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Fluorogenic sensing of H_2S in blood and living cells *via* reduction of aromatic dialkylamino N-oxide

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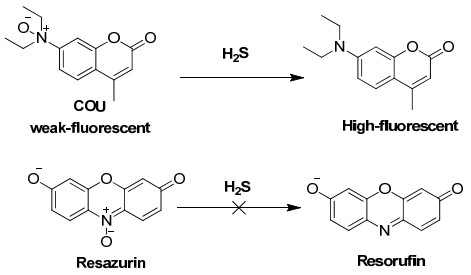
Fluorescent chemodosimeter, N-oxide of 7-diethylamino-4-methylcoumarin, was developed for H_2S detection based on a new water soluble aromatic dialkylamine N-oxide group and their selectivity and sensitivity of fluorescent detection of H_2S were evaluated in aqueous solution and blood media. Fluorescence sensing of H_2S was also achieved in HeLa cells.

Hydrogen sulfide (H_2S) is a well-known toxic gas that is often present in environments and industrial processing. Recent studies have demostreated that endogenous H_2S in living systems, is one of gaseous transmitters, along with nitric oxide (NO) and carbon oxide (CO) that exhibit beneficiary effects in a number of pathophysiological conditions.[1] Different endogenous sulfide concentrations have been reported in literature, suggesting large variation of the sulfide concentrations in blood due to the interference of other analytes and/or different sample processing methods.[2] Therefore, a direct and efficient method for sensitively and selectively probing H_2S in biological systems is highly required.

Current methods including colormetric,[3] electrochemical,[4] gas chromatagraphy assays[5] and metal-induced pecipation[6] were reported in literature for H_2S detection. Due to the high sensitivity, nondestructivity, and high spatiotemporal resolution, fluorescence-based assays have exitibited widespread applications especially in biological systems. In the past few years, fluorescent probes used for fast and real time H_2S detection in biological systems was based on H_2S-triggered spectific reaction including the reduction of azido,[7] the reduction of nitro or hydroxyamine groups,[8] the reduction of organic selenium oxide,[9] nucleophilic reaction,[10] high binding affinity towards copper ions[11] and efficient thiolysis of dinitrophenyl ether.[12] However, probes bearing these functional group usually have the low solubitiy in aqueous solution which needs organic solvents for H_2S detction. This limitation impeded their further applications in biological systems. Wu et al[13] reported two kinds of H_2S sensors in which chemofluorophores were respectively conjugated to



Scheme 1 Reduction of N-O chemical bond of fluorophore for H_2S detection

poly(ethylene glycol) and carbon dots to improve water-solublility. However, this conjugation increased the size of sensors. In addition, most previous sensors always involved two or more synthetic steps. Herein, we developed a new H_2S probe bearing an dialkylamine N-oxide group that was readily synthesized in single step with available fluorophores under a mild reaction condition and maintained high solubility in aqueous solution. Morever, detection of H_2S based on reduction of aromatic dialkylamine N-oxide group was not reported.

The rational design of this new H_2S sensitive functional group for commericallyavaliable fluorophores is depicted in Scheme 1. Two kinds of N-O functional moieties were introduced to 7-diethyl-4-methylcoumarin and resorufin, respectively. Since the formation of N-O bond consumes the electron pair of nitrogen atom, it breaks the electron conjugation of nitrogen and aromatic moiety which can quench the fluorescence of fluorophores. Upon reaction with H_2S in pH 7.4 PBS buffer, aromatic dialkylamine N-oxide was subsquently reduced and highly fluorecent species was observed. To determine the fluorescence species, the product of **COU** reaction with NaHS was purified and then analyzed by ^{1}HNMR and ^{13}CNMR which was in consistent with the NMR spectra of 7-diethylamino-4-methylcoumarin (Fig. S2 and S3, ESI†). Further HRMS analysis confirm to be 7-diethylamino-4-methylcoumarin (Fig. S4, ESI†). For commercially available resazurin, the addition of NaHS did not

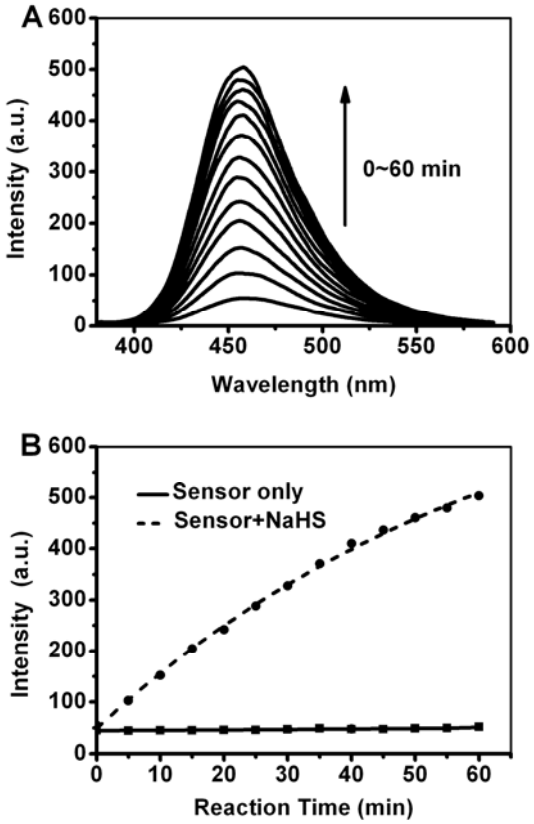


Fig. 1 (A) Fluorescence response of **COU** (5 μM) after the addition of NaHS (40 μM) in different incubation time: (B) Time-dependent fuorescence intensity change in the absence (black line) and presence (red line) of 40 μM NaHS. Time points represent 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 min. λ_{ex} = 360 nm, λ_{em} = 455 nm. Slits: 5/10 nm.

induce the change of absorbance and fluorescence spectra under physiological conditions, indicating that resazurin was more difficult to be reduced than aromatic dialkylamine N-oxide by NaHS and was not suitable for H_2S sensing (Fig. S7, ESI†).

To access the applicability of H_2S sensing assay with dialkylamine N-oxide moiety, **COU** (5 μM) was incubated with NaHS (40 μM) in pH 7.4 solution. As shown in Fig. 1, a roubst increase in fluorescence intensity was observed. Within 60 min of reaction time under above conditions, nearly 10-fold turn-on fluorescence response was observed with the probe **COU**.

Subsequently, we assayed the sensitivity of **COU** for H_2S detection. Various concentraitons of NaHS were added to a series of degassed PBS solutions contating the probe (100 μM), and the fluorescence intensity of the resulting reaction solution were analyzed. Standard curve for **COU** in degassed PBS was gained between fluorescence signal at 455 nm and the concentrations (μM) of H_2S in 60 min incubation. The regression equations were $F_{Ex/Em(360/455nm)}$ = 100.496 [H_2S] + 483.612 with R^2 = 0.9930, suggesting a good linear relationship between turn-on fluorescence intensity of **COU** at 455 nm and NaHS concentrations in degassed PBS buffer. (Fig. 2) The detection limit is nearly down to 0.2 μM.

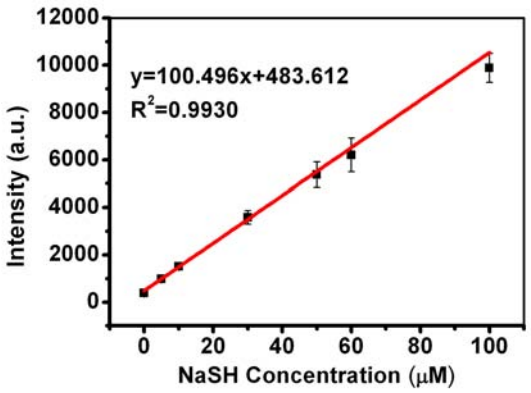


Fig.2 H_2S concentration-dependent fluorescence intensity of **COU** (100 μM) to NaHS. The fluorescence intensity at 455 nm was recorded. Data was acquired after incubation 60 min. λ_{ex} = 360 nm, λ_{em} = 455 nm, Cut off: 420 nm, PMT: medium.

Instead of PBS buffer, we also applied this fluorescent chemodosimeter to the determination of H_2S in blood. A standard addition procedure was used in the experiment (see supporting information for the detail). A liner relationship of **COU** turn-on fluorescence signal at 460 nm and the concentrations (μM) of H_2S in 60 min incubation was also obtained under 25 μM H_2S in mouse blood serum and human blood plasma (Fig. 3). The regression equations were $F_{Ex/Em(355/460\ nm)}$ = 67.672 [H_2S] + 101.290 with R^2 = 0.9680 and $F_{Ex/Em(355/460\ nm)}$ = 65.836 [H_2S] + 66.867 with R^2 = 0.9922 for mouse blood serum and human blood plasma, respectivly.

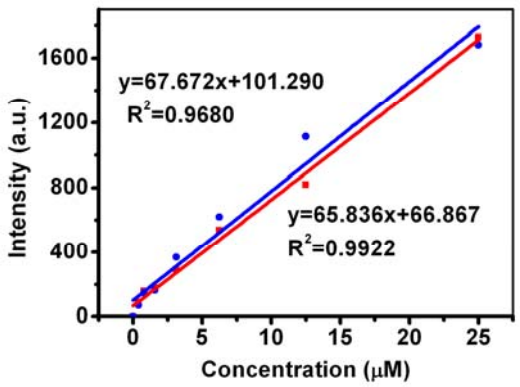


Fig. 3 H_2S concentration-dependent fluorescence intensity of **COU** (100 μM) in mouse blood serum (blue line) and human blood plasma (red line). λ_{ex} = 355 nm, λ_{em} = 460 nm; gain: 650.

To evaluate the specific selectivity of **COU** for H_2S, fluorescence enhancement (F/F_0) of **COU** incubated with relevant species including various anions (1 mM) and biothiols (5 mM, such as Cys, Hcy and GSH) were investigated. As can be shown in Fig. 4, no significant fluorescence change was found upon reaction with these species and only NaHS (40 μM) was found to induce dramatic increase in fluorescence intensity, suggesting high selectivity for H_2S over other biothiols with concentration up to 5 mM.

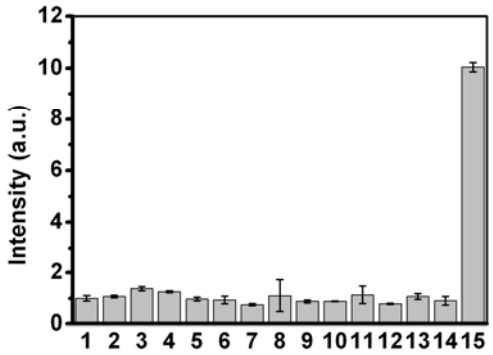


Fig. 4 Fluorescence enhancement (F/F_0) of **COU** (5 μM) in the presence of biological relevant species in pH 7.4 buffer (20 mM) : 1: Control, 2: Hcy (5 mM); 3: Cys (5 mM), 4: GSH (5 mM) 5: SO_4^{2-} (1 mM), 6: HCO_3^{2-} (1 mM), 7: N_3^- (1 mM), 8: $H_2PO_4^-$ (1 mM), 9: NO_2^- (1 mM), 10: CO_3^{2-} (1 mM), 11: Br^- (1 mM), 12: Cl^- (1 mM), 13: Citrate (1 mM), 14: AcO^- (1 mM), 15: HS^- (40 μM). Data was acquired after incubation 60 min. λ_{ex} = 360 nm, λ_{em} = 455 nm, Cut off: 420 nm, PMT: medium.

Cell viability indicated that no obvious toxicity was observed up to 200 μM **COU**.(Fig. S9) To further evaluate the feasibility of H_2S detection in living cells, MCF-7 cells were incubated with **COU** for 30 min, and were then treated with NaHS 250 μM for 30 min. The cells were washed to remove extracellular reagents and then visualized by fluorescence microscopy. As displayed in Fig 5, control cells treated with only **COU** remained very weak fluorescence in the absence of NaHS treatment. While fluorescence intensity in HeLa cells became much stronger after cells were treated with NaHS for 30 min, confirming the capability of **COU** in sensing of H_2S in living cells.

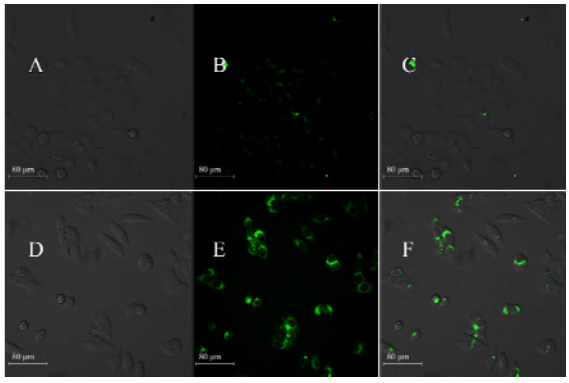


Fig. 5 Imaging of H_2S in MCF-7 cells with **COU** excitated at 405 nm. MCF-7 cells were seeded on glass coverslips and then incubated with **COU** (100 μM) for 30 min. The cells were washed and then treated with NaHS (250 μM) for 30 min. (A) DIC image of control cells with **COU**. (B) Fluorescence image of control cells with **COU**. (C) overlay of A and B. (D) DIC image of **COU**-loaded cells after treatment with NaHS. (E) Fluorescene image of **COU**-loaded cells in the presence of NaHS. (F) Overlay of D and E. Fluorescence images were set as pseudo-color green. Scale bar: 80 μm.

In summary, a novel selective fluorescent probe for H_2S based on dialkylamino N-oxide was developed for the detection of H_2S in aqueous solutions. This new H_2S sensitive functional dialkylamine N-oxide group with postive charge facilitate its high solubility in aqueous buffer and accumulation in cells. Synthesis of this kind of probe needs only one-step from commercially available fluorophore under mild conditions in the presence of mCPBA. The new sensor **COU** has the high solubility in aqueous solutions with no need of other solubable functionization. Fluorescence assay indicated that linear relationships between fluorecence intensity and sulfide concentrations were obtained in buffer, blood plasma or blood serum systems. This probe with high water solubility, sensitivity and selectivity has the potential to be a useful tool for detection of sulfide in many biological systems.

Notes and references

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