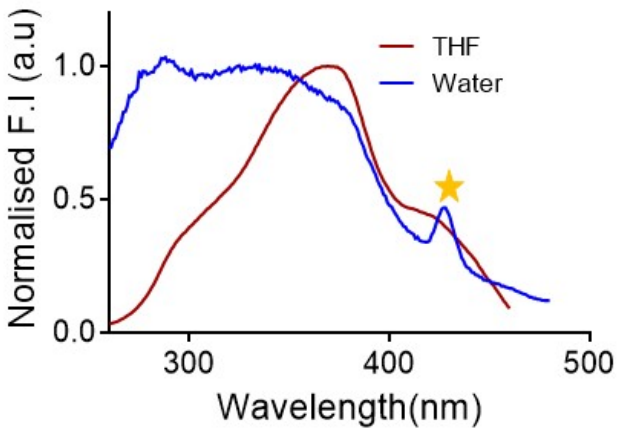


Fig. S8: Normalised excitation spectra of **CPN** (10 μM) in THF (λ_{em} = 480 nm) and Water (λ_{em} = 520 nm) . (★) represents solvent scattered light.

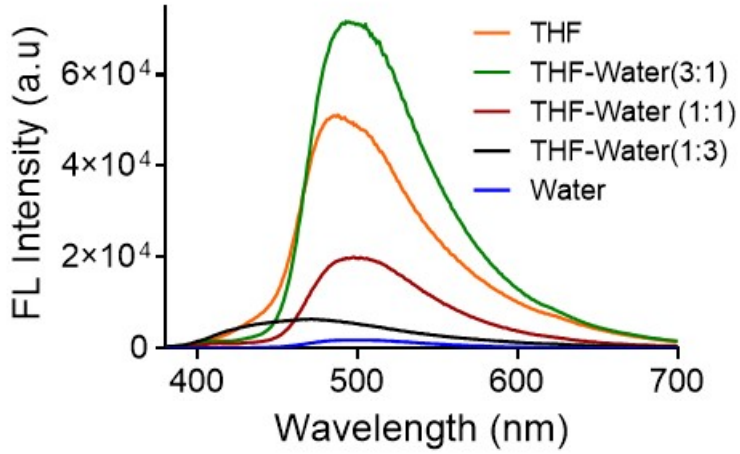


Fig. S9: Fluorescence spectra of **CPN** (10 μM, λex = 365 nm) in varying mixtures of THF: Water (pH 7).

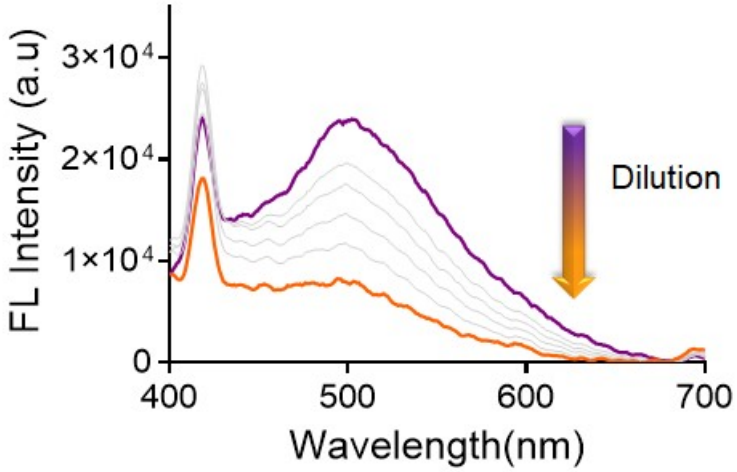


Fig. S10: Concentration-dependent fluorescence spectra of **CPN** (100 μM, λ_{ex} = 365 nm) in water medium.

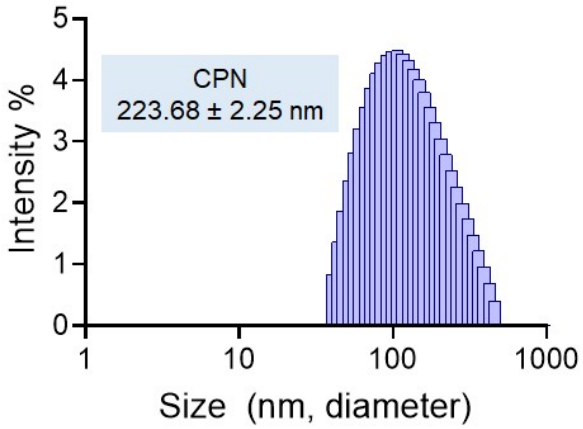


Fig. S11: Dynamic Light Scattering (DLS) experiment of **CPN** (10 μM).

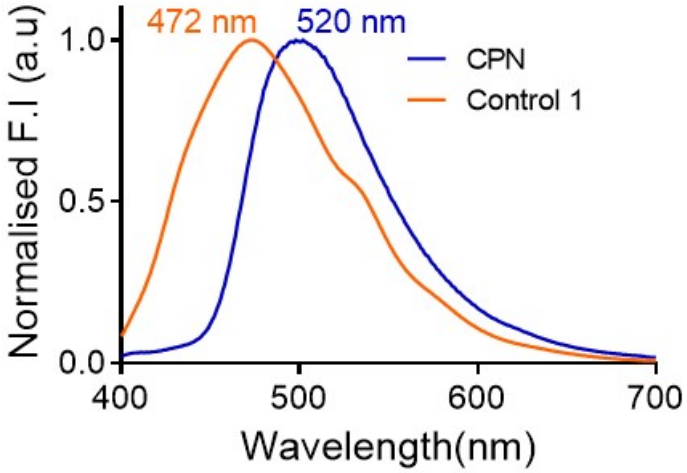


Fig. S12: Normalised fluorescence spectra of **CPN** (blue) (10 μM, λ_{ex} = 365 nm) and control 1 (orange) (10 μM, λ_{ex} = 365 nm) in THF-Water (1:1 pH 7) medium.

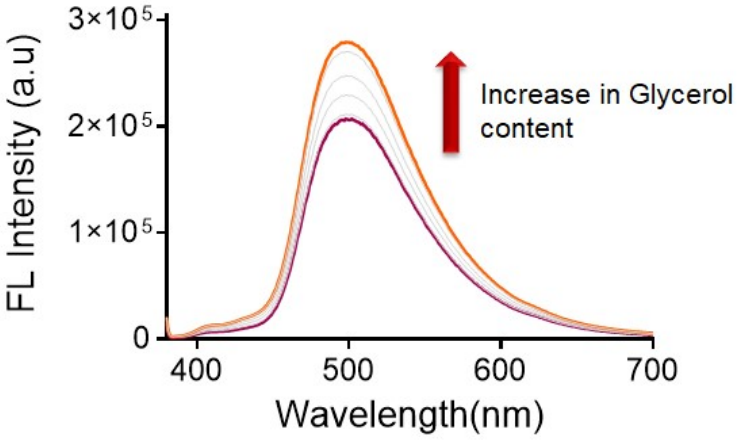


Fig. S13: Fluorescence spectra of **CPN** (10 μM, λ_{ex} = 365 nm) upon addition of glycerol in THF-Water (1:1 pH 7) medium.

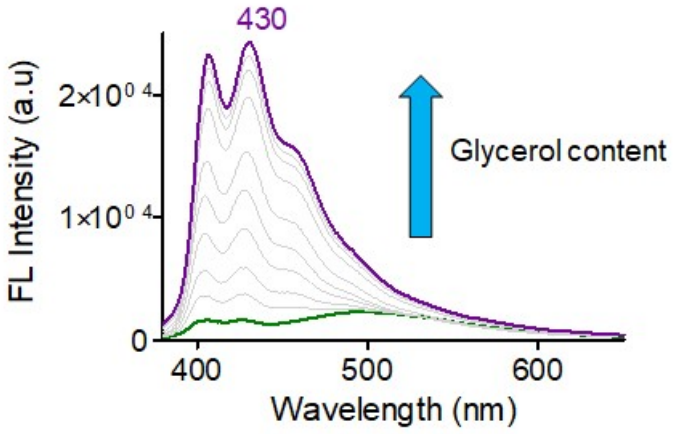


Fig. S14: Fluorescence spectra of **CPN** (10 μM, λ_{ex} = 365 nm) upon addition of glycerol in methanol.

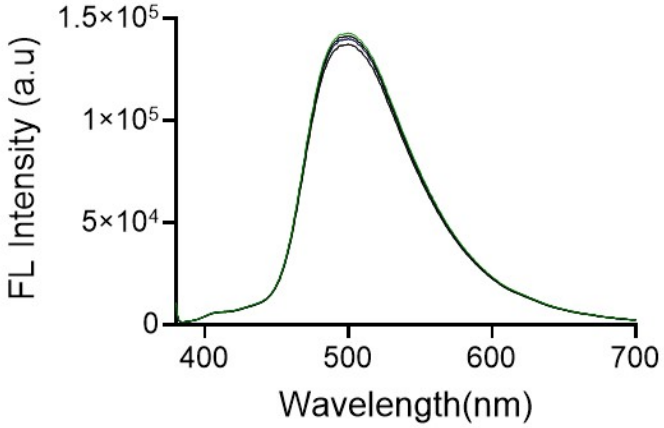


Fig. S15: Fluorescence spectra of control **1** (10 μM, λ_{ex} = 365 nm) upon hypochlorite addition (0 – 100 μM) in aqueous medium.

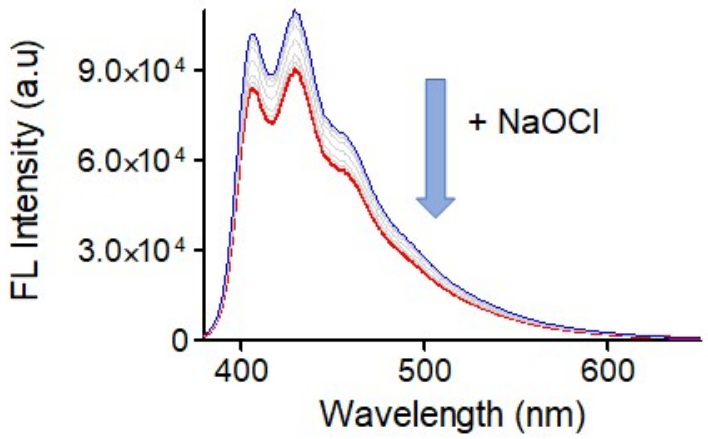


Fig. S16: Fluorescence spectra of **CPN** (10 μM, λ_{ex} = 365 nm) upon hypochlorite addition (0 – 100 μM) in ethanol medium.

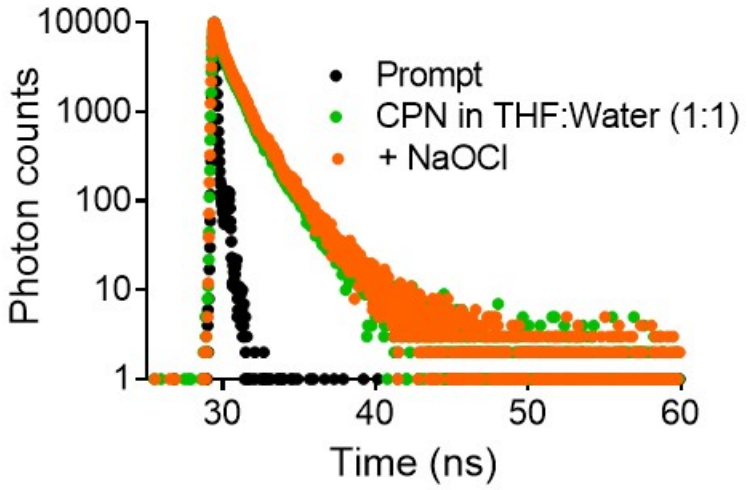


Fig. S17: Fluorescence lifetime spectra of **CPN** (10 μM, λ_{ex} = 365 nm) upon addition of hypochlorite (at 424 nm) in THF-Water (1:1 pH 7) medium.

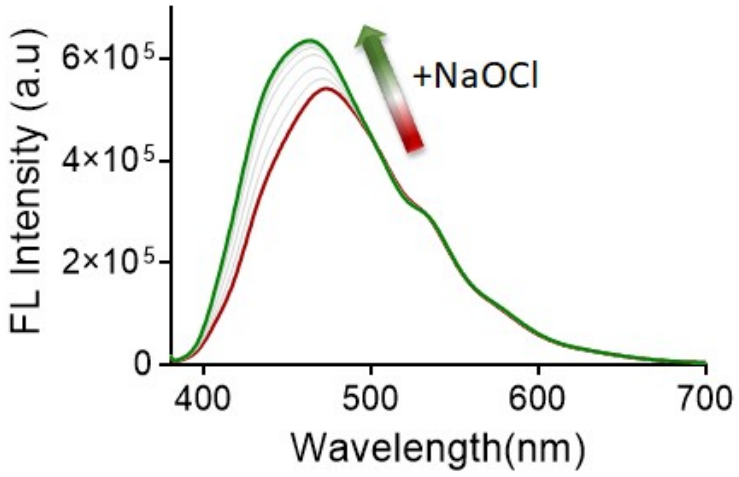


Fig. S18: Fluorescence spectra of control **1** (10 µM, λ_{ex} = 365 nm) with NaOCl (0 - 100 µM) in THF-Water (1:1 pH 7) medium.

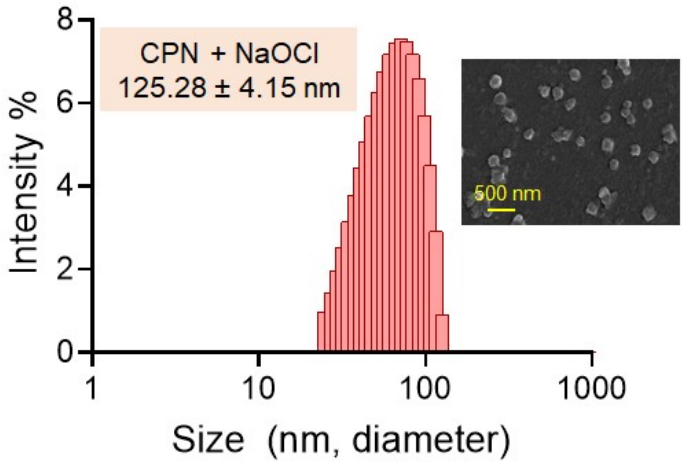
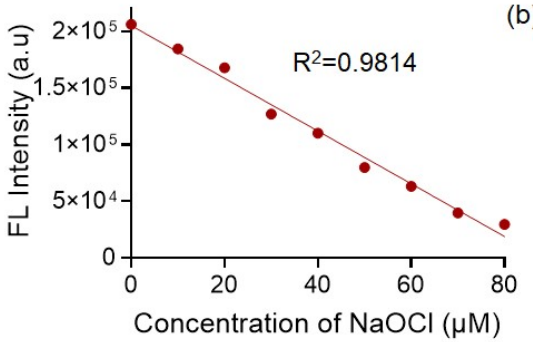


Fig. S19: Dynamic Light Scattering (DLS) experiment and FESEM image of **CPN** (10 µM) in the presence of NaOCl in THF: water (1:1) medium.

(a)



(b)

Samples	LOD (nM)	Recovery values (%)	RSD (%)
Tap water	1.3 nM	98.3 -100.2	2.4 - 3.1
Industrial wastewater	1.1 nM	97.8 - 102.6	2.1 - 3.2

Fig. S20: (a) Calibration curve of the fluorescence intensity of the **CPN** probe vs NaOCl concentrations in tap water. (b) Table summarizing the limit of detection (LOD), recovery values (%), and relative standard deviation (RSD) for NaOCl detection in tap water and industrial wastewater samples.

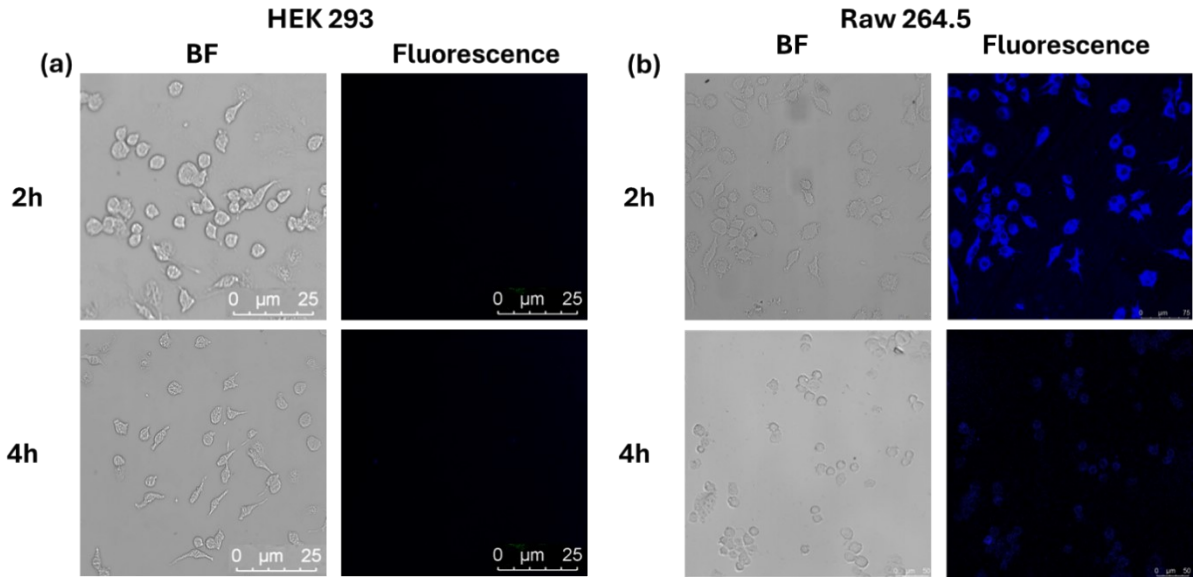


Fig. S21: Confocal fluorescence imaging of HEK-293 cells to evaluate the selectivity of probe CPN towards the RAW 264.7 cells. Cells were incubated with optimized concentration of the probe i.e., 70 µM for 2 h and 4 h. Blue channel images were obtained from 450 to 480 nm

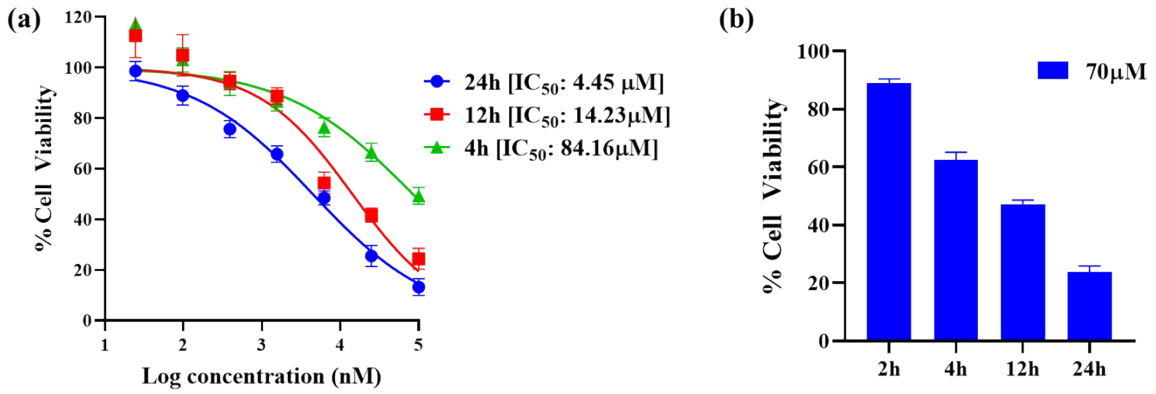


Fig. S22 (a) Graph representing the dose-response curves obtained from the MTT assay with the compound for 4 h, 12 h, and 24 h in RAW 264.7 cells. The cultured cells were treated with the compound at the concentration range from 0.024 μM to 100 μM (n = 6) for 4 h, 12 h and 24 h. (b) Bar graph representing the % cell viability of RAW 264.7 cells after treatment with the probe for various

time points (2 h, 4 h, 12 h, and 24 h). The graph was plotted using GraphPad Prism version 8.0.1. Data represent the mean ± SD (n = 6)

Table. S1: Table comparing the previously reported hypochlorite chemosensor with our present work

System	Medium	Mechanism	LOD	Time	Applications	Reference
Hydrazone linkage-based COF	Ethanol	H-bonding	1.4 μM	30s	Detection of ClO− in real water samples and detection of intracellular endogenous ClO^-	Spectrochim Acta A Mol Biomol Spectrosc. 2024, 306, 123577.
H-bonded organic frameworks (HOFs)	water	Redox balance (Oxidation)	1.32 μM	15s	Detection of ClO− in tap water samples	ACS Appl. Mater. Interfaces., 2022, 14(18), 21098-21105.
Bithiophene based probe	pH 7 buffer	Oxidation	4.2 μM		Detection of ClO− in zebrafish	Spectrochim Acta A Mol Biomol Spectrosc. , 2021, 261, 120059
Benzothiazole based probe	PBS buffer (pH 7.4)	Deprotection of dithiolane moiety	0.0089 μM	2 min	Ratiometric detection of the generation of endogenous hypochlorous acid in HeLa cells	Sens Actuators B Chem 2017, 243, 22-28.
Methoxy-hydroxybenzothiazole based	PBS buffer (pH 7.4)	ESIPT	0.00016 μM	>30 min	Detection of endogenously stimulated HOCl/ClO− in HeLa cells	ChemComm. 2018, 54(61), 8522-8525.
3-acetylcoumarin based	Tris-HCl buffer (pH 7.2)	Oxidation	0.00011 μM	20s	Detection of ClO− in real water samples and detection of intracellular endogenous ClO^-	RSC Adv., 2019, 9, 15926–15932
Conjugated polymer network (CPN)	THF: water (1:1)	Oxidation of sulfur functionality	0.0012 μM	12 min	Detection of NaOCl in water samples and fluorescent test-paper strips; Bio-imaging of endogenous hypochlorite in RAW 264.7 cells.	This work