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Construction of a turn-on probe for fast detection of H_2S in living cells based on a novel H_2S trap group with an electron rich dye

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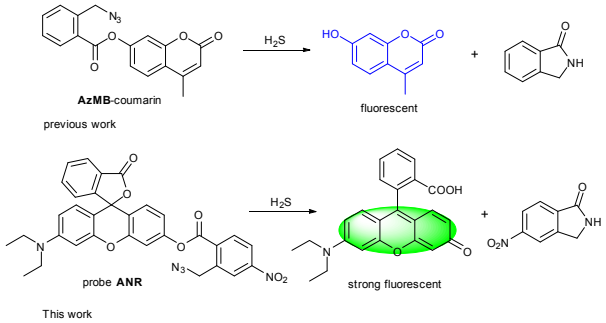
A turn-on probe (ANR) for fast detection of H_2S is constructed based on a 2-(azidomethyl)-4-nitrobenzoate moiety as a trap group. This group is very effective for the design of H_2S probes especially with electron rich dyes. The potential biological applications of ANR were proved by employing it for fluorescence imaging of H_2S in living cells.

Hydrogen sulfide (H_2S), known as a toxic pollutant, has been recently recognized as the third gaseous transmitter after nitric oxide and carbon monoxide.[1] Several endogenous enzymes in mammalian systems, including cystathionine β-synthase (CBS), cystathionine λ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (MPST), make a contribution to the production of H_2S.[2] These enzymes convert cysteine or its derivatives into H_2S in different organs and tissues, which play important roles in several pathophysiological processes, such as vasodilation, angiogenesis, regulation of cell growth, mediation of neurotransmission, inhibition of insulin signalling and regulation of inflammation.[3] Recent studies have shown that the deregulation of H_2S has been correlated with the symptoms of Alzherimer's disease, Down's syndrome, diabetes, and liver cirrhosis.[4] Obviously, accurate and real-time detection of H_2S concentrations in biological samples is highly required and would provide important information to understand the functions of H_2S.

Currently, several methods for H_2S detection have been established including colorimetric and electrochemical assays, gas chromatography, sulfide precipitation[5] and fluorescence-based assays.[6] Among these methods, fluorescence-based assays were useful because of their high sensitivities, non-destructive detection, and high spatiotemporal resolutions. A few fluorescent probes designed for H_2S detection in living systems have been reported since 2011.[7] Several significant characteristic properties of H_2S, such as its dual nucleophilicity,[8] excellent reducing property,[9] high binding affinity towards copper ions,[10] efficient thiolysis of dinitrophenyl ethers[11] as well as specific addition reactions toward unsaturated double bonds,[12] have been exploited for the design of fluorescent probes.

The fluorescent probes designed based on the strategy of the dual nucleophilicity of H_2S are especially attractive, which contain a potential fluorescent reporter and a H_2S trap group with two electrophilic reaction sites.[8] Another strategy which draws our attention is by using the reducing property of H_2S and the nucleophilicity of the produced amine. A designed trap group would be triggered by the reduction of an azido moiety via H_2S, and the resulting amine would attack the adjacent electrophilic reaction site through an intramolecular nucleophilic subsititution (SN_i).[13] Han used o-(azidomethyl)benzoate[14] as the probe trigger for their H_2S probe which can easily discriminate H_2S from the interfering biological thiols such as cysteine and glutathione (Scheme 1).[13a] The azido moiety in the probe 7-o-2'-(azidomethyl)benzoyl-4-methylcoumarin was reduced to the amino group which then attacked the benzoate, releasing the fluorescent 7-hydroxy-4-methylcoumarin.

Inspired by Han's design, we developed a fluorescent probe for discriminating detection of H_2S over thiols containing 2-(azidomethyl)-4-nitrobenzoate as the trigger (Scheme 1). This probe was synthesized from *N*,*N*-diethylrhodol, a new platform for the construction of fluorescent probes.



Scheme 1 The design of H_2S fluorescent probe based on a novel H_2S trap group with an electron rich dye.

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† Footnotes relating to the title and/or authors should appear here. Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

Firstly, a molecule named **AR** (Fig. 1) was synthesized following Han's design. However, when treated with 80 eq of Na_2S in CH_3OH/PBS buffer (10 mM, pH = 7.4, 5/95), no obvious fluorescence enhancement was observed. Some other interfering molecules containing sulfur were also examined. Unfortunately, the results showed that **AR** is not a proper probe for any of them (Fig. 1). A more extensive screening made to test **AR** was not promising as well (Figs. S1 and S2, ESI†).

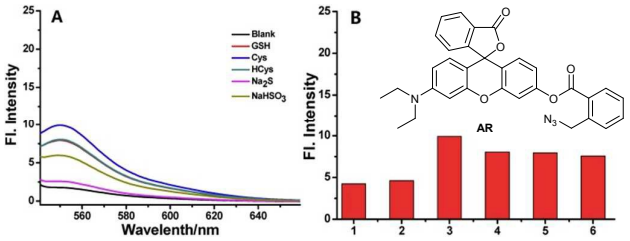
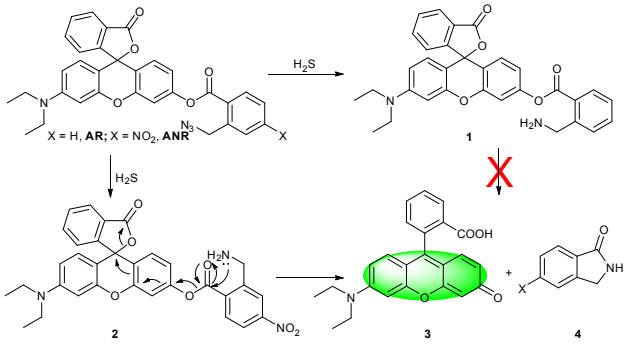


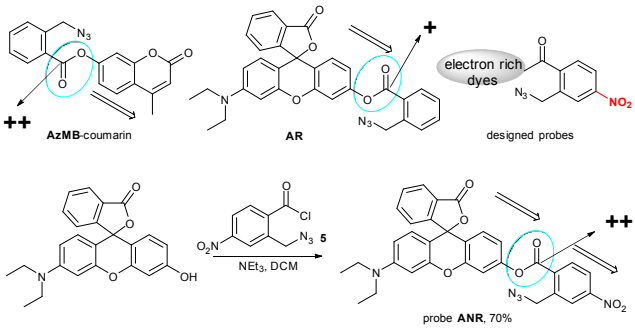
Fig. 1 (A) Fluorescence response of **AR** (5 μM) upon addition of various species (80 eq) in CH_3OH/PBS buffer (10 mM, pH = 7.4, 5/95). (B) Bar graph and the structure of **AR**. (1) Blank; (2) Na_2S; (3) Cys; (4) HCys; (5) GSH; (6) $NaHSO_3$. λ_{ex} = 519 nm, λ_{em} = 550 nm. Slits: 5/5 nm.

Perhaps the azido moiety was reduced to an amino group by H_2S, while the resulting amine was not able to undergo the subsequent substitution reaction (Scheme 2). We assumed that compound **1** was formed during this process. An HRMS-ESI test was taken to confirm our speculation. However compound **1** was not found when **AR** (5 μM in CH_3OH/PBS buffer, 10 mM, pH = 7.4, 5:95) was treated with 80 eq of Na_2S. Although the test failed to confirm our speculation, it proved that there neither **3** nor **4** were generated except a little **AR** ($[M + H]^+$ calcd for $C_{32}H_{27}N_4O_5^+$, 547.1976, found: 547.1901) was left in the solution (Fig. S3, ESI†).



Scheme 2 Speculation for the result of **AR** and **ANR** treated with H_2S and conformation for the result of **ANR**.

With this result in hand, we began to explore why molecule **AR** cannot act as a H_2S probe in contrast to **AzMB**-coumarin.[13a] The reason is perhaps that Han used an electron-withdrawing coumarin group as dye, which reduces the electron density of the ester carbonyl, facilitating the SN_i reaction. However, the Rhodol moiety is an electron rich ring, which we postulate will decrease the kinetics of the SN_i reaction. We believe that introducing an electron-withdrawing group in the other aryl ring would solve this problem (Scheme 3). 2-(azidomethyl)-4-nitrobenzoyl chloride **5** was synthesized according to a modified method.[14] Using this method, the probe **ANR** was obtained in 70% yield.



Scheme 3 Design and synthesis of probe **ANR** and possible explanation for the different results of probe **AzMB**-coumarin and **AR**.

When **ANR** (5 μM) was treated with 80 eq of Na_2S in CH_3OH/PBS buffer (10 mM, pH = 7.4, 5:95), the probe showed excellent response to H_2S. What's more, it could easily detect H_2S over biothiols and other nucleophiles (Fig. 2). As shown, the free probe **ANR** exhibited almost no fluorescence (fluorescence quantum yield: Φ = 0.0270, in CH_3OH/PBS buffer, 10 mM, pH = 7.4, 5/95, ESI†). When treated with Na_2S it elicited the obvious fluorescence turn-on at 550 nm (fluorescence quantum yield: Φ = 0.3520, in CH_3OH/PBS buffer, 10 mM, pH = 7.4, 5/95, ESI†). Then we evaluated the effect of pH on the fluorescence of **ANR** which showed the probe was very stable from pH 6 to 8, even in the presence of Na_2S (Fig. S4, ESI†).

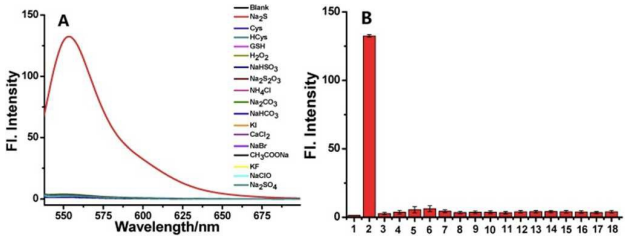


Fig. 2 (A) Fluorescence spectra of **ANR** (5 μM) upon addition of various species (80 eq) in CH_3OH/PBS buffer (10 mM, pH = 7.4, 5/95). (B) Bar graph. (1) Blank; (2) Na_2S; (3) GSH; (4) Cys; (5) HCys; (6) $NaHSO_3$; (7) H_2O_2; (8) $Na_2S_2O_3$; (9) NH_4Cl; (10) Na_2CO_3; (11) $NaHCO_3$; (12) KI; (13) $CaCl_2$; (14) NaBr; (15) CH_3COONa; (16) KF; (17) NaClO; (18) Na_2SO_4. λ_{ex} = 519 nm, λ_{em} = 550 nm. Slits: 5/5 nm.

The HRMS-ESI test proved that compound **3** ($[M + H]^+$ calcd for $C_{24}H_{22}NO_4^+$, 388.1543, found: 388.1547, Fig. S5, ESI†) was generated in the solution, which contributed to the fluorescence enhancement (Scheme 2).

The above results proved that **ANR** was a candidate for H_2S probe. The ester bond in **ANR** was stable enough with most of the nucleophiles. Only when the azido was reduced to an amine by H_2S, a SN_i reaction would happen and break the ester bond which released the fluorophore (Scheme 2). While the ester bond in **AR** was too stable to be broken even coexistent with H_2S. Considering the Rhodol moiety is an electron rich ring, it is obvious that 2-(azidomethyl)-4-nitrobenzoate is a good trap for the design of H_2S probe with electron rich dyes.

Since the catabolism of H_2S is extremely fast *in vivo*, time-based experiments were performed to study the kinetics of **ANR** reacting with H_2S. As was expected, the reaction time was as short as 4 minutes to reach a satisfied fluorescent intensity (Fig. 3). When

extended to 9 minutes, the fluorescent intensity only increased a minimal amount, thus we chose 4 minutes as test time.

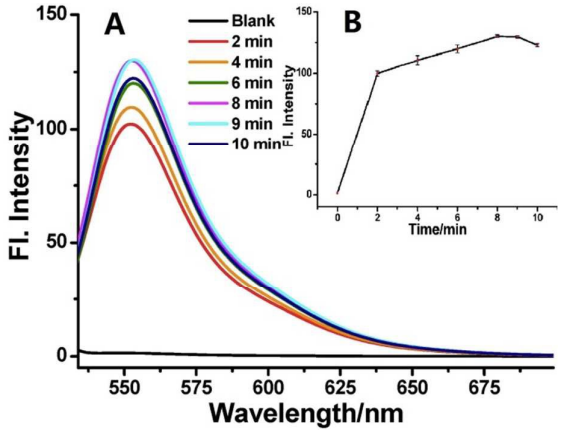


Fig. 3 (A) Time-dependent fluorescence spectral changes of **ANR** (5 μM) with H_2S (80 eq Na_2S, 400 μM) in CH_3OH/PBS buffer (10 mM, pH = 7.4, 5/95). Time points represent 2, 4, 6, 8, 9 and 10 min. (B) Line chart. λ_{ex} = 519 nm, λ_{em} = 550 nm. Slits: 5/5 nm.

Subsequently, we examined the reactivity of **ANR** (5 μM) towards different concentrations of Na_2S in CH_3OH/PBS buffer (10 mM, pH = 7.4, 5/95) at 25 °C. It turned out that the increasing of the probe's fluorescence intensity in PBS solution was linear to the concentration of Na_2S up to 600 μM, which indicated that **ANR** could monitor H_2S quantitatively in a wide concentration range (Fig. 4). Specifically, the detection limit of **ANR** was determined to be 0.4327 μM based on the 3σ/slope method (ESI†).

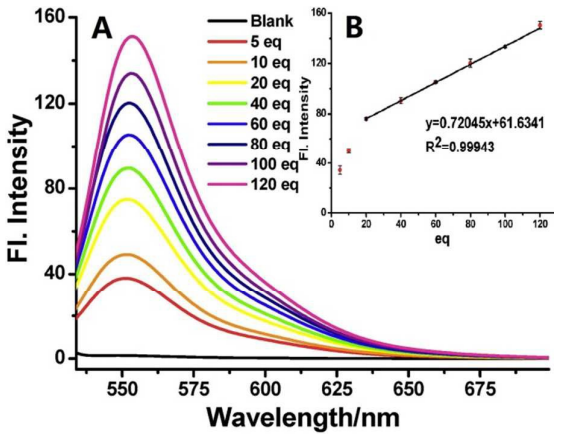


Fig. 4 (A) Fluorescence spectra of **ANR** (5 μM) upon addition of Na_2S (0–120 eq, 0–600 μM) in CH_3OH/PBS buffer (10 mM, pH = 7.4, 5/95). Spectra were recorded after incubation with different concentrations of Na_2S for 4 min. (B) Linear fitting chart. λ_{ex} = 519 nm, λ_{em} = 550 nm. Slits: 5/5 nm.

Next, we performed competition experiments in the presence of biothiols and other interfering molecules (Fig. 6). **ANR** was still able to respond to H_2S with strong fluorescence enhancements in the coexistence of other biothiols or other interfering molecules. What is worthy mentioning is that 4.0 mM of Cys or GSH had little interference to **ANR** (Fig. 5, the blue colour bar). The above results demonstrated the high selectivity of **ANR** towards H_2S and its feasibility to detect H_2S in the presence of other biologically relevant biothiols and other interfering molecules.

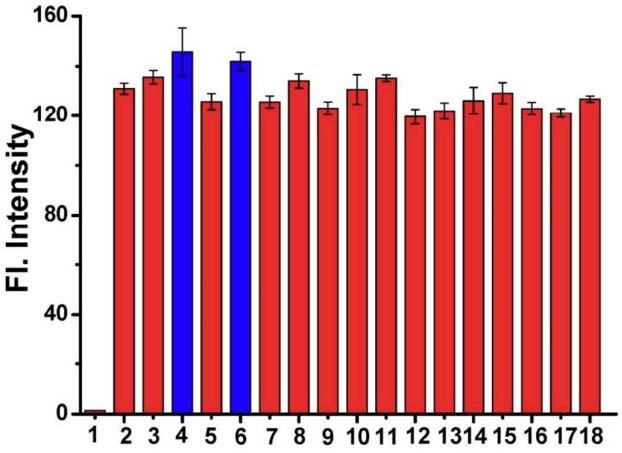


Fig. 5 Fluorescence responses of **ANR** (5 μM, 4 min after incubition) to Na_2S and other biologically relevant biothiols (80 eq for most of them except 2 samples with 800 eq) in CH_3OH/PBS buffer (10 mM, pH = 7.4, 5/95). (1) Blank; (2) Na_2S; (3) GSH; (4) 800 eq of GSH; (5) Cys; (6) 800 eq of Cys; (7) HCys; (8) $NaHSO_3$; (9) H_2O_2; (10) $Na_2S_2O_3$; (11) NH_4Cl; (12) Na_2CO_3; (13) $NaHCO_3$; (14) KI; (15) $CaCl_2$; (16) NaBr; (17) CH_3COONa; (18) KF. λ_{ex} = 519 nm, λ_{em} = 550 nm. Slits: 5/5 nm.

Encouraged by the above results, we subsequently explored the potential applications of **ANR** in biological systems. Firstly, the cytotoxicity of **ANR** was evaluated using MCF-7 cells and 3T3 cells by MTT assay (Fig. S6, ESI†). Probe **ANR** showed almost no cytotoxicity in the 0.1–30 μM range for MCF-7 cells (cancer cells, a human breast adenocarcinoma cell line, IC_{50}, 69.6 μM) and 3T3 cells (healthy cells, a standard fibroblast cell line, IC_{50}, 91.5 μM), implying that the probe is probably suitable for bioimaging of H_2S in living cells. Considering the regulation of H_2S on cancer cells, MCF-7 cells were chosen for the biology tests. MCF-7 cells incubated with **ANR** (5 μM) in culture medium for 30 min at 37 °C, showed almost no fluorescence (Fig. 6C). However, if the MCF-7 cells were pretreated with **ANR** (5 μM) for 30 min and then incubated with Na_2S (400 μM) for 30 min, strong fluorescence was observed (Fig. 6D). This result indicated that probe **ANR** has the potential to visualize H_2S levels in living cells.

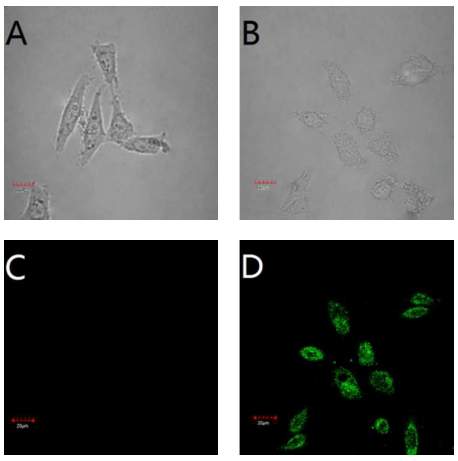


Fig. 6 Bright-field (A) and fluorescence image (C) of MCF-7 cells incubated with **ANR** (5 μM) for 30 min. Bright-field (B) and fluorescence image (D) of MCF-7 cells incubated with **ANR** (5 μM) for 30 min and washed with PBS three times. After replacement of

the medium, cells were incubated with Na_2S (400 μM) for another 30 min.

Conclusions

In summary, a novel reaction-type fluorescent probe **ANR** for fast detection of H_2S in aqueous solution was developed based on a novel H_2S trap group 2-(azidomethyl)-4-nitrobenzoate and an SN_i reaction mechanism. The novel H_2S trap group is very effective for the design of H_2S fluorescent probes especially with electron rich dyes. While the other trap group 2-azidomethylbenzoate was failed to be introduced to H_2S fluorescent probes with electron rich dyes. This probe shows high selectivity and sensitivity for H_2S even in the presence of micromole amounts. Probe **ANR** shows a linear fluorescence intensity enhancement with a wide range of concentrations of Na_2S. Preliminary fluorescence imaging experiments in cells indicate its potential to probe H_2S in biological systems.

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Construction of a turn-on probe for fast detection of H_2S in living cells based on a novel H_2S trap group with an electron rich dye

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