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A Highly Selective Fluorescent Probe for Detection of Hydrogen Sulfide in Living Systems: *in vitro* and *in vivo* Applications

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Dedication ((optional))

Abstract: A fluorescein based fluorescent probe **3** has been designed and synthesized which selectively detects H_2S in aqueous medium among the various analytes tested. The probe **3** has also been successfully utilized for real time imaging of exogenous and endogenously produced H_2S in cancer cells and normal cells. Moreover, the probe **3** can also detect H_2S in rat brain hippocampus at variable depth and in living nematodes.

Introduction

Hydrogen sulfide (H_2S) recognized as a toxic and inflammable gas regulates many biological processes in human body.[1-3] H_2S when present in low concentration can cause personal distress but its high concentration above 250 ppm can lead to cell death.[4] In mammals, H_2S is primarily produced from three endogenous enzymes: cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (3-MST) which convert cysteine and their derivatives into H_2S in different tissues and organs. The endogenously produced H_2S mediates several physiological processes such as regulation of cell growth, cardiovascular protection, vasodilation, anti-inflammation and antioxidation effects.[5]

Recently, a number of fluorescent probes have been reported for the detection of H_2S[6] but most of these probes suffer from the limitations of poor detection limit, slow response time, interference from biothiols and their limited biological applications. Further, there is no report of utilization of these probes for real time monitoring of endogenously produced H_2S in both cancer and normal cells. The monitoring of H_2S production in both types of cells is important as it helps in visualizing the distribution of H_2S receptors in these cells.

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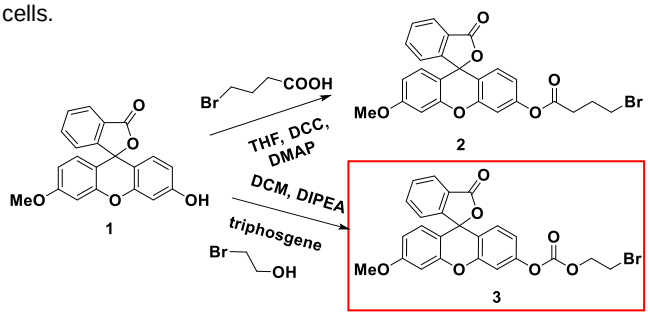
Recently, we reported a bodipy based probe for detection of H_2S but unfortunately the probe shows turn-off fluorescence behaviour and did not work in aqueous medium.[3b] Keeping in view the limitations of reported probes, we were then interested to design a probe which could selectively and rapidly detect H_2S in aqueous medium with turn-on fluorescence response. For achieving this target, we envisaged that if we could design a molecule that has dual nucleophilic center then, it will be possible to achieve selectivity and faster response and thus designed and synthesized fluorescein based probes **2** and **3** with 2-bromoethyl moiety linked *via* carbonate ester and ester linkages. We have introduced bromo groups in both the probes as these groups can be easily replaced by H_2S which will then facilitate the ring opening of fluorescein moiety. However, out of the two synthesized probes, only probe **3** shows selectivity towards H_2S and works well in aqueous medium. Moreover, the designed probe **3** has several advantages: (i) It shows fast turn-on response towards H_2S. (ii) The probe can detect upto 0.13 μM of H_2S which is sufficiently low for monitoring endogenously produced H_2S in living cells. (iii) It can also detect endogenously generated H_2S in living C6 and BV-2 cell lines. (iv) Moreover, probe **3** is used for real time monitoring of H_2S production in normal phenotype *vs* cancer cells. (v) Probe **3** was also utilized for the detection of H_2S levels in the hippocampus region of rat brain after stimulating the tissue with cysteine and for the detection of H_2S in nematodes. We believe that probe **3** will help in real time visualization of H_2S receptors in normal and cancer cells.



Scheme 1. Synthesis of compound 2 and 3

Results and Discussion

The coupling of 4-bromobutyric acid with methyl protected fluorescein **1** in the presence of DCC and DMAP furnished the compound **2** in 37% yield (Scheme 1). The reaction of 2-bromoethanol with methyl protected fluorescein **1** in the presence of DIPEA and triphosgene furnished the compounds **3** in 32% yield (Scheme 1). The structure of compounds **2** and **3** were confirmed from their spectroscopic data.

The molecular recognition behaviour of probes **2** and **3** was studied towards different analytes (H_2S, GSH, Cys, HSO_3^-, SO_3^-,

$S_2O_3^{2-}$, H_2O_2, $^tBuO^{\cdot}$ and $^{\cdot}OH$) in H_2O: DMSO (99.5:0.5, v/v) buffered with HEPES, pH = 7.4 by UV-vis and fluorescence spectroscopy. The absorption spectrum of probe **3** (5.0 μM) exhibits two very weak absorption bands at 497 nm and 460 nm (Fig. S5). Upon addition of H_2S (NaHS was used as H_2S source) to the solution of probe **3**, an increase in the absorption band at 495 nm as a function of H_2S concentration was observed. Similar absorption behaviour was observed for probe **2** (Fig. S6). The fluorescence spectrum of probe **3** exhibits no emission band when excited at 470 nm (Fig. 1a). However, upon addition of H_2S (0–80 μM) to the solution of probe **3**, a new emission band

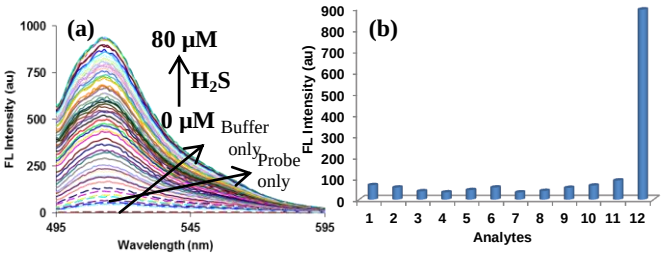
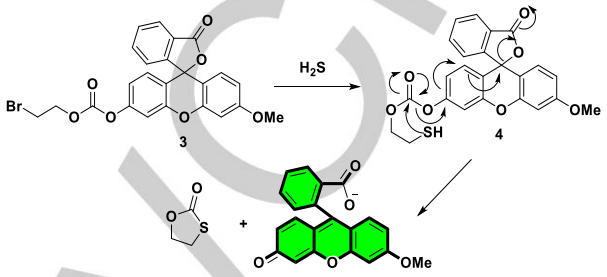


Figure 1. (a) Fluorescence emission spectra of probe **3** (1.0 μM) upon the addition of increasing concentration of H_2S (0–80 μM) in H_2O (0.5% DMSO used as co-solvent) buffer with HEPES (50 mM), pH=7.4. (b) Fluorescence change of probe **3** (1.0 μM) towards addition of different analytes (80 μM) in H_2O; 12 = H_2S, 11 = GSH (0.5 mM), 10 = Cys (0.5 mM), 9 = HSO_3^-, 8 = SO_3^-, 7 = $S_2O_3^{2-}$, 6 = H_2O_2, 5 = ^tBuO•, 4= HCO_3^-, 3= HO•,2= ClO^-, 1= 2-aminothiophenol.

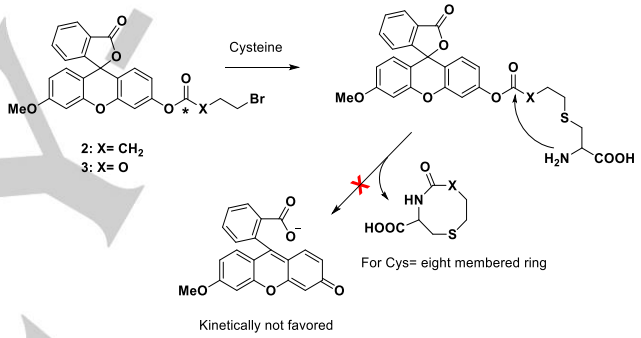
appears at 517 nm, the intensity of which increases with the increase in the concentration of H_2S. The fluorescence intensity of probe **3** increased dramatically by 32-folds on addition of H_2S (Fig. 1a). Similar fluorescence behaviour was observed for probe **2** (Fig. S7).

This increase in fluorescence emission at 517 nm with the addition of H_2S is attributed to double nucleophilic addition of H_2S on probe **3**. Initially, nucleophilic substitution (S_N2) mechanism operates where H_2S acts as a nucleophile and bromo group acts as leaving group.[7] After replacement of bromo group by SH (intermediate **4**), the 2nd nucleophilic substitution reaction operates and now thiol group of intermediate **4** acts as nucleophile which eliminates the thio-lactone and generates ring opened fluorescein moiety which is responsible for emission enhancement at 517 nm (Scheme 2).[8]

Under the same conditions as used for H_2S, we also carried out absorption and fluorescence studies of probes **2** and **3** with cysteine, no significant change in fluorescence emission was observed in the presence of cysteine. The selective response of probes **2** and **3** towards H_2S over cysteine is due to the rapid intramolecular attack of sulphur atom of thiol moiety to carbonyl carbon atom to form a five membered ring and ring opened fluorescein moiety (SI page S10). On the other hand on reaction with cysteine an intermediate **5** is formed in which intramolecular attack of nitrogen atom of amino group to carbonyl carbon does not takes place. This may be due to fact that formation of eight membered ring (**6**) is not kinetically favorable (Scheme 3 and SI page S10).[9] We have also carried out studies with reactive sulfur species like SO_3^-, $S_2O_3^{2-}$ and SO_4^{2-}; reactive oxygen species like H_2O_2, ^tBuO•, HO•, reducing agent like N_2H_4, NH_3 etc., biologically relevant metal ions (Na^+, K^+, Mg^{2+} and Ca^{2+}) (Fig. 1b) but no significant change was observed in the UV-visible and fluorescence spectra of these probes in the presence of these analytes except the response of probe **2** with N_2H_4 which shows increase in fluorescence emission at 517 nm in the presence of N_2H_4 (Fig. S8-S11). However, compound **3** does not show any significant fluorescence enhancement upon incremental addition of N_2H_4 which is probably due to the fact that the carbonate ester is less reactive towards basic analytes than the corresponding ester derivatives.[10] To test the practical applicability of probe **3** we also carried out the competitive experiments in the presence of H_2S; mixed with other analytes but no noticeable change in the fluorescence emission was observed in comparison with or without any other analytes (Fig. S12-13). Thus, from these



Scheme 2. Possible mechanism of H_2S induced ring opening of fluorescein moiety



Scheme 3. Possible mechanism of cysteine induced thioether formation

studies it is clear that only probe **3** is selective for H_2S. Thus, we carried out further studies using probe **3** only. We also studied the effect of pH on the recognition behaviour of probe **3** towards H_2S, which exists in equilibrium with HS^-/S^{2-} at pH 7. In basic pH (pH 7-10), probe **3** did not show fluorescence emission however fast fluorescence response was observed upon addition of H_2S as compared to fluorescence response in acidic pH (pH<6). This fast enhancement in fluorescence emission at pH 7-10 is due to the existence of H_2S as HS^- under basic conditions, which has more nucleophilicity in comparison to H_2S which exists in acidic pH (Fig. S14). From these studies, we may conclude that probe **3** does not show any interference of pH. Further, to experimentally verify the reaction mechanism, we carried out the mass spectral studies of probe **3** in the presence of H_2S. The appearance of peaks at m/z 347.08 and 105.09 (Fig. S15) in the mass spectrum confirms the formation of ring opened fluorescein moiety and 1,3-oxathiolan-2-one. Further, the ^1H NMR spectrum of probe **3** in the presence of H_2S also shows splitting and up field shift of aromatic protons which supports the formation of ring opened fluorescein moiety (Fig. S16). Next, we studied the time-dependent fluorescence response of the probe **3** in the presence of H_2S and cysteine. It was observed that within 10 min maximum fluorescence enhancement takes place in case of H_2S whereas no significant fluorescence

enhancement takes place in case of cysteine. This indicates the fast response of probe **3** towards H_2S (Fig. S17). The detection limit[11] of probe **3** for H_2S was found to be as low as 0.13 µM (Fig. S18), which is sufficiently low for monitoring H_2S level in biological systems. Further, to demonstrate the efficiency of probe **3** for quantitative detection of H_2S, we treated probe **3** with different concentrations of H_2S to obtain a standard curve of emission intensity *vs* H_2S concentration and regression analysis was done (Fig. S19). The concentration of probe **3** was maintained at 1.0 µM, while the concentration of H_2S varied from 0 to 80 µM. The regression curve indicates that the signal in the fluorescence spectrum was indeed linearly related to the concentration of H_2S. Thus, from these results, we may conclude that probe **3** can detect H_2S both qualitatively and quantitatively.

Having done all this, we were then interested to explore the applications of probe **3** in monitoring H_2S levels under different physiological conditions in living cells. For this, we used both *in vitro* (C6 Glioblastoma and BV-2 microglial cells) and *in vivo* rat model systems. We performed the MTT assay[12] to check whether probe **3** is non-toxic in nature. Cytotoxicity assay of the C6 glioma and BV-2 microglial cells treated with probe **3** showed that IC_{50} of the probe was around 2-4 µM and 1-2 µM for C6 glioma and BV-2 microglial cells respectively (Fig. S20). Based on this preliminary data, 1 and 0.75 µM concentrations of probe **3** were used for C6 glioma and BV-2 microglial cells respectively for further experiments. Firstly, we carried out exogenous detection of H_2S using probe **3**. For this, C6 glioma and BV-2 microglial cells were first exposed to 1.0 µM and 0.75 µM of probe **3** respectively for 1 hr, then exposed to 20.0 µM

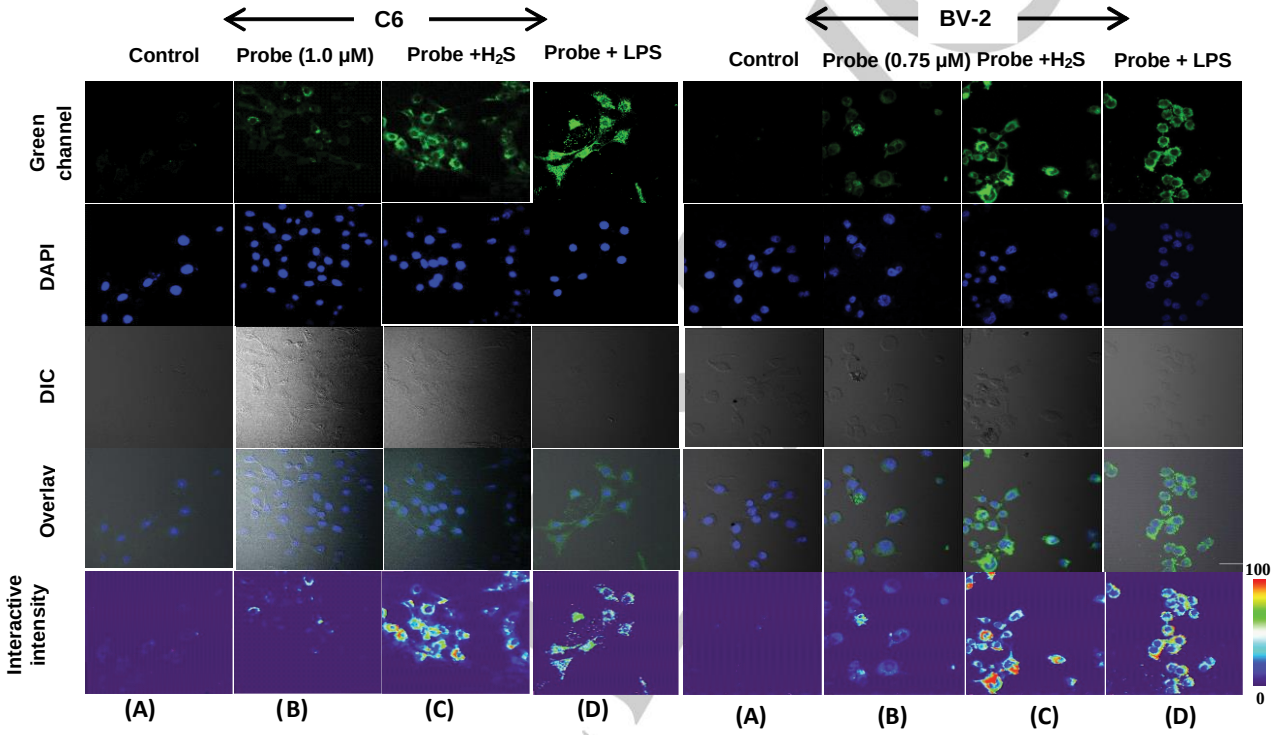


Figure 2. Confocal images of (I) C6 glioma and (II) BV-2 microglial cells treated with probe **3**: (Column A) Control (Column B) Probe alone: C6 glioma and BV-2 microglial cells were exposed to 1.0 µM and 0.75 µM of probe **3** respectively for 60 min at 37 °C. Column C: in it the probe **3** pre-treated cells were incubated with 20 µM of NaHS (exogenous source of H_2S) for another 60 min and column D: (I) C6 glioma and (II) BV-2 microglial cells were treated with LPS (2.0 µg/ml) and (100 ng/ml) for 24 hours respectively then incubated with probe **3** (1.0 µM for C6 glioma cells and 0.75 µM for BV-2 microglial cells) for another 60 min. Images were taken at λ_{ex} = 488 nm and λ_{em} range = 500-550 nm. Images were captured using A1R Nikon Confocal Microscope at 488 nm channel using 60X magnification.

exogenous H_2S source (NaHS) for another 1 hr. Cells treated with exogenous source of H_2S showed high fluorescence intensity than the probe alone as revealed by confocal imaging and intensity analysis (Fig. 2 & S21). Next, we studied the endogenous production of the H_2S in both C6 glioma and BV-2 microglial cells using activated bacterial cell wall component lipopolysaccharide (LPS). LPS is a known activator[13] that induces the production of H_2S by increasing cystathionine γ-lyase (CSE) expression.[14] For this, cells were pre-treated with LPS for 24 hrs and then exposed to probe **3** for 1 hr. Treatment with LPS increased the production of H_2S endogenously as indicated by increased fluorescent intensity in LPS treated cells in comparison to probe alone (Fig. 2 & S21).

Time dependent changes in the fluorescence of probe **3** were further monitored in the presence of H_2S in C6 glioma and BV-2 microglial cell lines, cells were pre-treated with probe **3** for 1 hr and then exposed to exogenous H_2S for different time intervals ranging from 0-60 min. Confocal imaging and intensity analysis showed the increase in fluorescence intensity of the probe **3** with increase in the exposure time of the exogenous H_2S source (Fig. 3). These studies clearly indicate that the probe **3** has the potential to monitor dynamic changes in H_2S levels in both cancer and normal cells. Further the specificity and selectivity of the probe **3** for H_2S was further confirmed by analyzing the fluorescence intensity of probe in the presence of the H_2S scavenger NEM (N-Ethylmaleimide). For this, H_2S scavenger treated C6 glioma

cells were exposed to probe **3** and change in fluorescent intensity was recorded at intervals of 10 min, for 60 min. The fluorescence intensity of the probe decreased by 56% in the presence of H_2S scavenger and was constant upto 50 min and then slightly increased at 60 min (Fig. S22). This slight increase in fluorescence emission at 60 min in comparison fluorescence emission at 50 min may be due to the more production of H_2S with time which reduces the quenching effect of the quencher and leads to higher availability of H_2S towards probe **3**. H_2S plays an important role in regulating CNS functions and has been reported to exert neuroprotective effects *via* various mechanisms like antioxidative, anti-inflammatory and anti-apoptotic.[15] In addition to its physiological role as a neuromodulator and neuroprotectant, higher concentration of H_2S is involved in the pathophysiology of the CNS[16]. For *in vivo* studies probe **3** was tested for monitoring the endogenous H_2S levels in dentate gyrus (DG) region of the hippocampus, a brain region which plays an important role in stress, depression, memory formation, and spatial behaviour.

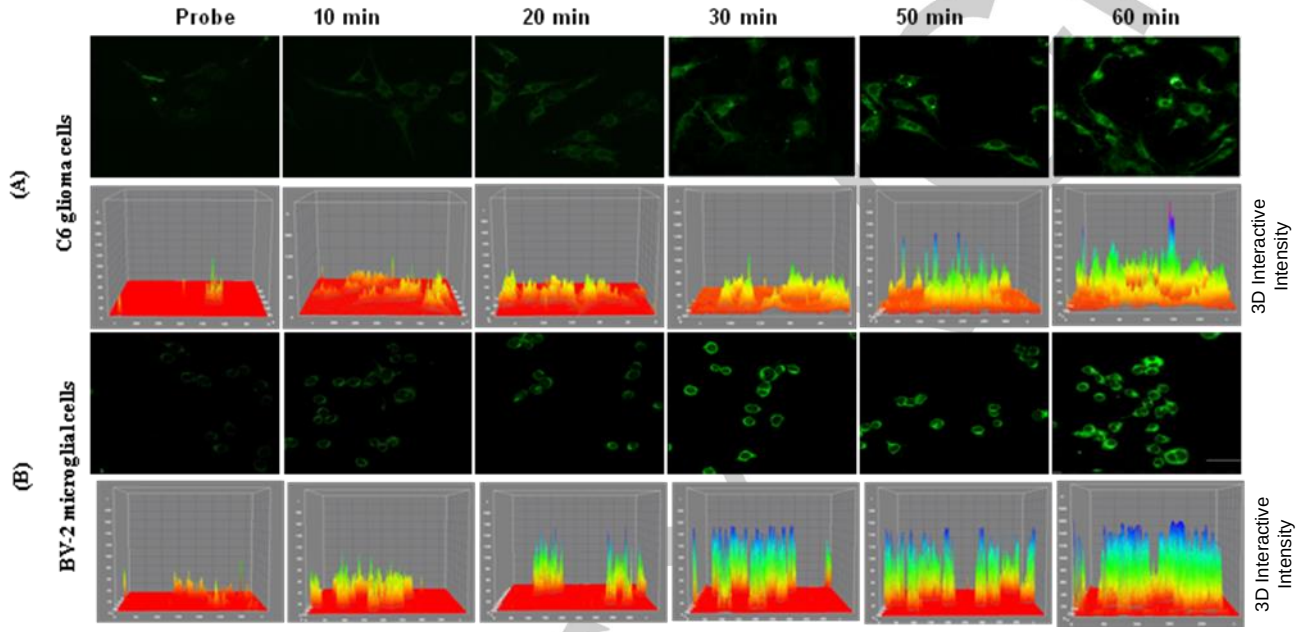


Figure 3. Confocal images of the (Row A) C6 glioma cells and (Row B) BV-2 microglial cells representing time dependent expression of the probe 3. Cells were first treated with probe **3**, and then incubated with H_2S (20 μM) for different time intervals of 10, 20, 30, 50, 60 min. Images were captured using the A1R Nikon Confocal Microscope at λ_{ex} = 488 nm (for probe) with λ_{em} range 500–550 nm.

To monitor H_2S levels in brain tissue, the brain was dissected and snap-frozen by using isopentane. Three groups were chosen; I, Probe alone: Brain sections (thickness: 60 μm)were incubated with probe **3** (5.0 μM) for 1 hr at 37 °C, II, Probe + H_2S: the probe **3** incubated sections were further treated with 20 μM of exogenous H_2S source (NaHS) for another 1 hr. III, Sections were first treated with 10 mM of endogenous source of H_2S, L-Cysteine (As Cysteine promoted the production of hydrogen sulfide in the brain tissue by stimulating CBS)[17,18] for 1 hr then exposed to probe for another 1 hr. Then the images were captured using confocal microscope at 488 nm wavelength. Tissue-imaging results showed increased fluorescence intensity in the sections treated with L-Cysteine and exogenous H_2S source as compared to probe **3** alone (Fig. 4 & S23).

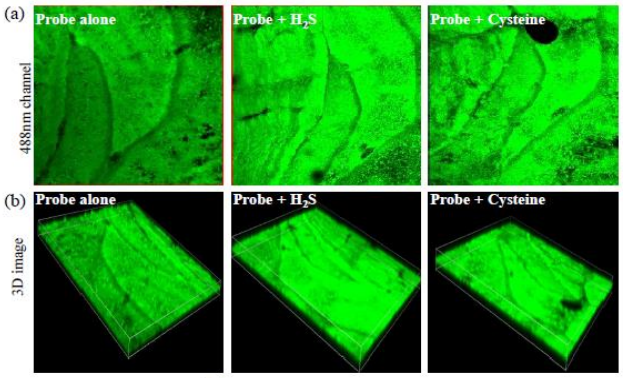


Figure 4. Fluorescent images of the 60 μm rat hippocampal slice labelled with 5.0 μM probe **3** (10X magnification). (a) Brain sections were incubated with 5.0 μM probe **3** for 1hr. (b) Probe pre-treated sections were exposed to 20 μM exogenous H_2S for 1hr. (c) Sections treated with 10 mM L-Cysteine for 1 hr then exposed to 5.0 μM probe **3** for 1hr. 3b represents the 3D images of the different treated groups.

From these tissue-imaging studies, it may be concluded that the probe **3** also has the potential to monitor hydrogen sulfide levels in tissues. Since the brain tissue is heterogeneous having different types of cells, optical sectioning was used to capture images at intervals of 5.0 μm along the z-axis to visualize the endogenous distribution of the H_2S in the brain tissue and the degree of penetration of probe **3** into the DG region of the brain slices at different depths.[19,20] The tissue images indicate that probe **3** has the ability to penetrate deeper inside the tissue. During sectioning, the cells on both the top and bottom surfaces of the brain slices may have been damaged due to slicing. An increase in fluorescence intensity in sections that were exposed to cysteine and the exogenous source of H_2S was observed in almost all the middle sections, thereby indicating that probe **3** effectively

monitored the production of hydrogen sulfide in the intact cells (Fig. S24).

Since the probe **3** could detect H_2S in living and intact cells at a depth of 60 µm, we envisaged that probe **3** may have the potential to detect H_2S under *in vivo* conditions as well and thus carried out *in vivo* studies on nematodes (*Distolabrellus species*) as *in vivo* model system.[21] To better understand the capability of probe **3** in tracing the accumulation of H_2S, *in vivo* imaging was done in nematodes. Confocal fluorescence imaging was examined by staining nematodes with probe **3** with different conditions. For detection of H_2S in nematodes, we choose three groups (i) probe alone: nematodes were exposed to 10.0 µM of probe **3** alone (ii) probe pre-treated nematodes were incubated with 0.3 mM exogenous H_2S source for 6 hrs; (iii) Firstly, the nematodes were incubated with 0.3 mM exogenous H_2S then 1 mM of NEM (N-Ethylmaleimide) was added and incubated for 6 hrs and thereafter exposed to the probe for another 3 hrs. The confocal images indicate that probe alone treated nematodes

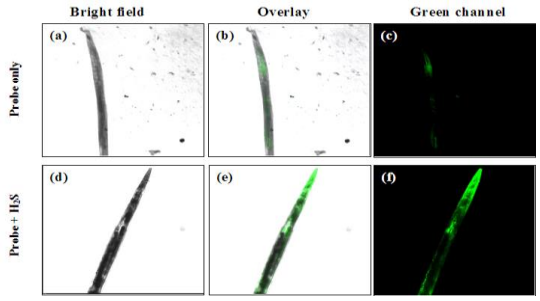


Figure 5. Confocal images of nematodes treated with probe **3** alone and probe **3** + H_2S. λ_{ex}= 488 nm channel using 10Xmagnification.

show very weak fluorescence emission (Fig. 5c). Exposure to exogenous H_2S source caused the increase in the fluorescence intensity of probe, which indicates that our probe **3** can be used for real time monitoring of H_2S in living systems (Fig. 5f). Moreover, the treatment with NEM caused the decrease in the fluorescence of probe **3** (Fig. S25). These studies clearly indicate that the higher fluorescence is due to the reaction with H_2S. From the above studies it is clear that probe is an efficient tool for monitoring H_2S in *in vivo* system.

Conclusions

In conclusion, we designed and synthesized probe **3** which is highly selective for H_2S and is used for monitoring exogenous and endogenous H_2S in both cancer and normal cells. Further, probe **3** was applied for imaging of H_2S in living tissues at variable depths and in nematodes. These studies designate probe **3** as an efficient platform for monitoring H_2S in living systems and it may be a useful tool for monitoring H_2S-related pathological responses in tissues as well as in *in vivo* system.

Experimental Section

Materials and Instrumentation:

All reagents were purchased from Aldrich and were used without further purification. HPLC grade DMSO 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. ^{1}H and ^{13}C spectra were recorded on a Bruker Avance III HD 500 MHz spectrophotometer using $CDCl_3$ and DMSO-d_6 as solvents and tetramethylsilane as the internal standard. Mass spectra were recorded on a Bruker MicroTOF QII mass spectrometer. 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).

Synthesis of Probe 2

Compound 1 (0.400 g, 1.156 mmol) dissolved in THF (10 ml) was added dropwise to a solution of 4-bromobutyric acid (0.212 g, 1.271 mmol), DCC (0.357 g, 1.734 mmol) and DMAP (0.014 g, 0.12 mmol) in THF (20 ml). After the addition the reaction mixture was further stirred at room temperature for overnight. The solvent was then evaporated under reduced pressure and the crude product was purified by column chromatography to give **2** as yellow solid in 37% yield (212 mg). ^{1}H NMR (DMSO-d_6, 500 MHz): 2.28 (m, 2H, CH_2), 2.81 (t, *J* = 7.5 Hz, 2 H, CH_2), 3.63 (t, *J* = 5 Hz, 2H, CH_2), 3.85 (s, 3H, CH_3), 6.70 (s, 1H, Ar-H), 6.76 (m, 1H, Ar-H), 6.85 (d, *J* = 10 Hz, 1H, Ar-H), 6.94 (m, 2H, Ar-H), 7.28 (s, 1H, Ar-H), 7.33 (d, *J* = 5 Hz, 1H, Ar-H), 7.76 (t, *J* = 7.5 Hz, 1H, Ar-H), 7.82 (t, *J* = 5 Hz, 1H, Ar-H), 8.04 (d, *J* = 5 Hz, 1H, Ar-H). ^{13}C NMR (DMSO-d_6, 125 MHz): 27.89, 32.61, 33.79, 56.19, 82.76, 101.18, 110.83, 110.99, 112.82, 116.88, 118.74, 124.49, 125.37, 126.10, 129.54, 130.89, 136.37, 151.48, 151.99, 152.26, 152.76, 161.68, 169.00, 171.13. MS (ESI) Calcd. for $C_{25}H_{19}O_6Br$: 494.03; Found: 495.0285 (^{81}Br isotope) and 497.0279 (^{83}Br isotope) [M+1].

Synthesis of Probe 3:

To a stirred solution of 2-bromoethanol (72 mg, 0.58 mmol) and DIPEA (290 mg) in dry DCM (10.0 mL) was added triphosgene (172 mg, 0.58 mmol). The reaction mixture was stirred at 0 °C under N_2 atmosphere. After 2 hours, the excess phosgene was removed from the reaction mixture by N_2 purge. Then the compound 1 (200 mg, 0.58 mmol) dissolved in dry dichloromethane with few drops of dry DMF was added in the reaction mixture and kept stirring for 2 days. After completion of the reaction (by TLC), the reaction mixture was diluted with EtOAc, washed twice with brine, dried over sodium sulphate, then filtered and the solvent was removed under reduced pressure. The crude product was purified by column chromatography to give probe **3** as orange yellowish solid in 32% yield (92 mg). ^{1}H NMR (500 MHz, DMSO-d_6): δ = 2.77 (t, *J* = 5 Hz, 2H, CH_2Br), 3.83 (s, 3H, CH_3), 4.26 (t, *J* = 5 Hz, 2H, CH_2CO), 6.70-6.75 (m, 2H, Ar-H), 6.86 (d, 1H, Ar-H), 6.94-6.97 (m, 2H, Ar-H), 7.28 (d, *J* = 5 Hz, 1H, Ar-H), 7.34 (d, *J* = 5 Hz, 1H, Ar-H), 7.75 (t, *J* = 5 Hz, 1H, Ar-H), 7.80 (d, *J* = 7.5 Hz, 1H, Ar-H), 8.04 (d, *J* = 5 Hz, 1H, Ar-H). ^{13}C NMR (DMSO-d_6 125 MHz): δ = 37.92, 56.20, 79.63, 101.30, 110.81, 111.05, 112.82, 116.99, 124.50, 125.36, 126.14, 129.48, 129.54, 130.88, 136.34, 151.53, 152.04, 152.28, 161.71, 168.96, 171.10. ESI-MS Calcd. for $C_{24}H_{17}O_7Br$: 496.0158 (^{81}Br) and 498.0137(^{83}Br); Found: 499.03 [M+1] for (^{83}Br) and 497.03 (^{81}Br).

Optical Studies:

UV-vis and fluorescence titrations were performed on 5.0 and 1.0 µM solution of ligand in H_2O (buffered with HEPES, pH = 7.4; at 25 °C) mixture. Typically, aliquots of freshly prepared $M(ClO_4)_n$ (M = Cu^{2+}, Zn^{2+}, Fe^{3+}, Fe^{2+}, Mg^{2+}, Ca^{2+}, Na^+ and K^+; n = 1 or 2 or 3), anions (SO_3^- as $NaSO_3$; $S_2O_3^{2-}$ as $Na_2S_2O_3$; SO_4^{2-} as Na_2SO_4; HCO_3^- as $NaHCO_3$), sulphur species such as H_2S as NaHS, cysteine, glutathione and ROS such as H_2O_2, OCl^- standard solutions (10^{-1} M to 10^{-3} M) were added to record the UV-vis and fluorescence spectra.

In titration experiments, each time a 3 ml solution of ligand was taken in a quartz cuvette (path length, 1 cm) and spectra were recorded after the addition of appropriate analytes.

Imaging of H_2S in living cells:

C6 glioma cells and BV-2 microglial cells was obtained from National Centre for Cell Sciences, Pune, and National Brain Research Center, Manesar, India respectively. Cells were maintained in DMEM supplemented with 1X PSN (GIBCO), 10% FBS (Biological Industries) at 37°C and humid environment containing 5% CO_2. For fluorescence detection, cells were seeded on 18 mm coverslips in 12 well plates. For the purpose of the study, three groups were chosen: (I) Control group; (II) Group in which Cells were treated with probe only; (III) Exogenous H_2S detection: Cells were pre-incubated with probe for 1 hr then treated with the 20.0 μM exogenous H_2S source- sodium hydrogen sulfide (NaHS); (IV) Endogenous H_2S detection: cells were treated with LPS (2.0 μg/ml for C6 glioma cells and 100 ng/ml for BV-2 microglial cells) for 24 hrs then exposed to the probe **3** for 1hour. (Concentration of the probe for C6 glioma cells was 1.0 μM and for BV-2 microglial cells was 0.75 μM).

Imaging of H_2S in Tissue:

Wistar strain rat was decapitated and its brain was carefully dissected. The freshly dissected brain was submerged in cryomatrix inside the mold and then snap-frozen in chilled isopentane for 5 min. Then, the cryomatrix-embedded brain was carefully removed from the mold, mounted, and thick coronal sections (60 μm) were cut directly on the microscopic glass slide by using a freezing cryomicrotome. For staining, the sections were washed three times with 1X PBS, for 5 min each time. For the analysis, the sections were divided into four groups: 1) a control group; 2) the brain sections were incubated with probe **3** alone (5.0 μM) for 1 h at 37 °C; 3) the sections were pre-treated with probe **3** and then exposed to an exogenous H_2S source for 1 h; 4) the sections were first treated with 10 mM L-Cysteine for 1 hour and then exposed to probe **3** for another hour. After treatment, images were recorded by using an A1R Nikon Laser Scanning Confocal microscope at 10X magnification at the 488 nm channel. Optical sectioning was performed to observe the changes in fluorescent intensity on changing the depth along the z axis at thickness intervals of 5.0 μm.

Formal permission to conduct animal experiments was obtained from the Institutional Animal Ethical committee, Reg. No. of Animal house: 226/CPCSEA.

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Keywords: Sensing • fluorescent probe • hydrogen sulfide • imaging in cells • detection in tissue and nematodes

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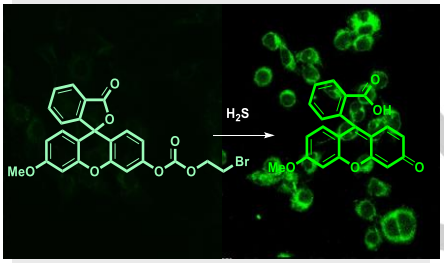
Layout 1:

FULL PAPER

A fluorescein based fluorescent probe **3** has been designed and synthesized which selectively detects H_2S in aqueous medium among the various analytes tested. The probe **3** was also successfully utilized for real time imaging of exogenous and endogenously produced H_2S in cancer cells, normal cells. Moreover, the probe **3** can also detects H_2S in rat brain hippocampus in variable depth and living nematodes.



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A Highly Selective Fluorescent Probe for Detection of Hydrogen Sulfide in Living Systems: *in vitro* and *in vivo* Applications