Organic & Biomolecular Chemistry

PAPER

View Article Online
View Journal | View Issue

Cite this: *Org. Biomol. Chem.*, 2014, **12**, 5629

Chemoselective reduction and self-immolation based FRET probes for detecting hydrogen sulfide in solution and in cells†

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Received 5th May 2014,
Accepted 4th June 2014

DOI: 10.1039/c4ob00923a

www.rsc.org/obc

Hydrogen sulfide (H_2S) has been regarded as the third gaseous transmitter. Based on the mechanism of chemoselective azido reduction and self-immolation, five fluorescence resonance energy transfer (FRET) probes for the detection of H_2S were designed and synthesized. The effect of functional substitution of the self-immolative moiety on azido reduction and quinone-methide rearrangement were investigated. Their fluorescence responses and chemoselectivity for H_2S detection were evaluated in solutions and in cells. This strategy may provide a general route for designing H_2S probes with many commercially available FRET pairs.

Introduction

Although H_2S has been recognized as a well-known toxic pollutant gas with an unpleasant smell, it has now been recognized as an important gaseous transmitter in addition to nitric oxide (NO) and carbon monoxide (CO).[1] It is involved in a variety of physiological and pathological processes in biological systems, such as vasodilation,[2] angiogenesis,[3] neuromodulation,[4] apoptosis[5] and inflammation.[6] The endogenous H_2S is synthesized in mammalian systems mainly with involvement of pyridoxal-5′-phosphate-dependent enzymes, such as cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE) and 3-mercaptopyruvate sulfur transferase (3-MST).[7] Due to the quick metabolism of H_2S *in vivo*, it was difficult to detect this transient gaseous signaling molecule selectively and sensitively. Several approaches, including colorimetric assay,[8] electrochemical assay,[9] gas chromatography[10] and sulfide precipitation,[11] have been developed for the detection of H_2S. However, they are usually time consuming and destructive with relatively high cost and not suitable for applications in biological samples.

Recently, several kinds of fluorescent probes for H_2S detection have been reported. In general, these probes were designed based on the different mechanisms of specific H_2S-involved reactions: (a) chemical reduction: the reduction of azide, nitro, hydroxyl amine group, disulfide or selenoxide;[12] (b) precipitation of heavy metal sulfide: fluorescence quenching of Cu^{2+} from Cu^{2+}-coordinated organometallic complexes was eliminated by the formation of CuS;[13] (c) nucleophilic reaction: the nucleophilic sulfide is utilized to react with fluorescent probes through Michael addition or nucleophilic substitution.[14]

Although much progress has been achieved on the development of fluorescent probes for H_2S, the sensitive moieties for H_2S (such as azido) are directly located on the reporter moieties which caused the limited options for available fluorophores and/or difficult and laborious synthesis of fluorescent probes. A general strategy with a universal H_2S sensitive moiety and many commercially available fluorophores will greatly expand the scope of fluorescent probes for H_2S detection. With this idea in hand, we designed a series of fluorescent probes based on fluorescence resonance energy transfer (FRET). These FRET probes contained the azido moiety as the sensor, and a fluorophore and a quencher as the FRET pair linked by a self-immolative linker which can dissociate through quinone-methide rearrangement after the reduction of the azido moiety,[15] as shown in Scheme 1.

Results and discussion

Synthesis and evaluation of FRET-based H_2S fluorescent probes

The FRET quencher–fluorophore pairs have been widely used in oligonucleotide probes and peptide probes for detecting nucleic acid mutation and enzymatic activities.[16] Fluorescein isothiocyanate (FITC) and [4′-(*N*,*N*′-dimethylamino)phenylazo] benzoyl (DABCYL), as an excellent FRET pair, were chosen as the fluorophore and quencher moieties due to their easy commercial availability and wide applications in sensors. Different

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†Electronic supplementary information (ESI) available: Synthetic details, NMR, MS and additional figures are provided. See DOI: 10.1039/c4ob00923a

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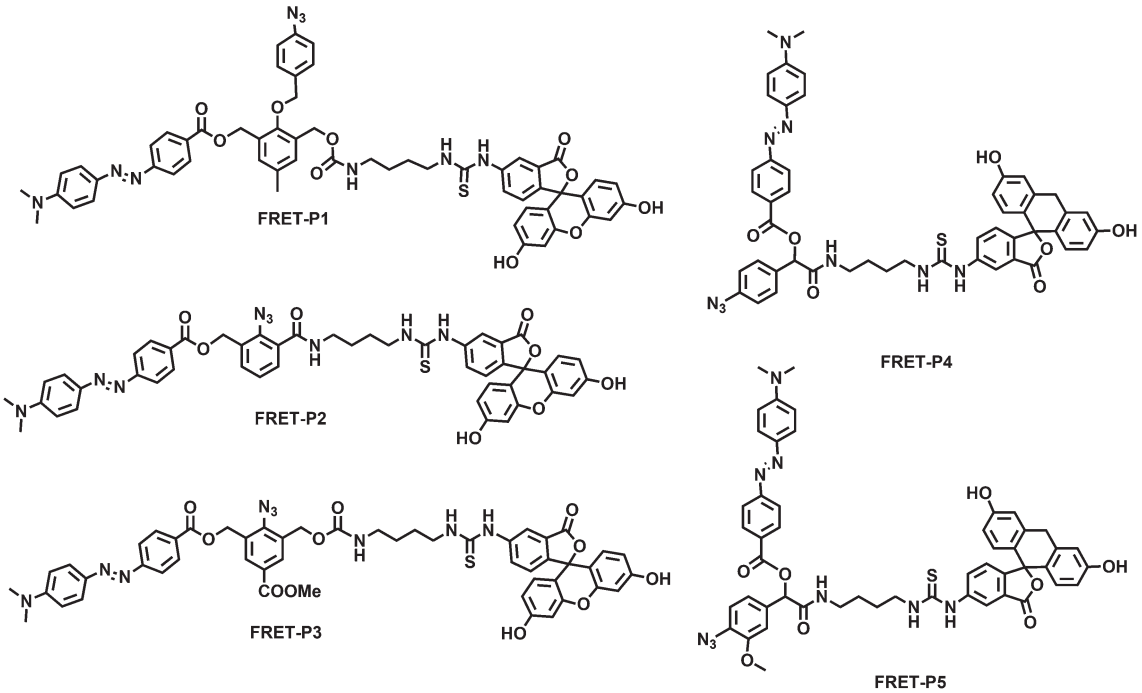
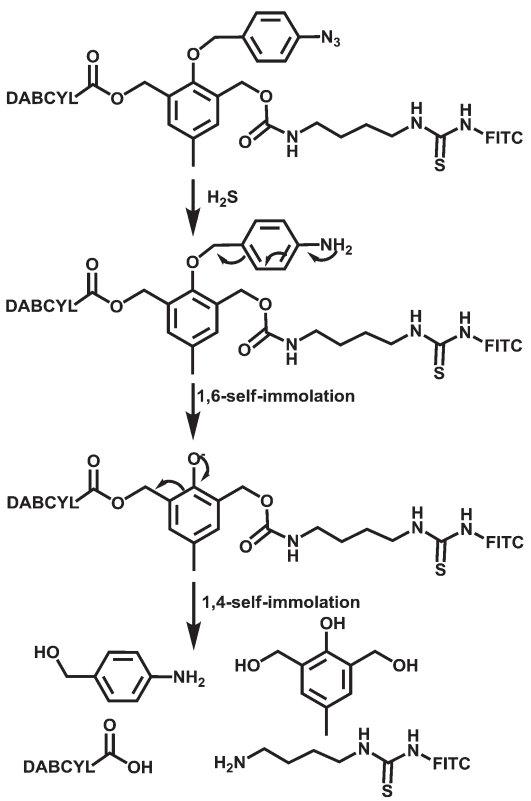


Scheme 1 The structures of FRET probes.

immolative linkers were developed, as shown in Scheme 1. For FRET-P1 (the synthetic route is shown in Scheme S1†), a *p*-azidobenzyl moiety was first linked to 2,6-bis(hydroxymethyl)-*p*-cresol whose two hydroxyl groups were then selectively coupled with a DABCYL and fluorescein through ester and carbomate formation respectively. With the existence of H_2S, the azido moiety could be reduced to the amine moiety that could further trigger self-immolation through quinone-methide rearrangement, as shown in Scheme 2; 1,4 or 1,6-quinone-methide rearrangement induced the cleavage of the chemical bond C–O and release the DABCYL. FITC was then excited to emit fluorescence thanks to the release of DABCYL. Initial evaluation of its response to the existence of H_2S indicated that a relatively slow increase in fluorescence intensity was observed with the excitation at 488 nm (Fig. S1†). This slow response may be due to the dual immolation of 1,6 and 1,4-quinone-methide rearrangement (Scheme 2). To shorten the two-step immolation, another two probes, FRET-P2 and FRET-P3, were designed and synthesized (Schemes S2 and S3†).

For these two probes, only one 1,4-quinone-methide rearrangement was needed to break the immolative linker and release the fluorescein moiety. Before the synthesis of FRET-P2 and FRET-P3, a preliminary experiment on the azide reduction of methyl 3-methyl-2-azide benzoate in the presence of H_2S was carried out. A quick response to H_2S was observed with the introduction of the electron acceptor on the phenyl moiety (Fig. S2†). Further coupling with DABCYL and fluorescein to this linker afforded two probes, FRET-P2 and FRET-P3. However, their responses to H_2S were still quite slow (Fig. S3



Scheme 2 The self-immolation mechanism of FRET-P1 for the detection of H_2S.

and S4†). Although the electron acceptor group on the phenyl moiety made the azido group easier to be reduced, it actually slowed down the 1,4-quinone-methide rearrangement. This may led to even slower responses of these two probes to H_2S.

Based on the above observations, we further developed another two FRET probes, FRET-P4 and FRET-P5 (Schemes S4 and S5†). These two probes have a 4-azidophenylhydroxyl acetic acid moiety. Once the azido moiety was reduced to the amine group, only one 1,6-quinone-methide rearrangement was needed to break the linkage of the fluorophore and the quencher. Time dependence of increase in fluorescence intensity indicated that FRET-P4 showed much faster response to H_2S than FRET-P1 (Fig. S5†). According to a previous literature report,[17] introducing an electron donor group on the *ortho-* or *para-* of the azidobenzyl moiety could promote the intramolecular electron transfer and 1,4 or 1,6-elimination. FRET-P5 containing the methoxy group on the phenyl moiety further enhanced the kinetics of H_2S triggered fluorescence emission, as shown in Fig. 1. By further simple separation and mass spectra analysis, we confirmed the existence of the product [4′-(*N*,*N*′-dimethylamino)phenylazo]benzoyl acid (DABSYL acid) (Fig. S9†).

After the evaluation of these FRET based probes, FRET-P5 showed the best sensitivity for the response of H_2S with a 12-fold enhancement in fluorescence intensity with 100 μM NaHS in 60 min, as shown in Fig. 1 and S6.† However, only 2.5, 1.3, 1.3 and 2.5 fold enhancements were observed for FRET-P1, FRET-P2, FRET-P3 and FRET-P4, respectively, with relative slower response under the same conditions.

Selectivity of FRET-P5 for the detection of H_2S

Due to the outstanding fluorescence response of FRET-P5 for the detection of H_2S, it was selected for further evaluation. Fig. 2 shows the selectivity for sulfide over biologically relevant reactive sulfur species (RSS), such as reduced GSH, L-Cys, $NaHSO_3$, $Na_2S_2O_3$ and $Na_2S_2O_5$. With the inclusion of 100 μM FRET-P5, the relative fluorescence intensity in the presence of 100 μM NaHS was 12 folds over probe solution without analytes and 6.4–8.7 folds over the presence of all other RSS species with high concentrations (1 mM for $NaHSO_3$, $Na_2S_2O_3$ and $Na_2S_2O_5$, and 5 mM for reduced GSH and L-Cys) in 60 min at room temperature. Even with higher concentration up to 10 mM of reduced GSH and L-Cys, FRET-P5 still showed good selectivity for H_2S. In addition to reactive sulfur species, we also evaluated the probe over other anions, reactive oxygen species (ROS), reactive nitrogen species (RNS) and 10% FBS in RPMI-1640. All these species and medium did not trigger fluorescence emission and had no obvious effect on the detection of H_2S with the probe (Fig. S7†).

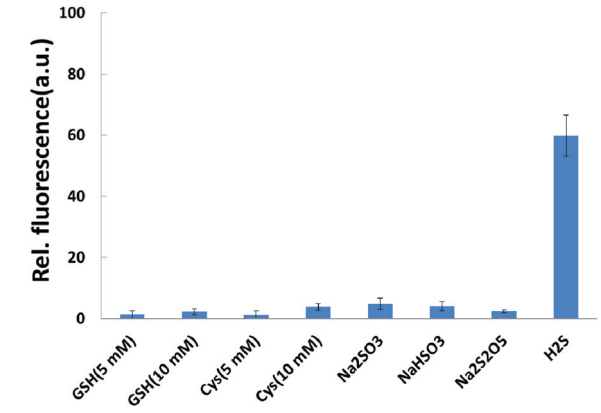


Fig. 2 Fluorescence responses of 100 μM FRET-P5 to relevant RSS species. Bars represent the mean fluorescence responses at 1 h after the addition of 100 μM NaHS or 1 mM of all other relevant RSS species (reduced GSH and L-Cys set at 5 mM or 10 mM). All measurements were done in fluorometer. Data were acquired at 37 °C in 10 mM degassed solution (PBS–DMF, v : v = 6 : 4, pH 7.4) with excitation at 488 nm.

Concentration dependence of FRET-P5 for fluorescence detection of H_2S

The concentration dependence of the triggered fluorescence intensity on H_2S was also evaluated, as shown in Fig. 3. The

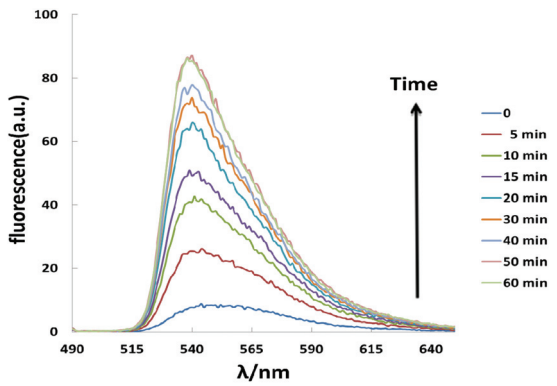


Fig. 1 Fluorescence response of 100 μM FRET-P5 to 100 μM NaHS. Data were acquired at 37 °C in 10 mM degassed solution (PBS–DMF, v : v = 6 : 4, pH = 7.4) with excitation at 488 nm. Emission was collected from 500 to 650 nm at 0, 5, 10, 15, 20, 30, 40, 50 and 60 min after the addition of 100 μM NaHS.

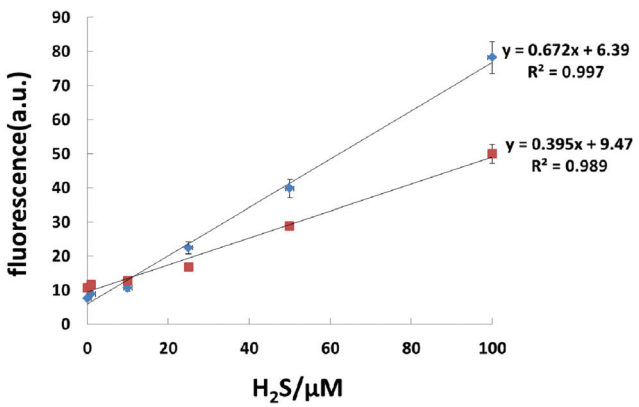


Fig. 3 Linear correlation of fluorescence intensity toward H_2S concentration using fluorospectrophotometer: FRET-P5, 100 μM; NaHS, 0, 1, 10, 25, 50, 100, μM, data were acquired at 37 °C in 10 mM degassed solution (PBS–DMF, v : v = 6 : 4, pH 7.4, diamond) and (commercial fetal bovine serum–DMF, v : v = 6 : 4, pH 7.4, square) with excitation at 488 nm. Emission was collected 60 min after the addition of NaHS.

probe FRET-P5 was treated with H_2S in degassed buffer and fetal bovine serum at various concentrations. The fluorescence enhancement of FRET-P5 solutions (100 μM) was monitored using a fluorescence microplate reader at 37 °C. Standard curves for FRET-P5 in degassed buffer and fetal bovine serum were obtained between fluorescence signal at 538 nm and the concentrations (μM) of H_2S for FRET-P5 in 60 min incubation. The regression equations were $F_{Ex/Em(488/538\ nm)} = 0.692\ [H_2S] + 6.39$ with $R^2 = 0.997$ and $F_{Ex/Em(488/538\ nm)} = 0.395\ [H_2S] + 9.47$ with $R^2 = 0.989$ for degassed buffer and fetal bovine serum respectively. The relatively low slope for fetal bovine serum may be due to the fact that proteins presented in plasma are scavengers for H_2S.[18]

Detection of H_2S in HeLa cells using a high content analyzer

We then tested the potential application of FRET-P5 for the detection of H_2S in HeLa cells. First, the cell viability was tested with FRET-P5. As indicated in Fig. S8,† no obvious toxicity was observed with the probe concentration up to 100 μM. HeLa cells were first incubated with 10 μM FRET-P5 PBS buffer for 30 min in a 96-well plate and then washed with PBS buffer. Cells were further incubated without or with 100 μM NaHS for another 60 min. After final washing, the cells were then subjected to imaging and analysis by a High Content Analyzer (Operetta, Perkin Elmer). As shown in Fig. 4, the addition of H_2S induced intense intracellular fluorescence emission than that without H_2S addition. Reduced glutathione was previously used as the substance by CBS for enzymatic production of endogenous H_2S.[14b] When HeLa cells were treated with reduced glutathione (250 μM), we also observed the increase in fluorescence emission of cells which indicated more generation of endogenous H_2S by the enzyme in cells. Further analysis indicated that the addition of exogenous H_2S (100 μM) induced about 2 fold enhancement in fluorescence emission, and the endogenous product of H_2S by glutathione induced about 1.6 fold enhancement in fluorescence emission.

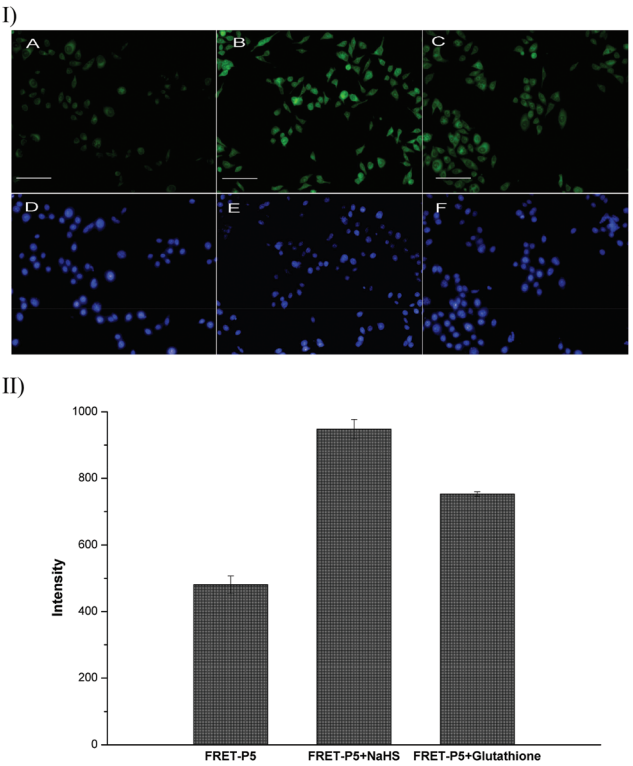


Fig. 4 Visualization (I) and quantification (II) of exogenous and endogenous H_2S with FRET-P5 in HeLa cells by a high content analyzer. A: cells were incubated with FRET-P5 (10 μM, 20 min) followed by PBS washing and further incubated for 60 min; B: cells pre-stimulated by PMA (1 μg ml^{-1}, 30 min) were treated with NaHS (100 μM, 60 min) after incubation with FRET-P5 (10 μM, 20 min); C: cells were treated with glutathione (250 μM, 60 min) after the incubation with FRET-P5 (10 μM, 20 min); D, E, F: the images of A, B, C with Hoechst staining at 4 °C, respectively; scale bars represent 100 μm.

Conclusions

We designed and synthesized a series of fluorescent probes (FRET-P1, FRET-P2, FRET-P3, FRET-P4 and FRET-P5) for the detection of H_2S based on FRET. Azido functionalized self-immolative linkers were used for the linkage of fluorescein and DABCYL. With the reduction of the azido moiety by H_2S, a 1,4 or 1,6-quinone-methide rearrangement triggered the breakage of linkers and the release of fluorescence, and induced the increase in fluorescence intensity. And quinone-methide rearrangement is the rate-limiting step for the quick response to H_2S with these FRET probes. An electron acceptor group on the aromatic moiety (FRET-P2 and FRET-P3) enhanced the reduction of the azido moiety by H_2S, but decreased the quinone-methide rearrangement, which led to less efficient fluorescence detection of H_2S. Shortening two steps (FRET-P1) to one step (FRET-P4) of quinone-methide rearrangement improved the kinetics of H_2S triggered fluorescence emission. Attachment of an electron donor (methoxy) on the aromatic moiety (FRET-P5) further increased the kinetics of the azido reduction by H_2S. Further evaluation of FRET-P5 showed its high sensitivity and selectivity for the detection of H_2S over other biologically relevant RSS, ROS, RNS and common ions. The linear relationships were well obeyed between the fluorescence intensity of released fluorescein and the concentrations of H_2S in both degassed buffer and fetal bovine serum. The study indicated that a general designing strategy may be used for sensors with many commercially available fluorophores. And the structure/function relationship provides more insights into designing more chemoprobes based on FRET fluorophores and self-immolative linkers.

Acknowledgements

This work was supported by the National Basic Research Program of China (973 Program; grant no. 2013CB933800 and 2012CB720600), the National Natural Science Foundation of China (grant no. 21372018) and the State Key Laboratory of Pharmaceutical Biotechnology (grant no. KF-GN-201305).

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