Fluorescence Imaging

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A Ratiometric Fluorescent Probe for Rapid Detection of Hydrogen Sulfide in Mitochondria**

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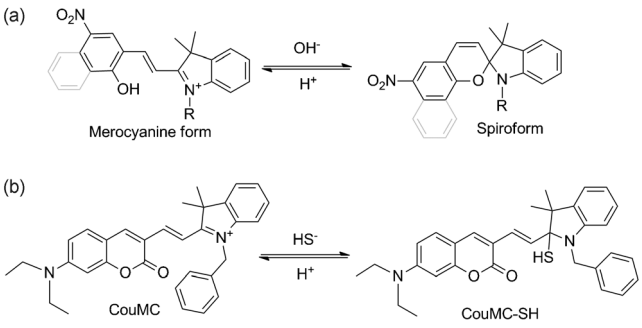
Traditionally considered as a toxic gas with unpleasant smell, hydrogen sulfide (H_2S) has emerged as the third endogenous gaseous signaling compound (gasotransmitter) after NO and CO.[1] Endogenous H_2S has been found in high concentrations (10 to 600 μM) in the brain of bovine, rat, and human.[2] H_2S has also been recognized to mediate a wide range of physiological processes, such as vasodilation,[3] antioxidation, anti-apoptosis and anti-inflammation,[4] and the abnormal H_2S level was correlated to diseases such as Alzheimer's disease and Down's syndrome.[5,6] The increasing interest in endogenous H_2S demand rapid, facile, and reliable detection techniques, since the current colorimetric and electrochemical assays, gas chromatography, and sulfide precipitation are difficult to implement for in situ detection.[7] Fluorescence imaging through staining with a fluorescent probe is now one of the most attractive molecular imaging techniques for the in vivo detection of biomolecules owing to its high sensitivity/selectivity, non-invasiveness, and aptness for living cells, tissues, and living small animals, and developing fluorescent probes for H_2S detection is now drawing much attention.

A few fluorescent turn-on probes for H_2S have been reported. Some could even be used for imaging of intracellular H_2S, while others were only applicable for in vitro detection of H_2S in blood samples.[8] They are chemodosimeters based on the specific H_2S-induced reactions, such as azide reduction,[8a–d] quencher (such as Cu^{2+}) removal,[8f,g] and nucleophilic reaction to achieve strong fluorescence.[8h-j] Most of them display a response time between 20 min to two hours, except for those that are based on the reduction of dansyl azide and Cu^{2+}-removal sensing mechanisms reported by Wang and Nagano, respectively.[8b,f] These probes, however, are not suitable for real-time imaging of quick H_2S-related biological processes, and searching for quick reactions that are sensitive to H_2S to enhance the response rate of H_2S probes has always been attractive and challenging. Of course, improving the H_2S selectivity over biological thiols is also essential for the sensing reaction. On the other hand, these reported turn-on probes are difficult to give quantitative information on the H_2S concentration, since molecular emission intensity can be distinctly affected by photobleaching, microenvironments, and local probe concentration. Therefore, ratiometric probes for H_2S are highly appealing owing to their ability in quantitative tracking, because they endow a self-calibration effect, which reduces most of the aforementioned interferences.[9] The only ratiometric H_2S probe reported so far is Cy-N_3,[10] but it responds to H_2S in 20 min. Moreover, fluorescent probes that are able to provide information on H_2S in an organelle of interest are especially appreciated, since studies have implied that H_2S biology is associated with certain organelles such as mitochondria.[11] Herein, we report a novel ratiometric fluorescent probe, CouMC, the function of which is based on the selective nucleophilic addition of HS^- to a specific merocyanine derivative in medium of near neutral pH value (Scheme 1). Besides the rapid and specific response to H_2S, this probe can also be applied for preferential imaging of H_2S in mitochondria of living cells.

Probe CouMC was constructed by connecting a coumarin fluorophore and an indolenium block through an ethylene group. CouMC can be considered as a hybrid fluorophore of coumarin and merocyanine. The merocyanine moiety was incorporated not only as fluorophore but also as the guest receptor, since its indolenium C-2 atom is an effective target for a nucleophilic analyte. Normally, simple spiropyran undergoes a reversible conversion between the merocyanine form and the spiroform, and displays a switch of color or emission, since the reversible nucleophilic addition of



Scheme 1. a) Reversible conversion between the merocyanine form and the spiroform of spiropyran; b) the proposed H_2S sensing mechanism and acid-induced retrieval of CouMC.

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a phenol anion to the indolenium C-2 atom alters the conjugated system and internal charge transfer (ICT) effect of the whole molecule (Scheme 1).[12] We envisioned that HS^-, the main stable form of H_2S in the physiological condition, can be added to the indolenium C-2 atom of CouMC as a nucleophile and lead to a ratiometric sensing behavior by eliminating merocyanine emission but retaining coumarin emission. Since biological thiols, such as cysteine (Cys), glutathione (GSH), and homocysteine (Hcy), possess high pK_a values (≥ 8.5),[13] H_2S is expected to be a better nucleophile than these biological thiols in neutral medium owing to its lower pK_a value (ca. 7.0). Therefore, CouMC was expected to have better selectivity for H_2S over these biological thiols, which are the main interference in intracellular H_2S detection.

This new probe shows fine aqueous solubility, and its fluorescence and sensing behavior to H_2S were tested in phosphate-buffered saline (PBS; 20 mM, pH 7.4) containing 2% DMSO. Free CouMC (10 μM) shows two well-resolved emission bands centered at 510 and 652 nm (λ_{ex}, 475 nm; Figure 1a), which can be assigned as the emission band of coumarin and merocyanine, respectively. The distinct gap between the two bands is over 140 nm, which makes this probe favorable for the dual emission ratiometric imaging owing to the minimum overlap between the two bands. Its quantum yield was determined as 0.03 with cresyl violet as reference.[14] Fluorescence titration of a CouMC solution with NaHS (0–200 μM) demonstrated that its emission band at 652 nm decreased distinctly, while the band at 510 nm underwent a noticeable increment simultaneously (Figure 1a). The intensity ratio of the two emission bands, F_{510}/F_{652}, increased from 0.17 to 21.5, and the final enhancement factor is over 120-fold. The detection limit according to the ratiometric fluorescent sensing was determined as approximately 1 μM (Figure S3 in the Supporting Information). The absorption spectrum of free CouMC (25 μM) displays a strong ICT band at 588 nm (ε, 67500 $M^{-1}cm^{-1}$, Figure 1b). This band decreased gradually upon HS^- titration, confirming the disruption of the ICT effect in the whole molecule caused by HS^- addition. The solution turned from dark blue to very pale blue, thereby suggesting HS^- can be detected with the naked eye when using CouMC. The titration profile according to the absorbance at 588 nm indicated that CouMC bind HS^- with a 1:1 stoichiometry.

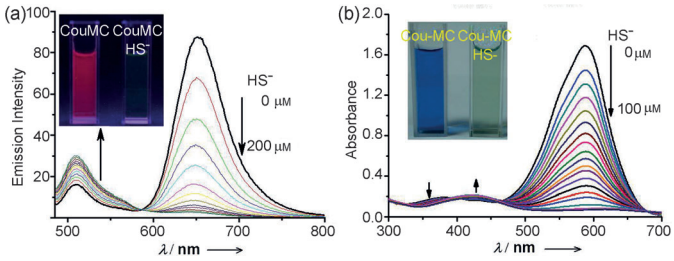

Figure 1. a) Fluorescence spectra of CouMC (10 μM) in PBS (20 mM, pH 7.40, 2% DMSO, v/v) obtained upon titration with HS^- from 0 to 200 μM. λ_{ex}, 475 nm. b) Absorption spectra of CouMC (25 μM) in PBS (20 mM, pH 7.40, 5% DMSO, v/v) obtained upon titration with HS^- from 0 to 100 μM. Inset in (a): photograph of CouMC solutions upon irradiation by a UV lamp (365 nm) in the absence or presence of HS^-. Inset in (b): photograph of CouMC solutions in the absence or presence of HS^-.

CouMC displays a very quick response to HS^-. Temporal emission tracking of CouMC (10 μM) in the presence of NaHS (10-200 μM) suggested that the sensing reaction could be completed within 30 s, even the total HS^- level was as low as 10 μM (Figure S4 in the Supporting Information). If the HS^- level was in the range from 100 to 200 μM, the reaction could be completed within seconds. In fact, probe CouMC is able to detect H_2S more rapidly than most of the reported H_2S probes (typically 20 min–2 h). This provides the probe an advantage in real-time intracellular imaging, when considering the variable nature and quick metabolism of endogenous H_2S in biological systems.

The ratiometric sensing selectivity of CouMC for HS^- was investigated in PBS buffer of pH 7.4 (Figure 2a). Only HS^- (200 μM) induces a dramatic increment of emission ratio F_{510}/F_{652}, while other anions (HCO_3^-, F^-, Cl^-, Br^-, I^-, CN^-, NO_3^-, 1 mM), inorganic reactive sulfur species (SO_4^{2-}, SO_3^{2-}, $S_2O_3^{2-}$, SCN^-, 1 mM), reactive oxygen species (H_2O_2, O_2^-, ClO^-, 1 mM), NO and NO producers (CysNO, *S*-nitrosoglutathione, 1 mM) only trigger very minor changes. The classical nucleophile CN^- induces almost no change of the ratio; the protonation of CN^- in neutral medium might be the origin, since HCN possess a p$K_a \approx 9.2$.[15] Other nucleophiles such as ethanol and ethylamine (1 mM) induce also no obvious change in CouMC emission. The ratiometric HS^- sensing ability of CouMC in the presence of other biological thiols was also investigated. GSH (1 mM) or Cys (1 mM) induce only a neglectable enhancement of emission ratio F_{510}/F_{652} (Figure 2b). The emission ratio enhancement induced by Hcy (200 μM) and BSA (200 μM, a protein with exposed Cys residues) is also very limited when compared to that induced by HS^-. The sensing selectivity of CouMC for H_2S over Cys, GSH, Hcy, and BSA is approximately 430-fold, 460-fold, 54-

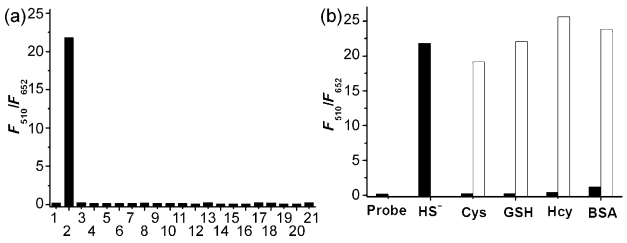

Figure 2. a) Emission ratio F_{510}/F_{652} of CouMC (10 μM) in PBS (20 mM, pH 7.40, 2% DMSO, v/v) in the presence of HS^-, various anions, or biologically relevant species. 1. Probe alone; 2. HS^- (200 μM); 3. HCO_3^-; 4. Cl^-; 5. Br^-; 6. I^-; 7. CN^-; 8. F^-; 9. NO_3^-; 10. SO_4^{2-}; 11. SO_3^{2-}; 12. $S_2O_3^{2-}$; 13. SCN^-; 14. $EtNH_2$; 15. EtOH; 16. NO; 17. *S*-nitrosoglutathione; 18. CysNO; 19. ClO^-; 20. O_2^-; 21. H_2O_2; (3–21: 1 mM). b) Emission ratio F_{510}/F_{652} of CouMC (10 μM) in the same buffer in the presence of HS^- (200 μM), Cys (1 mM), GSH (1 mM), Hcy (200 μM), and bovine serum albumin (BSA; 200 μM). For (b): black bars correspond to free CouMC, or CouMC with HS^- or CouMC with marked biological thiols; white bars to CouMC in the presence of both HS^- (200 μM) and the marked biological thiols.

fold, and 18-fold, respectively, and its HS^- sensing behavior in the presence of GSH, Cys, Hcy, or BSA indicated that CouMC is able to detect HS^- without any distinct interference from these biological thiols.

The color of the CouMC solution (25 μM) in the presence of NaHS (200 μM) can be recovered to dark blue when the pH value of the solution was adjusted to 1.0 (Figure S5a in the Supporting Information). However, a CouMC solution without HS^- displays stable absorption spectra in the pH range from 1.0 to 8.0. Similarly, the distinctly enhanced emission ratio F_{510}/F_{652} (21.5) of CouMC (10 μM) in PBS (1 mM, pH 7.4) upon NaHS addition (200 μM) can be reduced back to 0.32, when the pH value of the medium was adjusted to 2.5. If the pH value of the medium was adjusted again to 7.4, the emission ratio was enhanced back to approximately 15.0. The pH-dependent recovery of emission ratio can be observed for at least three cycles (Figure S5b in the Supporting Information). However, the emission ratio F_{510}/F_{652} of free CouMC exhibits very minor change in the pH range from 2.5 to 8.0 (Figure S6 in the Supporting Information), thus suggesting that the probe CouMC itself is stable in this pH range. All these results implied that CouMC might be retrievable in acidic condition after reacting with HS^-. Moreover, our attempt to separate HS^- nucleophilic addition product CouMC-SH was unsuccessful, and TLC analysis demonstrated that most product has been recovered to CouMC. The MALDI-TOF-MS spectrum of CouMC solution in the presence of excessive NaHS at pH 7.4 showed one major signal with m/z of 477.165 and another minor peak with m/z of 533.129, which can be assigned as $[CouMC]^+$ and $[CouMC\text{-}SH + Na]^+$, respectively (Figure S7 in the Supporting Information). This result implied a recovery of CouMC from CouMC-SH in the MS determination procedure. Moreover, MS determination of this solution after the pH value was adjusted to 2.5 gave only the signal of CouMC. The ^1H NMR and ^{13}C NMR spectra of CouMC in the presence of HS^- (1.2 equiv) are given in Figures S8 and S9 in the Supporting Information. As can be seen in Figure S8, almost all proton signals from the coumarin/merocyanine conjugated system underwent an obvious up-field shift after HS^- addition; this shift is consistent with the proposed product where the electron-withdrawing ability of indolenium N^+ has disappeared. In the meantime, the HS^- addition to $C{=}N^+$ resulted in the formation of a chiral center at C-2, thereby changing the singlet signals of the C-3 dimethyl groups into multiplets. Similarly, as can be seen from the ^{13}C NMR spectrum (Figure S9), the conversion of sp^2 to sp^3 hybrids of the C-2 atom resulted in a distinct up-field shift of the lowest signal of the indolenium C-2 atom at 182 ppm, which support the suggested indolenium C-2 atom as HS^- addition site.

Imaging with CouMC was investigated in MCF-7 cells with a confocal microscope Zeiss LSM710 (Figure 3). When the mono-emission mode imaging was used, the CouMC-stained cells showed a bright densely punctuated pattern in the cytoplasm, and the nonstained cells showed only a dim background, thus indicating the fine cell membrane permeability of CouMC. A colocalization assay with mitochondria dye Mito-marker Deep Red 633 and CouMC displayed that fluorescence of CouMC was colocalized with that of Deep Red (overlap coefficient 0.95), thereby implying a preferential distribution of CouMC in mitochondria (Figure 3a–c). The ratiometric imaging for intracellular H_2S was carried out with a dual-emission mode upon excitation at 488 nm. The ratiometric images (Figure 3f,i) were obtained by mediating the green channel image (band path: 500–560 nm; Figure 3d,g) with the related red channel image (band path: 640–700 nm; Figure 3e,h) by using the software of the microscope. MCF-7 cells stained by CouMC alone showed very dim fluorescence in the green channel and a strong

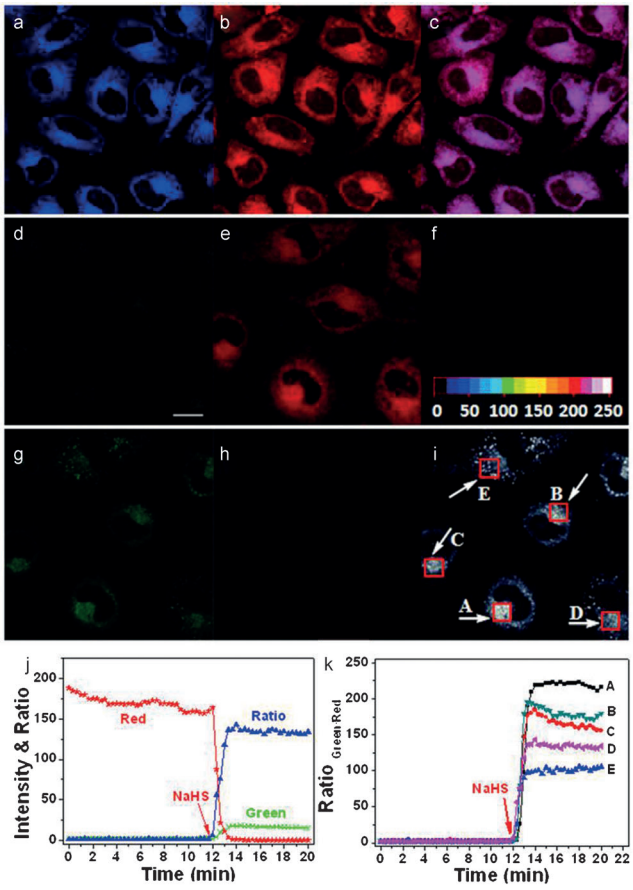

Figure 3. a–c) Confocal fluorescence images of MCF-7 cells costained by CouMC (10 μM, 30 min) and Mito marker Deep Red 633 (10 μM, 30 min). a) Pseudocolored image obtained with band path of 660–750 nm upon excitation of CouMC at 488 nm; b) image from band path of 665–750 nm upon excitation of Deep Red at 633 nm; c) overlay of (a) and (b). d–k) Confocal fluorescence imaging of MCF-7 cells by using a dual emission mode upon excitation at 488 nm. d–f) Images of cells stained by CouMC (10 μM, 10 min); g–i) images of cells preincubated with CouMC (10 μM) followed by incubation with NaHS (200 μM) for 8 min. d,g) Green-channel images collected with band path of 500–560 nm; e,h) red-channel images collected with band path of 640–700 nm; f,i) ratiometric images obtained by mediating green channel image with the related red channel image. j) Temporal profiles of average green and red channel fluorescence in region of interest D shown in (i), and the related profile of average fluorescence ratio (F_{green}/F_{red}); the values on the y-axis correspond to the fluorescence intensity of green and red channels, as well as the ratio of (F_{green}/F_{red}); k) temporal profile of average fluorescence ratio of (F_{green}/F_{red}) in regions of interest A, B, C, D, and E shown in (i), NaHS (200 μM) was added at the 12th min. Scale bar in (d): 20 μm.

fluorescence in the red channel. The related ratiometric image of dim background implies a very low H_2S level. Incubation with NaHS (200 μM) at the 12th min led to a distinct increase in green channel fluorescence, accompanied by the dramatic drop of red channel fluorescence, and a drastic enhancement of emission ratio (green/red) can be observed in the related ratiometric image. The NaHS-induced increase of the ratio is finished after approximately 80 s (Figure 3k), thus indicating that the H_2S level in mitochondria can be enhanced very quickly by incubation with 200 μM NaHS. The enhancement process was visualized and recorded in a video (see the Supporting Information) in which NaHS was added at the 12th second; 1 s video time equals to 1 min of real imaging process. Figure 3j showed clearly the response process of region of interest D upon incubation with NaHS according to the fluorescence recorded in both green and red channels. This preliminary imaging study suggested that CouMC can be used for ratiometric tracking of H_2S in mitochondria, which is of great importance to clarify the physiological roles of H_2S in the organelle.[11] Moreover, owing to the quick response rate of this probe it is possible to effectively track the quick increase of the H_2S concentration, which is finished in 80 s.

In summary, the new probe constructed by combining coumarin and merocyanine can be used for specific ratiometric sensing of H_2S. It preferentially targets mitochondria and has a quick response rate for intracellular H_2S, thus favoring the real-time intracellular H_2S imaging. The visualization of the increase of the mitochondrial H_2S concentration upon NaHS incubation by using CouMC staining is promising for the practical exploration of H_2S-related processes in mitochondria. This study provides an effective rationale to design ratiometric probes for H_2S that profit from the quick HS^- nucleophilic addition to a merocyanine derivative in medium of near neutral pH value. In addition, the possibility to recover CouMC from the nucleophilic addition product in acidic conditions ($pH < 3.0$) together with the quick sensing behavior shows the potential of this reaction in the design of H_2S probes.

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