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An iminocoumarin benzothiazole-based fluorescent probe for imaging hydrogen sulfide in living cells

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ABSTRACT

Hydrogen sulfide (H_2S) has recently been identified as the third gaseous signaling molecule that is involved in regulating many important cellular processes. We report herein a novel fluorescent probe for detecting H_2S based on iminocoumarin benzothiazole scaffold. The probe displayed high sensitivity and around 80-fold increment in fluorescence signal after reacting with H_2S under physiological condition. The fluorescent intensity of the probe was linearly related to H_2S concentration in the range of 0–100 μM with a detection limit of 0.15 μM (3σ/slope). The probe also showed excellent selectivity towards H_2S over other biologically relevant species, including ROS, RSS and RNS. Its selectivity for H_2S is 32 folds higher than other reactive sulfur species. Furthermore, the probe has been applied for imaging H_2S in living cells. Cell imaging experiments demonstrated that the probe is cell-permeable and can be used to monitor the alteration of H_2S concentrations in living cells. We envisage that this probe can provide useful tools to further elucidate the biological roles of H_2S.

1. Introduction

Hydrogen sulfide (H_2S) has recently emerged as the third gaseous signaling molecule after carbon monoxide and nitric oxide [1]. Recent biological studies have established the important role of H_2S in various physiological and pathological processes [2–6]. For example, H_2S has been shown to regulate inflammation, relax smooth muscles and act as a vasodilator. It has strong implications in neurodegeneration, and studies have shown that it might be able to protect neurons from secondary neuronal injuries through multiple biological mechanisms, such as anti-oxidation and anti-apoptosis [7]. In mammalian cells, H_2S can be produced through both enzymatic and non-enzymatic biosynthesis. In enzymatic biosynthesis, cysteine or its derivatives can be converted to H_2S through the catalysis of several enzymes, such as cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS), cysteine aminotransferase(CAT) and 3-mercaptopyruvate sulfurtransferase (MST) [8–10]. On the other hand, glutathione and polysulfides can be transformed to H_2S through non-enzymatic pathway [11,12]. Increasing evidence has shown that abnormal levels of H_2S is closely linked to several important diseases, such as Alzheimer's disease, Down's syndrome, diabetes and liver cirrhosis [13–15]. For example, it was observed that the concentrations of H_2S are severely decreased in the brains of Alzheimer's disease patients compared with those in the brains of healthy individuals. In recent years, it has spurred substantial interest in scientific community to develop useful chemical tools to accurately measure H_2S levels in biological systems.

Till date, many different methods have been developed for detecting H_2S, including electrochemical methods, colorimetric methods, gas chromatography and polarographic sensors [16–21]. Among these methods, fluorescence-based methods have attracted intensive interest due to their highly sensitive and non-invasive properties. In addition, fluorescence-based methods also allow one to monitor the molecules of interest in a native environment, thereby providing detailed spatial and kinetic information. Since the first H_2S probe was reported in 2011, a number of H_2S fluorescent probes have been designed and applied to imaging H_2S in live cells. These fluorescent probes can be mainly categorized into three types. 1) Probes containing an azide/nitro group that can effectively quench fluorescence [22–30]. When H_2S reduces the azide/nitro group, fluorescence will be turned on. 2) Probes that utilize the double

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nucleophilicity property of H_2S to switch on fluorescence [31–34]. 3) Probes containing a chelating unit that forms complex with Cu^{2+} [35–37]. Addition of H_2S can release Cu^{2+}, leading to fluorescence enhancement.

Biological research on H_2S has been carried out intensively by the scientific community. Notwithstanding, the production process, tissue specificity and action mechanism of H_2S still need to be further elucidated. We envisage that H_2S fluorescent probe with innovative design, high kinetics and improved fluorescent property will be highly useful to further elucidate the biological roles of H_2S.

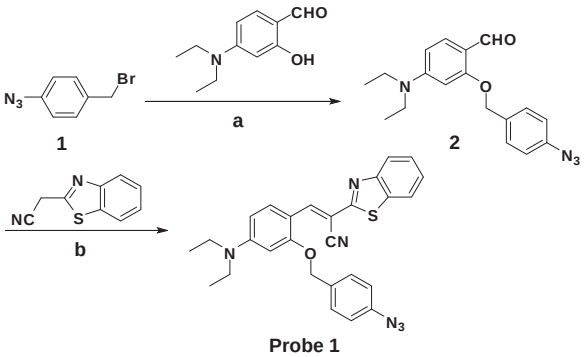
Inspired by the work of Kim's team [38], we designed and synthesized a new fluorescent probe 1 based on iminocoumarin benzothiazole to detect H_2S. The iminocoumarin-based fluorophore has been proven to possess excellent photophysical properties, such as good photostability and high quantum yield. In addition, our probe can be excited at long wavelength (465 nm). Compared with probes used under short wavelength irradiation, our probe can minimize cell damage and is more suitable for bioimaging studies. Furthermore, the probe has high signal-to-noise ratio, which is attributed to its special chemical structure. The probe itself has little fluorescence due to fast non-radioactive decay of the singlet excited state through internal bond rotations [39]. Upon reacting with H_2S, it will form a cyclized iminocoumarin benzothiazole structure that is highly fluorescent.

Probe 1 was synthesized through the following synthetic route (Scheme 1). *P*-azidebenzyl linker 1 was first prepared according to published procedure. It was then used to react with the free phenol hydroxyl group of 4-(diethylamino) salicylaldehyde under refluxing condition. The resulting compound 2 was subsequently subjected to Knoevenagel condensation with 2-benzothizoleacetonitrile to afford the final probe 1. The structures of probe 1 and the synthetic intermediates were all characterized by ^1H NMR, ^{13}C NMR and ESI-MS respectively (supporting Information).

2. Experimental details

2.1. Apparatus

^1H NMR and ^{13}C NMR spectrums were recorded on a Bruker 400 MHz NMR spectrometer. Fluorescence emission spectra were obtained with FluoroMax-4 fluorescence photometer. UV absorption spectra were obtained on Shimadzu 1700 UV/Vis Spectrometer. Mass spectra were obtained using a PC Sciex API 150 EX ESI-MS system. Fluorescence images were acquired with a Leica TCS SPE Confocal Scanning Microscope. pH value was recorded with a FiveEasy ™ Fe20 pH meter.



Scheme 1. Synthetic route of probe 1.a) K_2CO_3, acetone, reflux, 3 h, 78%; b) Piperidine, EtOH, rt, 5 h, 80%.

2.2. Reagents

4-aminobenzylalcohol, 4-(diethylamino)salicylaldehyde, sodium nitrite and sodium azide were purchased from Acros. Tribromophosphane, pipiridine and 2-(1,3-benzothiazol-2-yl)acetonitrile were obtained from J&K Chemical. Dulbecco's modified Eagle's medium (DMEM), PBS, fetal bovine serum (FBS), trypsin-EDTA and penicillin/streptomycin were purchased from Invitrogen. Other reagents were all of analytical grade and were used without further purification. Milli-Q water was used in all experiments.

2.3. Synthesis of 1-azido-4-(bromomethyl)benzene (1)

1-azido-4-(bromomethyl) benzene was synthesized according to literature. Details of synthesis are shown in Scheme S1 (Supporting Information).

2.4. Synthesis of 2-((4-azidobenzyl)oxy)-4-(diethylamino) benzaldehyde (2)

4-(Diethylamino)salicylaldehyde (0.096 g, 0.50 mmol), K_2CO_3 (0.138 g, 1.00 mmol) and 1-azido-4-(bromomethyl)benzene (0.084 g, 0.40 mmol) were dissolved in dry acetone (5.0 mL) under nitrogen atmosphere. The mixture was stirred at 60 °C for 3 h. After filtration, the solvent was evaporated under reduced pressure. The crude product was purified by silica gel column chromatography with hexane/ethyl acetate=3/1 to obtain compound **2** as a yellow solid (0.100 g, 77.2% yield); ^1H NMR (400 MHz, $CDCl_3$): δ=10.19 (s, 1H), 7.70 (*d*, *J*=8.0 Hz, 1H), 7.41 (*d*, *J*=8.0 Hz, 2H), 7.01 (*d*, *J*=8.0 Hz, 2H), 6.27 (*d*, *J*=8.0 Hz, 1H), 6.03 (*s*, 1H), 5.10 (*s*, 2H), 3.36 (*q*, 4H), 1.15 (*t*, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ=186.8, 163.0, 153.7, 139.8 133.3, 130.5, 128.7, 119.2, 114.3, 104.6, 94.0, 69.5, 44.8, 12.5; ESI-MS: calcd. for $C_{18}H_{21}N_4O$ $[M+H]^+$ 325.2; found, 325.4.

2.5. Synthesis of probe 1

One drop of piperidine was added to a stirred solution of 2 (0.097 g, 0.30 mmol) and 2-(1,3-benzothiazol-2-yl)acetonitrile (0.052 g, 0.30 mmol) in ethanol (5.0 mL) under nitrogen atmosphere at room temperature. The reaction mixture was stirred for 5 h at room temperature. The solvent was evaporated under reduced pressure. The crude product was purified by silica gel column chromatography with hexane/ethyl acetate=5/1 to obtain the probe 1 as a red solid (0.115 g, 79.9%). ^1H NMR (400 MHz, $CDCl_3$): δ=8.60 (*s*, 1H), 8.44 (*d*, *J*=8.0 Hz, 1H), 8.02 (*d*, *J*=8.0 Hz, 1H), 7.84 (*d*, *J*=8.0 Hz, 1H), 7.47 (*m*, 3H), 7.35 (*t*, 1H), 7.08 (*m*, 2H), 6.43 (*d*, *J*=8.0 Hz, 1H), 6.18 (*s*, 1H), 5.18 (*s*, 2H), 3.40 (*q*, 4H), 1.18 (*t*, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ=165.8, 160.3, 154.0, 152.5, 141.1, 140.0, 134.4, 133.4, 130.6, 128.7, 126.5, 125.0, 123.0, 121.4, 119.4, 118.6, 110.2, 105.6, 96.7, 95.1, 70.1, 45.1, 12.7; ESI-MS: calcd. for $C_{27}H_{24}N_6OS$ $[M+H]^+$ 481.2; found, 481.4.

2.6. Procedure of fluorescence measurement

Probe 1 stock was diluted in Tris buffer (50 mM, pH=8.0, 50% DMF) to a final concentration of 5 μM. For the selectivity experiment, 50 mM of biologically relevant analytes were prepared as stock solutions in PBS buffer. Appropriate amounts of biologically relevant analytes were mixed thoroughly with 5 μM of Probe 1 and then incubated at 37 °C for 60 min. For the time course experiment with Na_2S, 100 μM of Na_2S was incubated with 5 μM of probe 1 in Tris buffer at 37 °C, and the fluorescence intensity was measured at different time points. Fluorescence emission spectra were collected using a FluoroMax-4 fluorescence photometer with a 10 mm quartz cuvette. The excitation wavelength was set to

465 nm and the emission wavelength was set in the range of 480 nm to 650 nm. The slit width was set to 5 nm.

2.7. Cell culture and fluorescence microscope imaging

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and recommended working concentrations of antibiotics (penicillin and streptomycin). Approximately 10^5 cells were seeded in a confocal dish (35 mm). After 24 h, the cells were treated with probe 1 (5 μM) at 37 °C for 30 min and then incubated with Na_2S for another 30 min. Cells treated with probe 1 (5 μM) alone were used as a control. Fluorescence images were then taken using a Leica TCS SPE Confocal Scanning Microscope.

3. Results and discussion

3.1. Proposed mechanism

Based on the mechanism of several azide-containing probes, we propose the following mechanism whereby probe 1 reacts with H_2S. First, the benzyl azide group is reduced to benzyl amine group by H_2S. The resulting *p*-aminobenzyl moiety is unstable and can undergo self-immolation through 1,6-elimination. After self-elimination of the linker, the hydroxyl group is released. It will then undergo intramolecular cyclization to yield an iminocoumarin-benzothiazole molecule, resulting in a highly conjugated structure. This cyclized iminocoumarin-benzothiazole displays significant fluorescence enhancement compared with its unreduced form (Scheme 2).

To confirm our proposed mechanism, probe 1 (50 μM) and Na_2S (1 mM) were incubated in Tris buffer (50 mM, pH=8.0, 50 % DMF) at 37 °C. The resulting solution was then monitored using ESI-MS. After incubating for 20 min, three peaks at *m/z* 481.3, 350.2 and 106.3 were observed, which corresponds to the molecular weight of probe 1, iminocoumarin-benzothiazole molecule and 1,6-eliminated product respectively (Fig. S1a, Supporting Information). The peak of *m/z* 481.3, which belongs to probe 1, could not be observed after 60 min of incubation, indicating the reaction has completed (Fig. S1b, Supporting Information).

3.2. UV-vis absorption and fluorescence emission of probe 1

Next we investigated the optical properties of probe 1 in the absence and the presence of H_2S (100 μM, using Na_2S as an equivalent). Probe 1 exhibited a maximum absorbance at 455 nm. After reacting with H_2S, the maximum absorbance was shifted to 465 nm, an increase of 10 nm (Fig. 1a). For the fluorescence emission spectrum, probe 1 showed very weak fluorescence intensity when excited at 465 nm. After reacting with Na_2S, however, probe 1 displayed strong fluorescence at 520 nm (Fig. 1b, Fig. S2, Supporting Information).

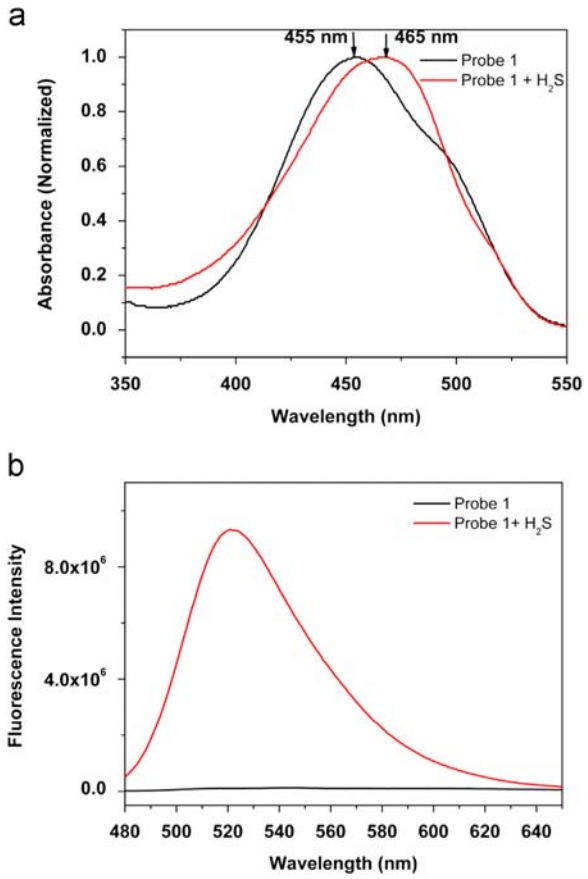


Fig. 1. (a)Absorption and (b) emission spectrum of probe 1 (10 μM) in the absence and the presence of Na_2S (100 μM) at 37 °C for 60 min.

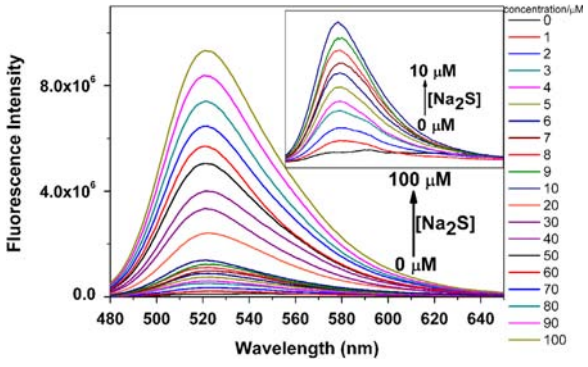
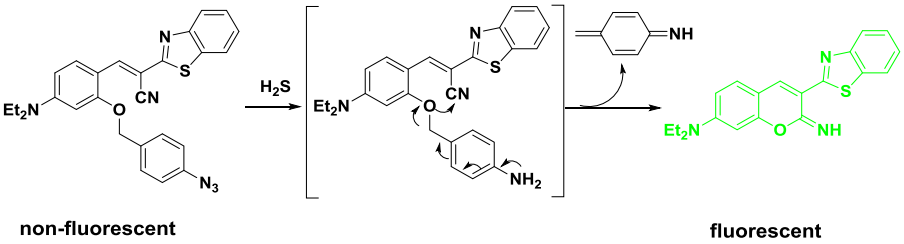


Fig. 2. Fluorescence response of probe 1 (5 μM) in Tris buffer (50 mM, pH=8.0, 50 % DMF) with addition of increasing concentrations of Na_2S (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 μM, λ_{Ex}=465 nm, λ_{Em}=480–650 nm) after 1 h incubation at 37 °C. The inset figure shows that the fluorescence signal increases linearly with increasing concentrations of Na_2S when the concentration is between 0–10 μM.



Scheme 2. Proposed "turn-on" mechanism of the probe reacting with H_2S.

3.3. Sensitivity and detection limit

We next carried out detailed study to examine the sensitivity of probe 1 towards H_2S. Fig. 2 showed the fluorescence response of probe 1 when different concentrations of Na_2S (0–100 μM) were added. Approximately 80-fold increase in fluorescence intensity at 520 nm could be observed when the reaction between probe 1 and Na_2S was complete. Further data analysis revealed an excellent linear relationship (R^2=0.99748) between the fluorescent signal at 520 nm and Na_2S concentrations in the range of 0 to 100 μM (Fig. S3 and S4, Supporting Information). The detection limit of probe 1 was determined to be 0.15 μM (based on the equation of 3σ/m, where m is the slope of fluorescence intensity against Na_2S concentration and σ is the relative standard deviation of the blank measurements) [40–42]. This value is comparable with the detection limits of other H_2S probes (Table 1.). Taken together, these data prove that the new probe described here can serve as a highly sensitive sensor to detect H_2S in a quantitative manner.

3.4. Kinetics

Subsequently we examined the reaction kinetics of probe 1 and Na_2S. Probe 1 (5 μM) was incubated with Na_2S (100 μM) at 37 °C in Tris-HCl buffer (50 mM, pH=8.0, 50 % DMF). The fluorescence intensity at 520 nm was recorded and plotted as a function of time for data analysis. As shown in Fig. 3, the fluorescence intensity increased rapidly at the beginning and plateaued after around 60 min. The pseudo-first-order rate constant of the reaction was calculated as $k_{obs}=8.5\times10^{-4}\,S^{-1}$ by fitting the fluorescence intensity data into the following equation: Ln $(F_{max}-F_t)$=LnF$_{max}$−$k_{obs}\times t$. The reaction rate (k_2) was found to be 8.50 $M^{-1}\,S^{-1}$, which is similar to other azide-based probes reported previously.

Table 1
The summary of recently developed H_2S probes.

Probe	λ_{ex} (nm)	λ_{ex} (nm)	Linear range (μM)	pH condition	LOD (μM)	Increased fold	Ref.
SF1	490	525	~	7.4	5–10	7-fold	[22]
DNS-Az	340	535	5–100	7.4	1	40-fold	[23]
HSN2	432	542	~	7.4	1–5	60-fold	[24]
1	535	620	1–40	7.4	0.1	30-fold	[27]
Probe 1	465	515	0–10	7.4	1–10	55–70-fold	[31]
HSip-1	491	516	~	7.4	10	50-fold	[35]
Cy-N3	625	750	0–100	7.4	0.08	3.3-fold	[43]
L1Cu	495	534	~	7.2	5	4-fold	[44]
NHS1	360	480	5–10	7.4	0.1	67-fold	[45]
1	520	670	25–250	7.4	3.05	65-fold	[46]
C-7Az	340	445	0.25–100	7.4	~	108-fold	[47]
1	465	520	1–100	8.0	0.15	80-fold	This work

Note: ~means not mentioned.

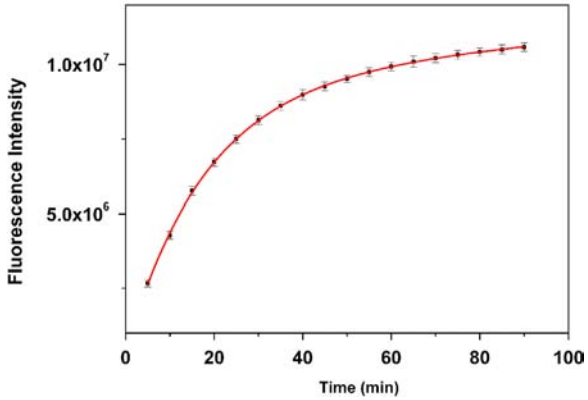


Fig. 3. Time course experiment of probe 1 (5 μM) reacting with Na_2S (100 μM) in Tris buffer (50 mM, pH=8.0, 50% DMF) at 37 °C.

3.5. Selectivity

Selectivity test is a critical step to ensure that our probe will not be interfered by other biomolecules in the complex biological system. To perform the selectivity test, various biologically relevant analytes, including reactive oxygen species (ROS), reactive nitrogen species (RNS), reactive sulfur species (RSS) and other reactive anions (Fig. 4, Fig. S5, S6, Supporting Information) were examined under the following assay conditions: different biologically relevant analytes (1 mM) were added respectively to a solution containing 5 μM of probe 1 (100 μM of Na_2S was incorporated as a control). The fluorescence was measured after 60 min of incubation. As shown in Figs. 4 and S6, all the biologically relevant analytes tested did not show any obvious fluorescence increase except cysteine (2.6 fold) and I^- (2.4 fold). The selectivity ratio towards Na_2S over cysteine is as high as 31-fold. The excellent selectivity of probe 1 towards Na_2S over other analytes suggests that probe 1 is a promising tool for specifically detecting H_2S in a complex biological environment.

3.6. Bioimaging application in living cells

For the bioimaging applications of the probe, we first performed MTT assay to examine whether the probe is cytotoxic to cells. Results indicated that 5 μM of probe 1 did not show any significant cytotoxicity after 24 h (Fig. S7, Supporting Information). We then moved on to carry out imaging experiment with Na_2S in live cells. The cells were incubated with 5 μM of probe 1 for 30 min and then treated with different concentrations of Na_2S at 37 °C for another 30 min. As shown in Fig. 5, the probe displayed negligible fluorescence (Fig. 5c). For the cells treated with Na_2S, a significant fluorescence increase can be observed in the cytoplasm (Fig. 5f). In addition, increasing level of green fluorescence could be observed with increasing concentrations of Na_2S (Fig. S8, Supporting Information). These experiments clearly showed that probe 1 can serve as a useful tool for monitoring the change of H_2S levels in living cells.

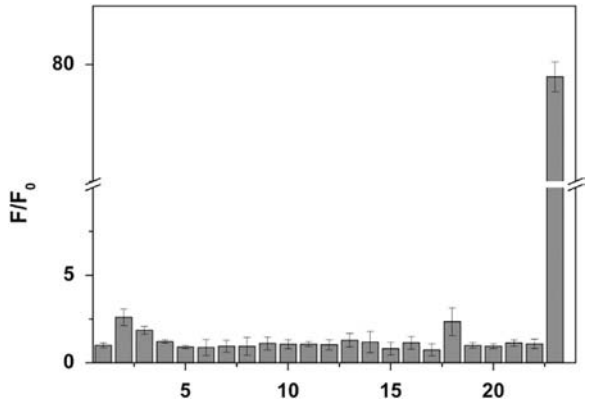


Fig. 4. Fluorescence responses of probe 1 (5 μM) after adding various ROS, RNS, RSS and other analytes and incubating at 37 °C for 60 min in Tris-HCl buffer (50 mM, pH=8.0, 50 % DMF). The bars represent fluorescence response at 520 nm. (1) probe only; (2) cysteine; (3) GSH; (4) homocysteine; (5) SO_3^{2-}; (6) $S_2O_3^{2-}$; (7) $S_2O_4^{2-}$; (8) $S_2O_5^{2-}$; (9) SCN^-; (10) NO_2^-; (11) NO_3^-; (12) N_3^-; (13) H_2O_2; (14) HOCl; (15) F^-; (16) Cl^-; (17) Br^-; (18) I^-; (19) HPO_4^{2-}; (20) $H_2PO_4^-$; (21) HCO_3^-; (22) CO_3^{2-}; (23) Na_2S. (the concentration of all the analyte is 1 mM except that of Na_2S, which is 100 μM).

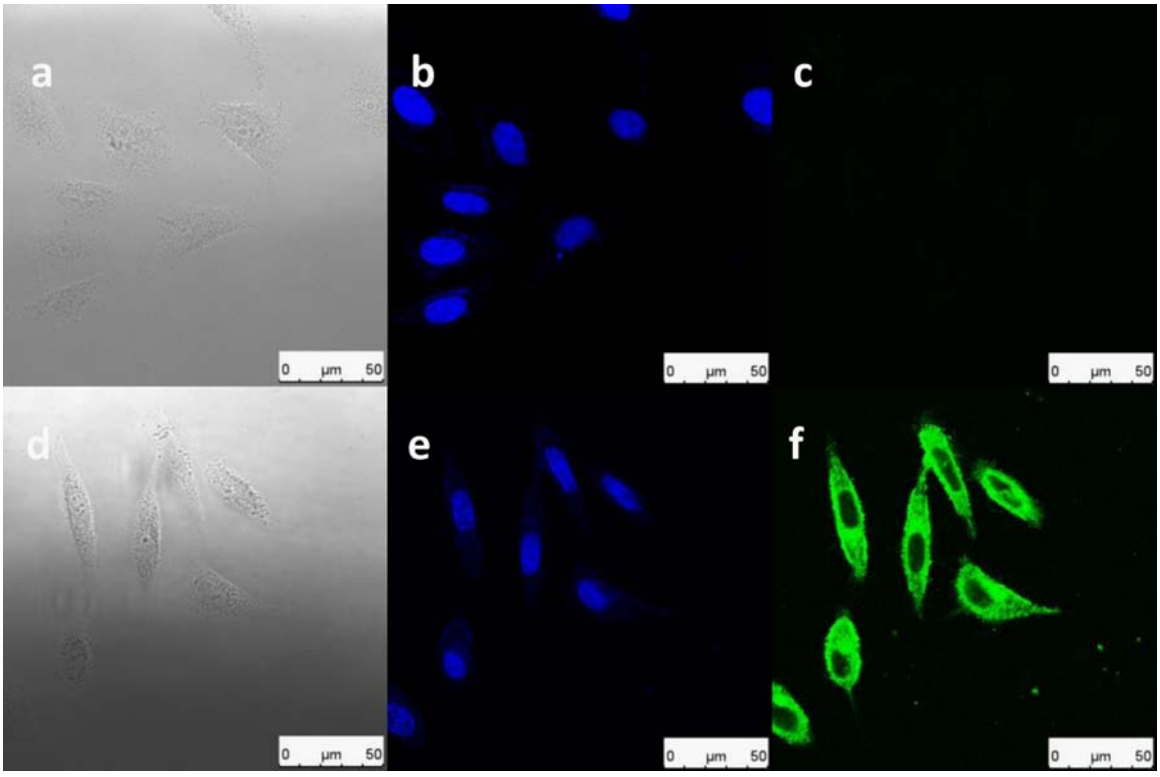


Fig. 5. Fluorescence images of H_2S with probe 1 in HeLa cells. Hela cells were incubated with probe 1 (5 μM) for 60 min. (a) Brightfield image, (b) Fluorescent image (using filters for Hoescht), (c) Fluorescent image (using filters for Fluorescein). Hela cells were incubated with probe 1 for 30 min and then incubated with Na_2S (250 μM) for another 30 min. (d) Brightfield image, (e) Fluorescent image (using filters for Hoescht), (f) Fluorescent image (using filters for Fluorescein). Scale bars represent 50 μm.

4. Conclusions

In conclusion, we have developed a new fluorescent probe based on iminocoumarin benzothiazole scaffold for detecting H_2S. The probe utilizes a H_2S-induced cascade reaction consisting of three consecutive steps: reduction, elimination and cyclization. Our studies show that the probe possesses high sensitivity and selectivity towards H_2S. Furthermore, the bioimaging experiments of H_2S prove that the probe can serve as a promising tool to further explore the biological roles of H_2S in complex biological environment.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.12.044.

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