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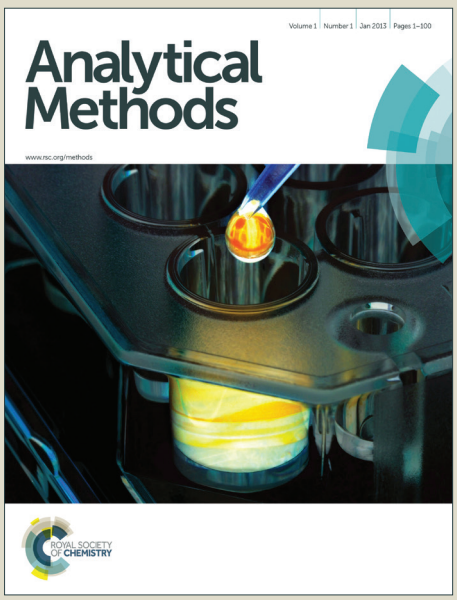

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A Rhodamine-Formaldehyde Probe Fluorescently Discriminate H_2S from Biothiols

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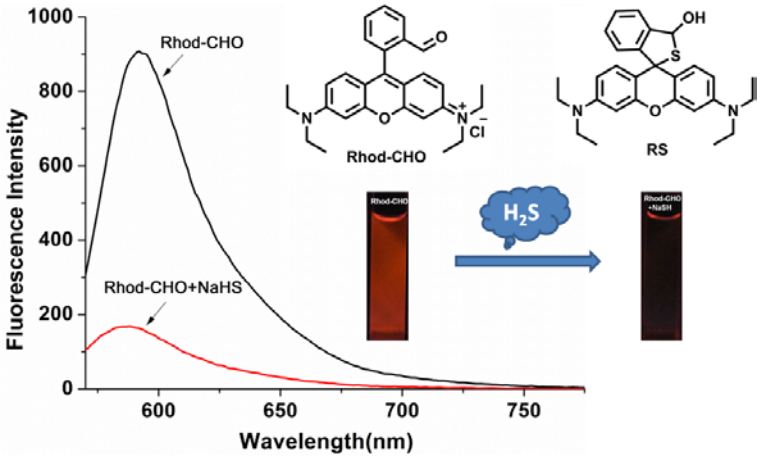

A novel mechanism-based fluorescent probe **Rhod-CHO** which involved the initial nucleophilic addition of H_2S to aldehyde followed by the subsequent intramolecular nucleophilic addition process.

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ARTICLE TYPE

A Rhodamine-Formaldehyde Probe Fluorescently Discriminate H_2S from Biothiols

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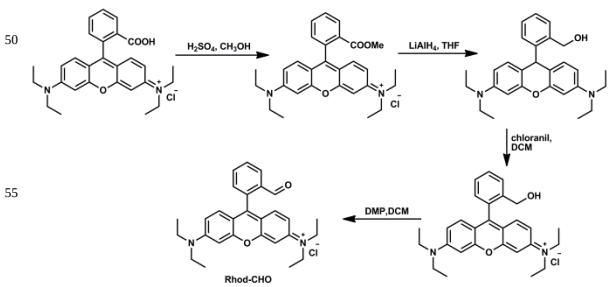
A new rhodamine-formaldehyde probe based on fluorescence on-off strategy for hydrogen sulfide detection was developed. The probe shows a fast and highly selective response to hydrogen sulfide over other reactive thiol-containing species both in aqueous media and in living cell system. Its potential utility for biological applications was confirmed by fluorescence imaging of H_2S in live cells.

Biological SH-containing molecules, such as cysteine (Cys), homocysteine (Hcy), glutathione (GSH) and hydrogen sulfide (H_2S), elicit diverse physiological responses in cellular processes.[1] For instance, endogenous biothiols, that is, Cys, Hcy, and GSH, are of crucial importance in maintaining redox balance of biological systems, while abnormal level of these biothiols is closely associated with a variety of diseases such as cancer, AIDS, cardiovascular and Alzheimer's diseases, Parkinson's disease and neurodegenerative diseases.[2] In addition, H_2S, the gasotransmitter endogenously generated from *L*-cysteine in mammalian cells, is recently recognized as a signaling molecule exhibiting significant effects in both the cardiovascular and neuronal systems,[1b,3] and it is also believed to be related with diseases such as diabetes, Alzheimer's disease and Down's syndrome.[4] Therefore, developing efficient methods for SH-containing molecules detection is of great importance and helpful to elucidate the physiological and pathological roles of biological thiols.

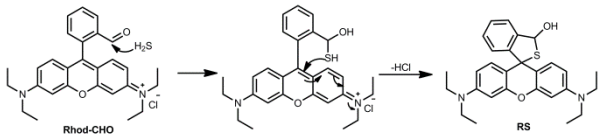
Recently, fluorescence bioimaging based on chemical probe has become a powerful tool for biomolecules detection in living systems owing to its high sensitivity, selectivity and especially, non-destructive intracellular detection.[5] To date, various fluorescence probes have been developed for H_2S and biothiols individually. In general, the design strategies of these probes are mainly based on the characteristic chemical reactions of H_2S or biothiols such as nucleophilic attack to unsaturated double bonds or aldehyde,[6] reducing azides or nitro groups,[7] binding affinity towards metal ions,[8] thiolysis of dinitrophenyl ether,[9] and so on. Only a few fluorescence probes discriminating H_2S from biothiols (i.e. Cys, Hcy and GSH) in biological systems have been reported so far.[6a-b,7a-b,8c,10] However, there exist some limitations in discriminating H_2S from biothiols because of the similarity in reactivity. For example, as a reductant, the amine reaction product generated from H_2S or thiol-mediated reduction is nondistinctive, thereby causing non-specific detection of the fluorescence probe. As for the nucleophilicity of both H_2S and biothiols, the initial nucleophilic addition to the electrophilic center can occur equally, thus resulting in intricate fluorescence changes, decreasing the H_2S detection capacity. In view of these facts, the development of innovative chemical probes that effectively differentiate H_2S from biothiols is eagerly needed. Therefore in recent years, intelligent mechanism-based chemical probes for H_2S selective detection have been increasingly reported. Xian et al. reported the fluorescein-based H_2S detection probe which undergone the nucleophilic attack of H_2S to disulfide bond followed by intramolecular nucleophilic addition of adjacent ester group to release the fluorophore.[6c,11] Similarly, Qian et al. conjugated the adjacent disulfide benzoic moiety with 2-(2'-hydroxyphenyl)-benzothiazole and achieved specific detection of H_2S.[10b] He, Zhao and Jiao et al. respectively used the aldehyde and α,β-unsaturated acrylate moiety (*ortho* to each other) as H_2S trap and elucidated the mechanism in which the initial nucleophilic addition of H_2S to the electrophilic center produced a thiol intermediate and the subsequent thiol nucleophilic attack to aldehyde resulted in cyclization, thus inducing fluorescence change and detecting H_2S.[6a,6b] Following the above mechanism, biothiols such as Cys, Hcy and GSH, owing to the lack of dual nucleophilicity, would not proceed. In view of these, further design strategies based on the dual nucleophilicity of H_2S are highly required.



Scheme 1. Synthesis of fluorescent rhodamine-formaldehyde probe (**Rhod-CHO**).

Herein, we present the design and biological application of a fluorescent rhodamine-formaldehyde probe (**Rhod-CHO**, Scheme 1). It includes a H_2S trap group formaldehyde and a fluorescent reporter rhodamine. The probe was synthesized as shown in Scheme 1, and the structure was confirmed by ^1H NMR, ^{13}C NMR, and HRMS spectra (ESI, †).

The rhodamine scaffold has excellent fluorescence



Scheme 2. The proposed detection mechanism of **Rhod-CHO** for H_2S.

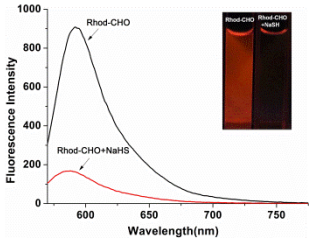


Fig. 1 Fluorescence spectra of **Rhod-CHO** (10 μM) in PBS (20 mM, pH 7.4, 1% CH_3CN, v/v) upon titration with NaHS (100 μM). λ_{ex}: 560 nm. Slits: 5/5 nm. Inset: The visual fluorescence color of **Rhod-CHO** (10 μM) before (left) and after (right) addition of 10 equiv. of NaHS (UV lamp, 365 nm).

characteristics, high quantum yield, favourable membrane permeability and good solubility in aqueous phosphate-buffered solution.[12] In practice, it can transform reversibly between the nonfluorescent spirocyclic form and the fluorescent open-ring form.[13] We speculate that, in **Rhod-CHO** probe, the initial nucleophilic addition of H_2S to aldehyde will produced a thiol intermediate, and then intramolecular nucleophilic attack of the SH-containing compound on xanthene framework results in the nonfluorescent spirocyclic form. As for other biothiols (i.e. Cys, Hcy and GSH), owing to the lack of dual nucleophilicity, the second nucleophilic attack will not perform. Furthermore, the relatively high p*K*a values of these endogenous biothiols compared with H_2S in physiological media (p*K*a: for Cys, Hcy and GSH, ≥ 8.5; for H_2S, ca. 7.0)[16d,11] will make them be inefficient in nucleophilicity. Because the probe **Rhod-CHO** exists as fluorescent open-ring form, such addition-cyclization cascade reaction induced by H_2S will make the probe exhibit "on-off" type fluorogenic behavior. Therefore we anticipate that the **Rhod-CHO** probe will discriminate H_2S from biothiols with high selectivity. The proposed detection mechanism of probe **Rhod-CHO** for H_2S is depicted in Scheme 2.

To verify the proposed detection mechanism, we examined the reactivity of probe **Rhod-CHO** (10 μM) towards NaHS (a commonly employed H_2S donor) in phosphate buffer (20 mM, pH 7.4, containing 1% CH_3CN) at 25 °C. As shown in Fig. 1, free

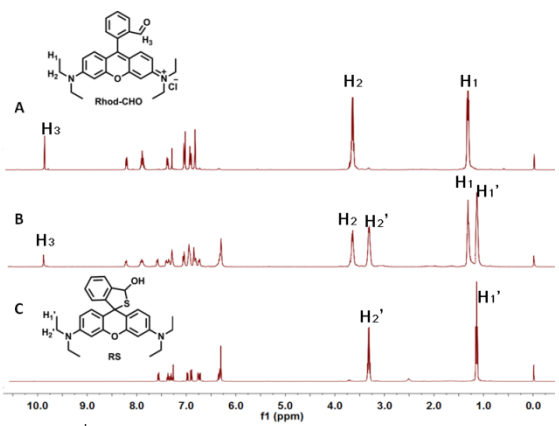


Fig. 2 ^{1}H NMR titration experiment in $CDCl_3$. A) **Rhod-CHO**; B) **Rhod-CHO** + NaHS; C) Compound **RS**.

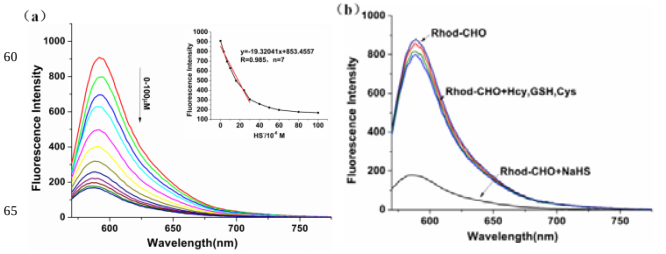


Fig. 3 (a) Fluorescence spectra of **Rhod-CHO** (10 μM) in PBS (20 mM, pH 7.4, 1% CH_3CN, v/v) upon titration with NaHS from 0-100 μM. λ_{ex}: 560 nm. Slits: 5/5 nm. Inset: The linear relationship between the fluorescence intensity and the concentration of NaHS (0-30 μM). (b) Fluorescence spectra of **Rhod-CHO** (10 μM) in PBS (20 mM, pH 7.40, 1% CH_3CN, v/v) upon addition of 10 equiv. of Hcy, GSH, Cys and NaHS. λ_{ex}: 560 nm. Slits: 5/5 nm.

Rhod-CHO displayed strong fluorescence band at 592 nm (fluorescence quantum yield, 0.541). Upon addition of H_2S, the fluorescence intensity at 592 nm dramatically decreased with a slight blue-shift of emission. The result was attributed to the consumption of the open-ring fluorophore and simultaneous generation of spirocyclic. Moreover, the H_2S- induced fluorescence quenching was obviously observed under UV lamp (365 nm) (Fig. 1, inset). This mechanism was proved by ^{1}H NMR titration experiment (Fig. 2). The ^{1}H NMR spectrum of **Rhod-CHO** in $CDCl_3$ completely turned into the same spectrum as spirocyclic compound RS in the presence of NaHS, and during the course of transformation, i.e. 10 min after addition of NaHS, the coexistence of both **Rhod-CHO** and **RS** was found in the NMR spectrum (Fig. 2B). For example, the aldehyde proton (CHO), methylene proton (CH_2) and methyl proton (CH_3), which belonged to **Rhod-CHO** and **RS** respectively, appeared simultaneously in the spectrum (Fig. 2B), and this clearly indicated the transition. In addition, the structure of spirocyclic compound **RS** was confirmed by ^{1}H NMR, ^{13}C NMR, and HRMS spectra (ESI, †). These results coincide with our proposed mechanism mentioned above.

Next, we investigate on the sensitivity and the selectivity of **Rhod-CHO** in response to H_2S. The reactivity of **Rhod-CHO** (10 μM) towards different concentrations of NaHS was examined in phosphate buffer (20 mM, pH 7.4, containing 1% CH_3CN) at 25 °C. As shown in Fig. 3a, upon addition of increasing amount of NaHS into the **Rhod-CHO** solution, the fluorescence intensity at 592 nm sharply decreased. A linear relationship was found between the fluorescence quenching and the concentration of NaHS in the range of 0 to 30 μM (Fig. 3a, inset), with the detection limit being 0.12 μM. The results demonstrate that hydrogen sulfide could induce fluorescence quenching of **Rhod-CHO** in a concentration-dependent manner. The reaction time profile of **Rhod-CHO** and H_2S was detected in PBS buffer solution (20 mM, pH 7.4, 1% CH_3CN, v/v) and the results showed that there was a linear response within 5 min and the fluorescence intensity decreased with nonlinearity in the range of 5 to 20 min (Fig. S3). To evaluate the selectivity, we examined the effect of other biologically relevant analytes on **Rhod-CHO**. As shown in Fig. 3b, compared to H_2S, the addition of 10 equiv. of Cys, Hcy and GSH induce much smaller fluorescence quenching. Moreover, other biologically relevant species such as HSO_3^-, AcO^-, $S_2O_3^{2-}$, SO_4^{2-}, CO_3^{2-}, Cu^{2+}, Zn^{2+} and Hg^{2+}, performed in a similar way (Fig. S4). Therefore these results

confirmed the sensitive and selective nature of **Rhod-CHO** towards H_2S.

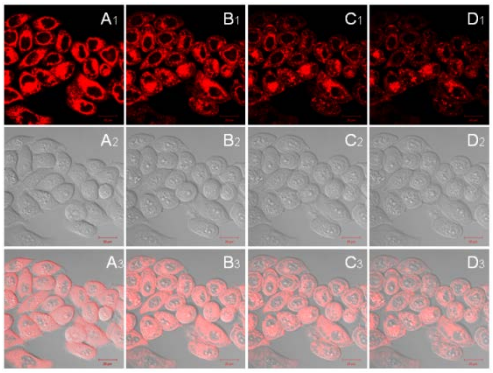


Fig. 4 Confocal fluorescence images of living HepG2 cells incubated with NaHS at different time fluorescence image (A_1, B_1, C_1, D_1), bright field image (A_2, B_2, C_2, D_2) and overlayed image (A_3, B_3, C_3, D_3). (A) The cells incubated with **Rhod-CHO** (5 μM) for 30 min in the presence of 20 mM PBS (pH 7.4), incubation was performed at 37 °C under a humidified atmosphere containing 5% CO_2. (B) The cells pre-treated with 5 μM Rhod-CHO for 30 min, and incubated with 100 μM NaHS for 5 min at 37 °C, (C) The cells pre-treated with 5 μM **Rhod-CHO** for 30 min, and incubated with 100 μM NaHS for 10 min at 37 °C. (D) The cells pre-treated with 5 μM **Rhod-CHO** for 30 min, and incubated with 100 μM NaHS for 20 min at 37 °C. Scale bar: 20 μm.

With these data in hand, we perform the confocal fluorescence imaging of **Rhod-CHO** in biological system. HepG2 cells, incubated with **Rhod-CHO** (5 μM) in culture medium for 30 min at 37 °C, exhibited strong fluorescence (Fig. 4A_1). By contrast, the fluorescence intensity decreased gradually after the cells were further incubated with NaHS (50 μM) for 20 min (Fig. 4B_1, C_1 and D_1), indicating the potential application of **Rhod-CHO** in visualizing H_2S levels of living cell.

In conclusion, a mechanism-based fluorescent **Rhod-CHO** for H_2S selective detection was developed. The probe exerts highly sensitive and selective "on-off" fluorescence detection towards H_2S over other biothiols such as Cys, Hcy and GSH. Preliminary fluorescence imaging in living cells indicates its potential for biological applications. Moreover, the interesting reaction mechanism may provide new design strategy for fluorescent probes for H_2S selective detection.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Electronic Supplementary Information (ESI) available: Experimental procedures, supplemental spectra, and the ^{1}H-, ^{13}C-NMR, and HRMS spectrum. See DOI: 10.1039/b000000x/

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