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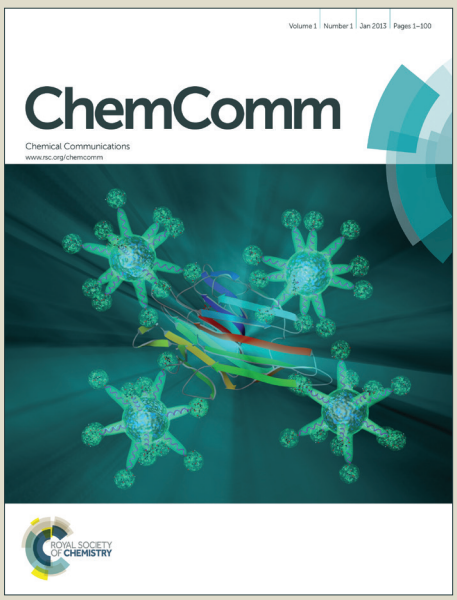

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A bodipy based dual functional probe for the detection of hydrogen sulfide and H_2S induced apoptosis in cellular system

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Cite this: DOI: 10.1039/x0xx00000x Received 00th January 2012,
Accepted 00th January 2012DOI: 10.1039/x0xx00000x
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A bodipy based dual functional probe 1 has been designed and synthesized which selectively detects H_2S as well as monitors H_2S induced apoptosis in cells.

Hydrogen sulfide (H_2S), being a gaseous signalling molecule is involved in a wide range of physiological and pathological responses. The physiological concentration of H_2S ranges from nano to milli-molar levels.[1] Once this concentration level of H_2S is disturbed, it leads to various diseases like chronic kidney disease, liver cirrhosis, Down's syndrome and Alzheimer's disease.[2] H_2S is also a well-known apoptosis inducer[3] and activates the key apoptotic enzymes caspase-9, caspase-8, and caspase-3[3c-4] and initiates cell death by causing nuclear condensation and cytoplasmic shrinkage which ultimately leads to increase in intracellular viscosity.[5] The apoptosis inducing property of H_2S is vital to study as it is also linked with anti-cancer properties of H_2S.[6] Thus, visualization and monitoring of H_2S induced apoptosis in cellular systems would be important to understand the biological effects of H_2S.

Recently, a number of fluorescent probes have been reported for the detection of H_2S but most of these probes suffer from the limitations of poor detection limit and their biological application is limited to exogenous detection of H_2S only and furthermore, in none of these reports H_2S induced apoptosis has been studied[7]. Based on these observations, we were then interested to design a probe which performs dual function *i.e.,* detects H_2S as well as monitors H_2S induced apoptosis in cellular systems. For achieving this target, we envisaged that if we could design a molecule that have molecular rotation property and an effective H_2S reaction centre then it will be able to perform dual function. The molecular rotation property is required to monitor H_2S induced apoptosis as the rotation gets restricted in viscous medium created during apoptosis.[8] Keeping this in mind, we designed and synthesized a meso-substituted bodipy derivative **1** which has a phenyl ring connected to bodipy core. Further, an azide group is introduced in this derivative as it is known to undergo reduction in the presence of H_2S. We envisaged that the resulting reduced product will act as a fluorescence molecular rotor (FMR) since it will have donor-π-spacer-acceptor arrangement,[8c,9] a pre-requisite for a molecule to behave as FMR. To our pleasure, the reduced product **2** responded to changes in medium viscosity and acts as fluorescent molecular rotor. To the best of our knowledge this is the first report where H_2S induced apoptosis phenomenon is investigated by a fluorescent probe. Moreover, the designed probe **1** has many advantages: (i) It performs dual function *i.e.,* detects H_2S as well as monitors H_2S induced apoptosis. (ii) Probe **1** can detect H_2S upto 35 nM, which is one of the best among the reported probes. (iii) It can detect both exogenous and endogenously generated H_2S in C6 glial cells. (iv) Probe **1** undergoes complete intracellular reduction with exogenous and endogenous H_2S which was proved by carrying out mass spectral studies of cell fluid.

The synthesis of the probe **1** is given in scheme S1 (ESI†, Page S5-S6) and structure of the probe **1** was confirmed by its spectroscopic data (ESI†, Fig. S1 to S8). The molecular recognition behaviour of probe **1** towards H_2S was studied by UV-vis and fluorescence spectroscopy in H_2O: DMSO (8:2, v/v) buffered with HEPES, pH = 7.02. The absorption spectrum of probe **1** (5.0 μM) exhibits two absorption bands at 495 nm and 350 nm (ESI†, Fig. S9 and S10) corresponding to the S_0-S_1 and S_0-S_2 transitions with molar extinction coefficients of 4.65 $\times 10^4$ and 1.45×10^4 M^{-1} cm^{-1} respectively.

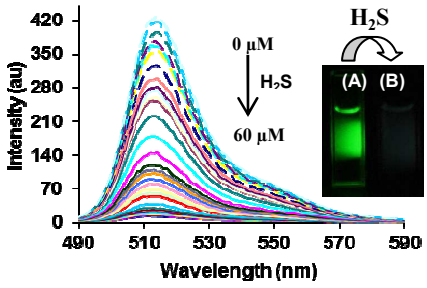
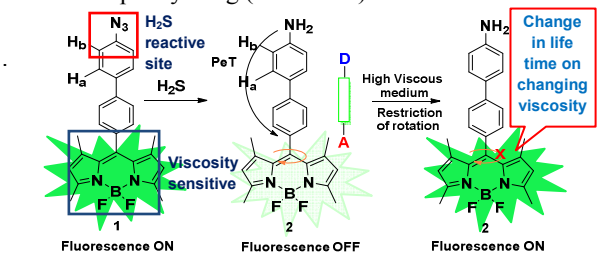


Fig. 1: Fluorescence response of probe **1** (5.0 μM) in H_2O: DMSO (8:2, v/v) buffered with HEPES, pH=7.02; on addition of H_2S (0-60 μM); λ_{ex}= 470 nm and λ_{em} = 515 nm.

Upon addition of H_2S (Na_2S was used as H_2S source) to the solution of probe **1**, the decrease in the absorption band at 495 nm as a function of H_2S concentration is observed (ESI†, Fig. S11). The fluorescence spectrum of probe **1** exhibits an emission band at 515 nm when excited at 470 nm. Upon addition of H_2S (0-60 μM) to the solution of probe **1** the emission band at 515 nm decreases with the increase in the concentration of H_2S (**Fig. 1**). This decrease in fluorescence emission with addition of H_2S at 515 nm is attributed to (i) photo-induced electron transfer (PeT) from nitrogen atom to photo-excited bodipy moiety which leads to quenching of fluorescence emission. (ii) The product formed after reduction acts as fluorescent molecular rotor and the energy is dissipated by non-radiative pathways due to the free rotation of meso-substituted phenyl ring (**Scheme 1**).



Scheme 1: Designing strategy for monitoring H_2S and H_2S induced apoptosis.

To investigate whether the probe **1** can selectively detect H_2S under physiological conditions, the absorption and fluorescence response of the probe **1** was also studied with reactive oxygen species like ClO^-, H_2O_2, $^tBuO•$, HO• and biothiols like cysteine, glutathione and homocysteine but no significant change was observed in the presence of these analytes in the UV-visible and fluorescence spectra (ESI†, Fig. S12, S13, S14 and S15). To test the practical applicability of the probe **1** 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 (ESI†, Fig. S16). Thus, probe **1** acts as an efficient fluorescent probe for the selective detection of H_2S in mixed aqueous environment. Further, to confirm the reduction of probe **1**, we also carried out the mass and ^1H NMR studies of probe **1** in the presence of H_2S. The appearance of a peak at m/z 416.21 (ESI†, Fig. S17) in the mass spectrum confirms the formation of reduced product **2**. The ^1H NMR spectrum shows an appearance of new peak at 3.7 ppm corresponding to amino protons and up-field shift of 0.1 and 0.4 ppm for the H_a and H_b protons which confirms the conversion of azide group to amino moiety in the presence of H_2S (ESI†, Fig. S18 and S19). We also studied the effect of pH on the recognition behavior of probe **1** towards H_2S which exists in equilibrium with HS^-/ S^{2-} at pH 7. It was found that fluorescence quenching occurs at a faster rate in basic pH than in acidic pH (ESI†, Fig. S20). This fast quenching in fluorescence emission at pH > 7 is due to the existence of H_2S as HS^- under basic conditions which has more reducing power in comparison to H_2S which exists in acidic pH.[7e,10] Further, we also studied the effect of pH on the fluorescence behaviour of probe **2**. There was a negligible fluorescence enhancement on lowering the pH from 7.4 to 4 (ESI†, Fig. S21a). On further lowering the pH from 4 to pH 2, there was slight enhancement in fluorescence intensity of probe **2** (0.91 folds). To find out the reason why there was a negligible fluorescence enhancement on lowering the pH from 7.4 to 4, we determined the p*Ka* value of probe **2** which was found to be 3.87 (ESI†, Fig. S21b). From this p*Ka* value and pH studies, it is clear that probe **2** is not much sensitive to the pH of the environment.[11,13]

Further, we also studied time dependent response of the probe **1** towards H_2S. It was observed that 80% of fluorescence quenching takes place within 20 min of addition of 60 μM of H_2S at a time (ESI†, Fig. S22). The time dependent fluorescence quenching indicates that probe **1** is an efficient probe for monitoring the changes in H_2S level in living systems. The detection limit of probe **1** for H_2S was found to be 35 nM (ESI†, Fig. S23). Having done all this, we were then interested to study the H_2S induced apoptosis and for this, we studied the fluorescence and fluorescence lifetime behaviour of probe **2** in different methanol: glycerol fractions and it was observed that the fluorescence intensity and decay time increases with increase in viscosity of the medium which proves that reduced product probe **2** acts as FMR (ESI†, Fig. S24 and S25). We then studied the fluorescence spectra of probe **1** in the presence of H_2S (60 μM for 30 min) in different methanol: glycerol fractions. A significant increase in fluorescence intensity was observed with increasing glycerol fractions (ESI†, Fig. S26). However, in the absence of H_2S no significant enhancement in fluorescent intensity and fluorescence lifetime of probe **1** was observed with increasing glycerol fractions (ESI†, Fig. S27 and S28). These results clearly indicate that the fluorescence enhancement is due to reduced product **2** which may be used for monitoring H_2S induced apoptosis in biological systems.

Since, it has been reported that viscosity increases during apoptosis in biological systems,[5b,12] so we were interested to study the H_2S induced apoptosis in C6 glial cells. At first, we performed MTT assay which showed that probe **1** is non-toxic in nature and may be safely used for biological studies (ESI†, Fig S29). Initially, we carried out experiment for exogenous detection of H_2S and for this purpose; the cells were first incubated with probe **1** for 20 min at 37 ºC and then treated with 20 μM H_2S for 20 min. The quenching of fluorescence emission was observed in cells, which was due to the reduction.

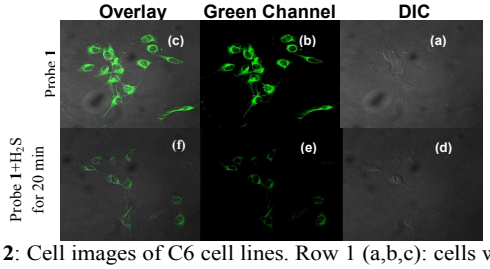


Fig. 2: Cell images of C6 cell lines. Row 1 (a,b,c): cells were treated with probe **1** (1.0 μM) for 20 min at 37 °C. Row 2 (d,e,f); cells were treated with probe **1** (1.0 μM) for 20 min and then incubated with H_2S (20 μM) for 20 min. Images were taken at λ_{ex} =488 nm and λ_{em} range 500-550 nm. All images are taken at 40X magnification.

of azide group to amino moiety (**Fig. 2**) and (ESI†, Fig. S30). Further to monitor the H_2S induced apoptosis; we increased the incubation period and concentration of H_2S in cells. When we increased the incubation period of H_2S from 20 to 60 min, then instead of quenching, an enhancement in fluorescence emission intensity was observed (**Fig. 3**) and (ESI†, Fig. S30). From the above results, it may be concluded that when cells were incubated with exogenous H_2S for longer period then apoptosis gets initiated in cells and as a result of which, the intracellular viscosity gets increased leading to enhancement in fluorescence emission of reduced product **2** *via* restriction of rotation. Further, on increasing the concentration of H_2S from 20 μM to 100 μM and incubation for 20 min, again fluorescence enhancement was noticed in C6 cells (ESI†, Fig. S31) which may be due to the fact that higher concentration of H_2S enhanced the rate of apoptosis. Thus, we observed an enhancement in fluorescence intensity in both cases *i.e.,* on longer incubation period of H_2S and at higher concentration of H_2S. On the other hand, the other possibility of increase in fluorescence emission in cells may be due to the binding of azide moiety of probe **1** with intracellular proteins which will prevent azide to undergo reduction in the presence of H_2S.

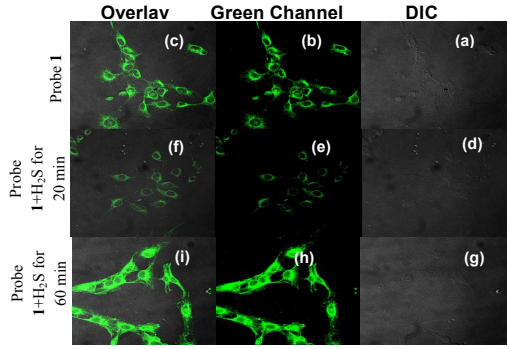


Fig. 3: Cell images of C6 cell lines. Row 1 (a,b,c): cells were treated with probe **1** (1.0 μM) for 20 min at 37 °C. Row 2 (d,e,f): cells were treated with probe **1** (1.0 μM) for 20 min and then incubated with H_2S (20 μM) for 20 min. Row 3 (g,h,i): cells were treated with probe **1** (1.0 μM) for 20 min and then incubated with H_2S (20 μM) for 60 min. Images were taken at λ_{ex} =488 nm and λ_{em} range 500-550 nm.

To rule out this possibility, we carried out mass spectral studies of cell cytosol. Cells were treated with probe **1** and incubated with 20 μM of H_2S for 30 min and then cells were lysed and centrifuged to obtain the cell supernatant. The mass spectrum of this supernatant solution showed a peak at 416.23 corresponding to reduced product **2** (**Fig. 4**) and (ESI†, Fig. S32).

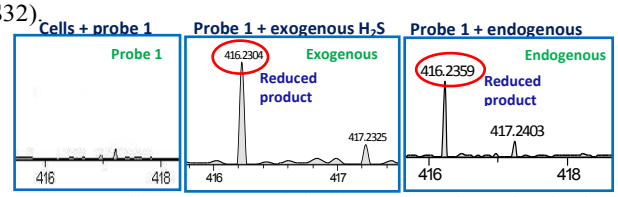


Fig 4: Intracellular mass analysis for confirmation of reduction of probe **1** with exogenous and endogenous sources of H_2S in cells.

Thus, the mass spectral studies prove that probe **1** undergoes reduction with H_2S in cells and thus rule out the possibility of binding of azide to proteins. Further, to detect endogenously generated H_2S in living cells, the cells were stimulated with 2μg/ml lipopolysaccharide (LPS) for 24 hrs. LPS stimulated cells were then incubated with probe **1** for 20 min and cell imaging was performed. The cell imaging results showed an enhancement in fluorescence emission instead of quenching (**Fig. 5**) and (ESI†, Fig. S33). The enhancement in fluorescence intensity is due to the fact that LPS treatment stimulated cells to generate H_2S and this process continued for 24 hrs and during this time, the endogenously generated H_2S initiates apoptosis in cells as a result of which the viscosity of cells increases leading to enhancement in fluorescence emission of probe **2**. Further, to confirm the reduction of azide in cells by LPS stimulation, we carried out mass spectral studies of cells and observed a peak at m/z 416.23 which proved that the reduced product **2** is formed by reduction of probe **1** by endogenously generated H_2S (**Fig. 4**). Thus, from the above results, we propose that probe **1** shows turn off response with H_2S before apoptosis and turn on response after initiation of apoptosis.

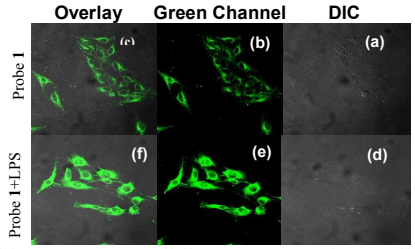


Fig. 5: Cell images of C6 cell lines. Row 1 (a,b,c): cells were treated with probe **1** (1.0 μM) for 20 min at 37 °C. Row 2 (d,e,f); cells were stimulated with LPS (2μg/ml LPS) for 24 hrs at 37 °C and then treatment with probe **1** (1.0 μM) 20 min. Images were taken at λ_{ex} =488 nm and λ_{em} range 500-550 nm.

Further, to confirm that the fluorescence turn on response is due to H_2S induced apoptosis, we performed nuclear condensation studies by staining the cells with DAPI (4',6-diamidino-2-phenylindole), which selectively stains the nucleus and the changes in nuclear morphology during apoptosis could be observed by this stain[12]. The cells were first incubated with

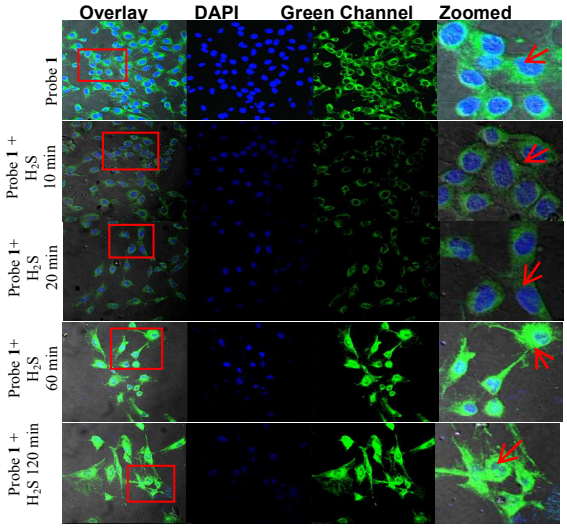


Fig. 6: Cell images of C6 cell lines showing nuclear condensation by H_2S induced apoptosis. Cells were first treated with probe **1** (1.0 μM) then incubated with H_2S (20 μM) for different time intervals of 10, 20, 60, 120 min. Images were taken at λ_{ex} =488 nm (for probe) and 405 nm (for DAPI) with λ_{em} range 500-550 and 430-480 nm.

probe **1** for 20 min and then treated with H_2S for different time intervals of 10, 20, 60 and 120 min. Finally, cells were washed and then stained with DAPI, which selectively stains the nucleus and depicts the changes in nucleus morphology associated with apoptosis. Cell imaging results showed that upto 20 min, no significant change in nuclear morphology was observed and the fluorescence intensity of cells decreased which is due to the reduction of azide to amine. Further, on increasing the incubation period to 60 and 120 min, the cells showed prominent nuclear condensation as well as increase in fluorescence intensity (**Fig. 6**). The intensity analysis also showed that at 120 min maximum fluorescence intensity was observed (ESI†, Fig. S34). The nuclear condensation proves that cells underwent apoptosis. We also carried out fluorescence lifetime imaging (FLIM) studies to prove the phenomenon of apoptosis. FLIM studies were carried out in C6 glial cells in the presence of probe **1** and then incubating the cells with 20 μM of H_2S for different time intervals of 10, 20, 60 and 120 min. We observed that upto 20 min, no significant change in decay time was observed in comparison to control (cells incubated with only probe **1**). Further, on increasing the incubation time to 60 and 120 min, the decay time was increased dramatically (**Fig. 7** and ESI†, Fig. S35). This result justifies that the reduced product **2** acts as FMR and shows an

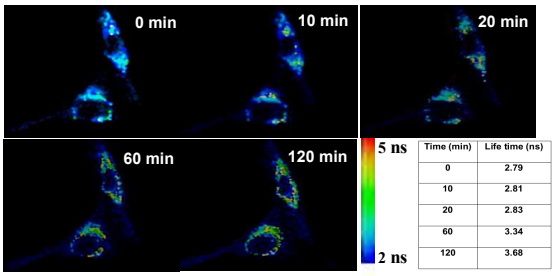


Fig. 7: Fluorescence life time imaging (FLIM) in C6 cell lines showing change in life time of probe **1** by H_2S induced apoptosis. Figure showing the changes in life time when the cells treated with probe **1** (1.0 μM) then incubated with H_2S (20 μM) for different time intervals of 10, 20, 60, 120 min. Images were taken at λ_{ex} =488 nm (for probe) with λ_{em} range 500-550 nm.

increase in decay time with increasing viscosity during apoptosis[13]. Therefore, the reported probe **1** is an efficient tool for detecting H_2S as well as for monitoring H_2S induced apoptosis phenomenon in living cells.

In conclusion, we designed and synthesized a bodipy based dual functional fluorescent probe **1** which selectively detects H_2S and can also monitor H_2S induced apoptosis in cellular systems. Further, the potential of the probe **1** to explore H_2S induced apoptosis phenomenon was studied by FLIM studies which showed that during apoptosis the decay time of probe **2** (amine) increases due to restriction of rotation in viscous intracellular matrix. Hence, the designed probe **1** reported herein is an efficient tool for the detection of H_2S and H_2S induced apoptosis in cellular system and will attract more attention of the scientists to discover more sensors which can detect H_2S as well as monitor H_2S induced apoptosis.

MK & VB acknowledge the financial support from DST and CSIR, New Delhi. NG, SIR and MG acknowledges UPE/UGC/CSIR program for their fellowship. UGC, grants under UPE and CPEPA schemes and DBT, GOI grant under Sub-DIC are acknowledged for providing infrastructural facilities to carry out the research work.

Notes and references

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Electronic Supplementary Information (ESI) available: [Experimental details, NMR, mass spectra, fluorescence spectra and time resolved fluorescence spectra]. See DOI: 10.1039/c000000x/

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