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ARTICLE TYPE

d-PET coupled ESIPT phenomenon for fluorescent turn-on detection of hydrogen sulfide

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A d-PET coupled ESIPT based probe has been designed and synthesized for the selective detection of H_2S among the other ions and various sulfur species such as cysteine, glutathione in aqueous media *via* the hydrogen sulfide induced reduction of nitro functionality and have further been utilized for the imaging of H_2S in intracellular systems.

Introduction

Hydrogen sulfide (H_2S) is an endogenously produced gaseous molecule along with nitric oxide (NO), carbon monoxide (CO) which induces unique signaling response in cellular system.[1] The production of H_2S is enzymatically regulated in mammals which led to increased interest to evaluate the biological as well as physiological role of H_2S. For example, it acts as a physiological vasodilator, signal transmitter in the brain and in antitumor activity of the immune system. Further, H_2S is considered as highly toxic and hazardous pollutant to the environment besides its numerous physiological functions. Any imbalance in the H_2S has also been recognized to mediate a wide effects of physiological processes, such as vasodilation, anti-oxidation, anti-apoptosis, anti-inflammation and the abnormal H_2S level was connected to diseases such as Alzheimer's and Downs syndrome including carcinogenesis as well as neurodegradation.[2] Thus, from both physiological and pathological point of view, it is desirable to develop sensitive, specific and rapid techniques for the detection of hydrogen sulfide. A number of techniques including polarography, gas chromatography and colorimetry are available for the detection of gasotransmitters.[3] However, these techniques require complicated procedures which did not allow the temporal monitoring and are destructive in nature. However, the use of fluorescence approach provides unique advantages including high sensitivity and simplicity.[4] Moreover fluorescence imaging is the best technique for the determination and measurement of intracellular molecules without the destruction of tissues or cells which makes fluorescence approach superior to other analytical methods.[5] In literature a few fluorescent chemosensors for H_2S have been reported which involve H_2S mediated reduction of azides to amines,[6] nucleophilic addition of H_2S[7] and copper sulfide precipitation approach[8] but, H_2S promoted reduction of nitro group to amino group has not been explored much.[9] Further, a key challenge for the selective detection of H_2S within the cellular system is comparatively high concentrations of biological sulfur species such as glutathione and cysteine.

Our research work involves the design and synthesis of fluorescent chemosensors for the detection of soft metal ions and evaluation of their switching behaviour.[10] Recently, we reported a charge transfer assisted fluorescent probe for selective detection of hydrogen peroxide.[11] In continuation of this work, we have now synthesized compound **2** based on the 2-hydroxynaphthalene moiety appended with nitrophenylimino functionality as a fluorescence turn-on chemosensor for H_2S under physiological conditions. Furthermore, biological application of probe **2** is evaluated for *in vitro* detection of H_2S in prostate cancer (PC3) cell lines. We envisioned that the fluorescence properties of compound **2** could be modulated *via* a photo-induced electron transfer (PET) process from the excited fluorophore to a strong electron-withdrawing group (donor-excited PET or d-PET).[12] Therefore, nitro group, a strong electron-withdrawing moiety was selected as the signal modulator for the probe **2**. The presence of nitro group favours the d-PET from the excited fluorophore to the nitro group responsible for the quenched fluorescence emission in the aqueous media. The nitro group can be reduced to amino group under physiological conditions by H_2S which will then trammel the d-PET mechanism and thus will result in the enhancement of fluorescence emission owing to the excited state intramolecular proton transfer (ESIPT)[13] process.

Experimental

General information

All reagents were purchased from Aldrich and were used without further purification. Acetonitrile (AR grade) was used to perform analytical 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 25^0C. The fluorescence spectra were recorded with a SHIMADZU 5301 PC spectrofluorimeter. ^{1}H and ^{13}C spectra were recorded on a JEOL-FT NMR-AL 300 MHz spectrophotometer using $CDCl_3$ as a solvent and tetramethylsilane as the internal standard. Data are

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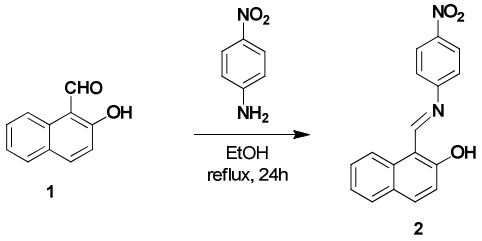
reported as follows: chemical shift in ppm (d), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad singlet), coupling constants *J* (Hz), integration and interpretation.

UV-vis and fluorescence titrations

UV-vis and fluorescence titrations were performed on 5.0 μM solution of ligand in H_2O:CH_3CN (99.5:0.5, v/v) buffered with HEPES, pH = 7.4; λ_{ex} = 320 nm. Typically, aliquots of freshly prepared anions (Cl^-, F^-, Br^-, I^-, CN^- and AcO^- as tetrabutylammonium salt; CO_3^{2-} as K_2CO_3; N_3^- as NaN_3), H_2S as Na_2S; Cysteine and Glutathione standard solutions (10^{-1} M to 10^{-3} M) were added to record the UV-vis and fluorescence spectra. Hydrogen peroxide (H_2O_2) and hypochlorite (OCl^-) were delivered from 30% and 5% aqueous solutions, 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.



Scheme 1 Synthesis of compound **2**.

Synthesis of compound 2

A mixture of compound 1 (0.1 g) and *p*-nitroaniline (0.08 g) were dissolved in ethanol and refluxed for 24 h at 80-90˚C. After the completion of the reaction, solvent was evaporated and the residue was crystallized from $CHCl_3$/CH_3OH to give compound **2** in 70% yield; mp 224 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): δ= 7.14 (d, 1 H, *J* = 9 Hz, ArH), 7.42 (t, 1 H, *J* = 6 Hz, ArH), 7.47 (d, 2 H, *J* = 9 Hz, ArH), 7.59 (t, 1 H, *J* = 9 Hz, ArH), 7.78 (d, 1 H, *J* = 6 Hz, ArH), 7.89 (d, 1 H, *J* = 9 Hz, ArH), 8.15 (d, 1 H, *J* = 6 Hz, ArH), 8.36 (d, 2 H, *J* = 9 Hz, ArH), 9.42 (d, 1 H, *J* = 3 Hz, CH=N), 14.92 (s, 1 H, OH) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 111.30, 117.69, 118.91, 120.92, 121.53, 122.39, 124.29, 125.43, 127.27, 128.70, 129.34, 137.66, 145.67, 151.96, 156.97 ppm. ESI-MS: m/z Calcd for $C_{17}H_{12}N_2O_3$ Calcd: 293.0921 $(M + H)^+$ Found: 293.0921 $(M+H)^+$.

Results and discussion

Condensation of β-hydroxynaphthaldehyde (**1**) with 4-nitroaniline in ethanol gave the desired compound **2** in 70% yield (Scheme 1). The ^{1}H NMR spectrum of compound **2** showed five doublets (1 H each) at 7.14, 7.78, 7.89, 8.15 and 9.42 ppm (*J* = 9, 6, 9, 6 and 3 Hz), two triplets (1 H each) at 7.42 and 7.59 (*J* = 6 and 9 Hz) ppm, two doublets (2 H each) at 7.47 and 8.36 ppm (*J* = 9 Hz each) corresponding to the aromatic protons and imino proton, one singlet (1 H) at 14.92 ppm corresponding to the –OH proton. The molecular ion peak at m/z 293.0921 $[M+H^+]$ corresponds to the condensation product **2** observed in the ESI-MS spectrum. These spectroscopic data corroborate structure **2** for this compound (see ESI S5-S8†).

The photophysical behavior of **2** was studied toward different analytes such as sulfur species, reactive oxygen species and anions (H_2S, GSH, Cys, $S_2O_3^{2-}$, H_2O_2, ClO^-, *t*-BuOOH, Cl^-, F^-, Br^-, I^-, N_3^-, CN^-, AcO^- and CO_3^{2-}) by UV-vis and fluorescence spectroscopy. The absorption spectrum of compound **2** (5.0 μM) in H_2O:CH_3CN (99.5:0.5, v/v) buffered with HEPES, pH = 7.4, is characterized by an absorption band at 322 nm (see ESI,† S9) with an extinction coefficient[14] of~6.4×10^4 M^{-1} cm^{-1}. However, the addition of H_2S (Na_2S was used as a hydrogen sulfide source in all experiments) results in a slightly blue shifted absorption

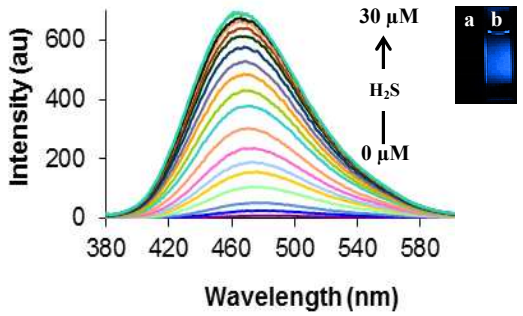


Fig. 1 Fluorescence spectra of **2** (5.0 μM) with H_2S (0-30 μM); spectra were recorded immediately after the additions of H_2S in H_2O:CH_3CN (99.5:0.5, v/v) buffered with HEPES, pH = 7.4; λ_{ex} = 320 nm at 25°C.

band at 314 nm along with the appearance of broad absorption band at 278 nm. The UV-vis behavior of compound **2** in the presence of H_2S is ascribed to the selective reduction of acceptor nitro to donor amino functionality, as a result it prevents the charge transfer from donor naphthalene to acceptor nitro benzene moiety leading to blue shift in the absorption band. The addition of other analytes such as cysteine, ROS and anions did not alter the absorption spectrum of compound **2** (see ESI,† S9).

In the fluorescence spectrum, receptor **2** exhibited no fluorescence emission when excited at 320 nm in H_2O:CH_3CN (99.5:0.5, v/v) buffered with HEPES, pH = 7.4 (Figure 1). Although, the phenomenon of excited state intramolecular proton transfer (ESIPT) could have resulted in the fluorescence emission of **2** but owing to the photo-induced electron transfer (PET) from the excited fluorophore to the strong electron-withdrawing group

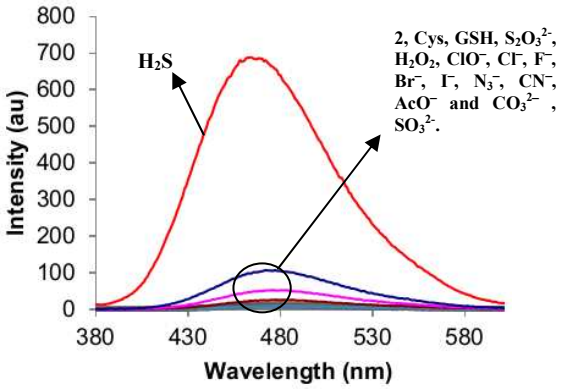


Fig. 2 Fluorescence spectra of **2** (5 μM) in the presence of H_2S (30 μM); spectra were recorded immediately after the additions of H_2S and other analyte (30 μM each but in case of glutathione and cysteine we used 5mM and 10mM) in H_2O:CH_3CN (99.5:0.5, v/v) buffered with HEPES, pH = 7.4; λ_{ex} = 320 nm. .

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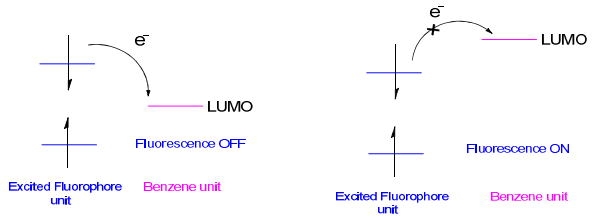
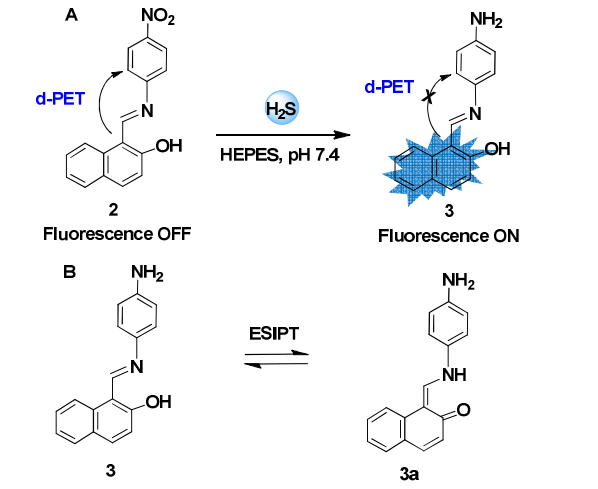


Fig. 3 Schematic diagram of d-PET mechanism

(donor-excited PET or d-PET)[15] makes it non-fluorescence. In general, nitro substituted ring has lower LUMO energy level due to its electron-withdrawing effect.[15b] Thus, intramolecular electron transfer occurs from excited fluorophore to the electron deficient benzene moiety.[16] Upon addition of increasing amounts of only H_2S to the aqueous solution of **2**, a remarkable enhancement in emission intensity was observed at 462 nm (Figure 1) along with the appearance of a blue coloured fluorescence emission (inset of Figure 1) due to conversion of nitro functionality to amino functionality which trammels the d-PET mechanism owing to higher LUMO energy level (Figure 3). The fluorescence quantum yield (Φ_s) of the compound **3** is found to 0.194 as compared to that of receptor **2** (0.005) at 462 nm, which shows good agreement with fluorescence spectra obtained



Scheme 2. (A) H_2S-induced reduction of **2**; (B) Fast enol–imine to keto–amine tautomerism in **3**.

for receptor **2** in the presence of H_2S. Fluorescence enhancement observed for receptor **2** upon addition of H_2S is attributed to the reduction of nitro to amino group[9] (Scheme 2A) which is responsible for the inhibition of d-PET and hence fluorescence turn-on behaviour due to the excited state intramolecular proton transfer mechanism (Scheme 2B).

Thus, probe **2** acts as efficient platform for the fluorescence turn-on detection of H_2S in aqueous environment. In the mass spectrum peak at *m/z* 263.1173 (see ESI,† S10) confirmed the formation of **3** which clearly indicates the reduction of nitro to amino group in the presence of H_2S. As the reduction by H_2S results in the formation of compound **3** containing an electron-donating group instead of an electron-withdrawing group, the fluorescence emission also underwent around 12 nm blue shift (see ESI,† S11).[4e] Under the same conditions as used for H_2S in aqueous media, we also carried out the fluorescence studies of probe **2** toward other biologically relevant species (H_2O_2, ClO^-, TBHP, N_3^-, $S_2O_3^{2-}$, Cys and GSH). As shown in Figure 4, no significant change was observed with other species in comparison to the hydrogen sulfide. The time dependent fluorescence study indicates that the 35 fold fluorescence enhancement was obtained in 20 minutes after the addition of H_2S (Figure 4). Interestingly, the emission enhancement was observed immediately after the addition of H_2S which in turn indicates the highly reactive nature of probe **2** towards H_2S.

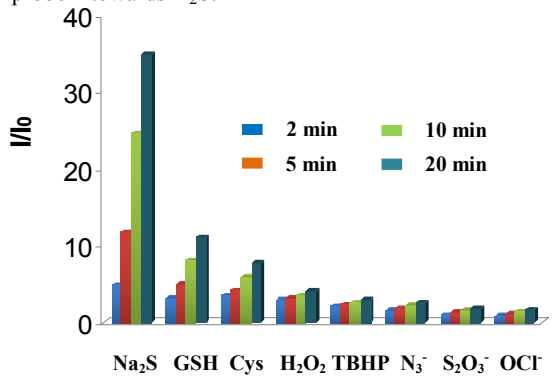


Fig. 4 Fluorescence response of **2** (5 μM) in H_2O:CH_3CN (99.5:0.5, v/v) buffered with HEPES, pH = 7.4 (λ_{ex}= 320 nm) with various analytes (30 μM each but in case of glutathione and cysteine we used 5mM and 10mM). Bars represent selectivity (I/I_0) (I_o = initial fluorescence intensity at 462 nm; I = final fluorescence intensity at 462 nm after the addition of analyte) of **2** upon addition of different analytes. Data were given after incubation with the appropriate analyte at 25°C after 2, 5, 10 and 20 minutes.

Next, we investigated the effect of pH on the recognition behavior of probe **2**. At the acidic pH conditions (pH = 5.0 or 6.0) probe **2** exhibits low reactivity towards H_2S and hence we observed small increase in the fluorescence emission (Figure 5). However, as we increase the pH = 7.0, the enhancement in the fluorescence emission was fast (Figure 5). On further increase of the pH (pH = 8.0), more fast emission enhancement is observed. This fast emission enhancement is due to the existance of H_2S as HS^- under basic conditions which has more reducing power.[17] Further, we also investigated the effect of temperature on the fluorescence behaviour and observed that at higher temperature the reduction of nitro group to amino group is faster in comparision to reduction at lower temperature conditions (see ESI,† S12). We also tested the fluorescence response of

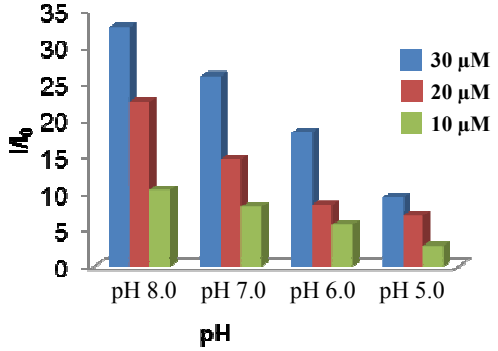


Fig. 5 Fluorescence response of **2** (5 μM) in H_2O:CH_3CN (99.5:0.5, v/v) buffered with HEPES in different pH value (λ_{ex}= 320 nm) to addition of different concentration of H_2S. Data were given after incubation with H_2S at 25 °C after 15 minutes.

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receptor **2** towards other analytes (Cl^-, F^-, Br^-, I^-, N_3^-, CN^-, AcO^-, CO_3^{2-} and SO_3^{2-}). No significant change in fluorescence occurred in the presence of any other analyte (Figure 2). Further, we investigated the practical ability of receptor **2** as a H_2S selective fluorescent chemosensor by carrying out competitive experiments in the presence of H_2S mixed with other analytes (see ESI,† S13). No significant variation in fluorescence emission was observed in comparison with or without the any other analyte. Thus, **2** acts as efficient fluorescence turn-on probe for the detection of H_2S in aqueous media. The detection limit of 10×10^{-7} mol L^{-1} (see ESI,† S14) of probe **2** for H_2S suggests this system as efficient sensing platform for H_2S.

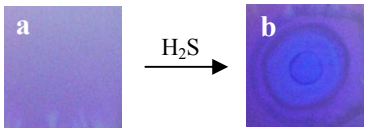

Fig. 6 Change in the fluorescence of **2** in the presence of H_2S. (a) Test-strips of **2**;(b) After adding 1 drop of H_2S solution (10^{-4} M in H_2O) onto the test-strips of **2**. All images were taken under 365 nm UV illumination.

In order to work as an efficient sensing system, a probe molecule must have some practical applications. Therefore, we introduced for the first time a fast and easiest method for the detection of H_2S. We prepared test-strips (see ESI,† S15) coated with probe **2** which showed change in fluorescence after treating with H_2S. The fluorescence enhancement was observed upon dropping the saturated aqueous solution of Na_2S (10^{-4} M) onto the test strips (Figure 6). These results show the practical applicability of probe **2** toward the instant visualization of traces of H_2S.

The potential biological application of the chemosensor **2** was evaluated for *in vitro* detection of H_2S in prostate cancer (PC3) cell lines. The prostate cancer (PC3) cell lines were incubated with receptor **2** (5.0 μM) in an RPMI-1640 medium for 20 min at 37°C and washed with phosphate buffered saline (PBS) buffer (pH 7.4) to remove excess receptor **2**. Microscopic images showed no intracellular fluorescence which indicated that receptor **2** is non-emissive in nature (Figure 7a). However, after treatment with H_2S (5.0 μM), the cells pre-treated with receptor **2**

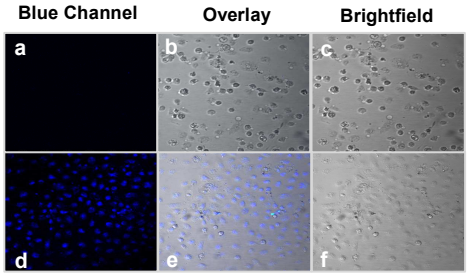

Fig. 7 Fluorescence and brightfield images of PC3 cell lines. (a) Fluorescence image of cells in blue channel treated with probe 2 (5 μM) for 20 min at 37 °C. (b) Overlay image of (a) and (c). (c) Brightfield image of (a). (d) Fluorescence images of cells in blue channel upon treatment with probe **2** (5 μM) and then Na_2S (5 μM) for 10 min at 37 °C. (e) Overlay image of (d) and (f). (f) Brightfield image of (d). λ_{ex} = 405 nm; fluorescence images are recorded at blue channel.

(5.0 μM) show fluorescence in blue channel (Figure 7d). The appearance of blue fluorescence is attributed to the amino form of the receptor **2** produced by reduction of nitro functionality of receptor **2** in presence of H_2S. These results suggest that receptor **2** is cell permeable and an effective intracellular H_2S imaging agent with turn-on blue coloured fluorescence emissions.

Conclusions

In conclusion, we developed a reaction based fluorescent chemosensor **2** which undergoes fluorescence enhancement in the presence of only H_2S among the various sulfur, reactive oxygen species and anions in aqueous media and living cells. The sensing mechanism is based on the selective reduction of nitro group to amino moiety under physiological conditions by H_2S. The addition of increasing amounts of H_2S to the aqueous solution of receptor **2** resulted in a remarkable enhancement in emission intensity along with the appearance of a blue coloured fluorescence emission. Confocal microscopy studies indicate that our probe can detect the changes of H_2S level in living cells. We envision that the fluorescent probe will be of great benefit for detection of H_2S to investigate the effects of H_2S in biological systems.

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† Electronic Supplementary Information (ESI) available: [Experimental details, ^{1}H NMR, ^{13}CNMR, mass spectra, UV-vis and fluorescence spectra]. See DOI: 10.1039/b000000x/

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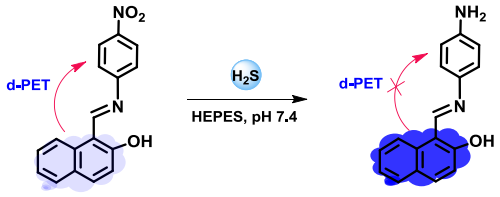
Graphical Abstract



A d-PET coupled ESIPT based probe has been designed and synthesized for the selective detection of H_2S among the other ions and various sulfur species such as cysteine, glutathione in aqueous media *via* the hydrogen sulfide induced reduction of nitro functionality and have further been utilized for the imaging of H_2S in intracellular systems.