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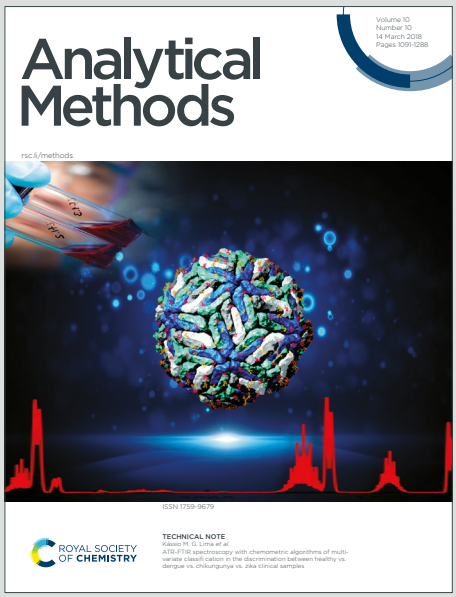

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Development of an effective H_2S-activatable fluorescent probe for imaging in drug-induced living cells†

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Hydrogen sulfide (H_2S) is a toxic gas signaling molecule which plays multiple physiological regulatory roles in both physiological and pathological processes. A novel fluorescent probe T1 is designed and synthesized for detecting H_2S in biological systems. Based on the ICT mechanism, thiolysis of the 2,4-dinitrophenyl ether which is used as the fluorescence quenching group occurs after the addition of H_2S, releasing the original fluorescent group and accompanied by the recovery of the fluorescence signal (emission peak at 615 nm) with the detection limit of 0.42 μM. Probe T1 displays low cytotoxicity and excellent ability to recognize exogenous and endogenous H_2S in living cells. It could recognize H_2S produced by LPS induced cell inflammation, monitor the release of H_2S by the H_2S prodrug ADT-OH in cells, and serve as an anti-counterfeiting dye for printing technology, confirming the reliability of the probe. Therefore, probe T1 can be used as a fast and effective tool for detecting H_2S in vitro and in vivo.

1. Introduction

Hydrogen sulfide (H_2S) is the third gas signaling molecule, playing a significant role in various physiological and pathological activities[1]. H_2S was introduced by enzymatic reaction of homocysteine and cysteine in mitochondria and cytoplasm[2-5]. The pathophysiological influences of H_2S exhibit bell-shaped effect, which shows significant cytoprotective effects through anti-apoptoticmeaning, anti-necrotic and cell proliferation pathways at low concentrations and demonstrates cytotoxic effects by generating free radicals and oxidants at high concentrations[6]. Involved in regulating vascular tension, stimulating angiogenesis and heart protection, H_2S may contribute to Down syndrome[7,8], Alzheimer's disease[9,10], Parkinsons disease[11,12], liver cirrhosis[13]and cardiovascular disease, and sometimes even be life threatening. Furthermore, studies have shown that H_2S is regarded as a classic anti-inflammatory mediator [14], demonstrating anti-inflammatory, anti-catabolic and antioxidant effects in various inflammatory diseases. In recent years, H_2S gas therapy is investigated actively to treat inflammatory diseases[15]. The anti-inflammatory effect of H_2S is mainly achieved by increasing the levels of anti-inflammatory and cytoprotective molecules and inhibiting the secretion of inflammatory cytokines[16]. Therefore, detecting endogenous H_2S within organs can provide valuable insights into pathological processes

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such as inflammatory responses, enabling early diagnosis and treatment of diseases.

Common detection methods for H_2S encompass colorimetry, electrochemical analysis, chemical titration, atomic emission spectrometry (AES), chromatography (GC-MS) and fluorometric analysis[17-20]. Among these, fluorescence-based detection has emerged as a dominant approach in diverse fields, including biomedical research, environmental monitoring, and industrial process control, owing to its unparalleled advantages: exceptional specificity, rapid response kinetics (millisecond-level detection), cost-effectiveness (low reagent consumption), and operational simplicity. This technique is particularly valuable for real-time tracking of H_2S dynamics in live-cell imaging and in situ pathological studies. Fluorescent probes with exceptional sensitivity and superior spatiotemporal resolution, have emerged as indispensable tools in biomedical research, environmental monitoring, and chemical analysis[21-23]. These probes enable real-time detection of target molecules, including ions, reactive oxygen species (ROS), and gaseous signaling molecules through dynamic changes in fluorescence signal intensity, emission wavelength, or lifetime. Capitalizing on the established roles of H_2S as a dual-function mediator in inflammatory pathways, a series of probes utilizing H_2S as a biomarker have been developed based on strategies such as azide reduction reactions, thiolysis reactions, and CuS precipitation mechanisms[24-30]. Zhang et al.[31] reported a coumarin-based red-emitting fluorescent probe for the detection and monitoring of H_2S, which featured several advantages, such as red emission (611 nm), reliability at physiological pH, low cytotoxicity, high sensitivity. The probe was successfully utilized to detect H_2S semi-quantitatively in real water and beer samples, food spoilage and mouse model.

Despite advancements in H_2S-sensing technologies, previously reported fluorescent probes still face critical limitations, including poor water solubility, laborious synthetic procedures, background interference, and suboptimal biocompatibility. Consequently, the development of "turn-on" fluorescent probes with low detection limits, simplified synthesis, high selectivity, robust anti-interference capability, and ultrahigh sensitivity remains a key research priority. Notably, there is a critical unmet need for rapid-response H_2S probes to achieve real-time tracking of H_2S, which is essential for early diagnosis of disorders and precision-guided therapeutic interventions.

Compared with cysteine (Cys) and glutathione (GSH), H_2S exhibits stronger nucleophilicity in biological systems. Capitalizing on this property, fluorescence probes operating via a thiolysis reaction mechanism demonstrate enhanced detection efficacy. Notably, the 2,4-dinitrophenyl ether structure displays exceptional H_2S-capturing capability, with its thiolytic reactivity under physiological pH conditions outperforming other reactive sulfur species (RSS), which can be used to construct responsive fluorescent probes.

In this work, a new fluorescence “turn-on” probe T1 is designed and synthesized for the recognition of H_2S in living cells. Probe T1 comprises two critical components: the resorufin derivative serving as fluorophore and a 2,4-dinitrophenyl ether moiety that functions dually as a

fluorescence quencher and a H_2S-specific recognition unit (Fig. 1). The quenching mechanism operates via intramolecular charge transfer (ICT), rendering the native probe essentially non-fluorescent. Upon addition to nucleophilic H_2S, thiolysis of the 2,4-dinitrophenyl ether occurs, leading to regeneration of the fluorophore and subsequent emergence of a prominent emission band at 615 nm. Both exogenous and endogenous H_2S in living cells, including LPS-induced H_2S production is monitored. Furthermore, probe T1 successfully tracks H_2S release from the ADT-OH prodrug, highlighting its substantial potential for pharmacological evaluation and real-time drug metabolism studies.

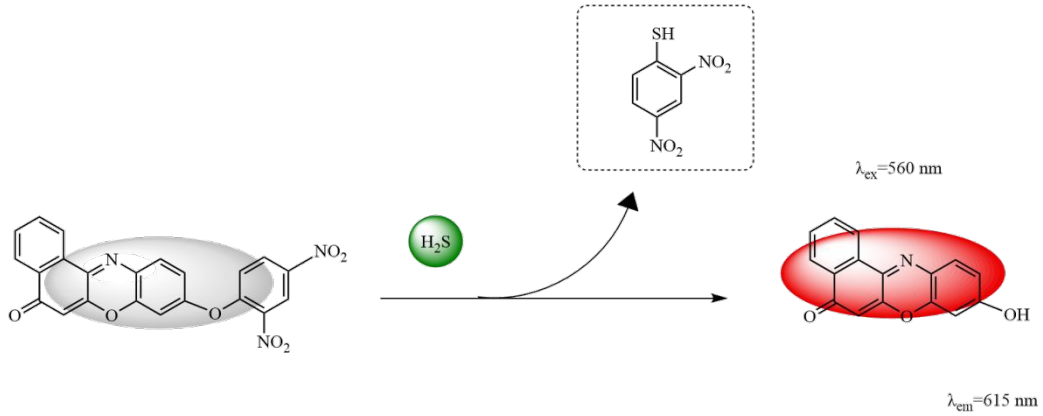

Fig.1 The response mechanism of T1 to H_2S.

2. Materials and methods

2.1 Reagents and instruments

^1H NMR and ^{13}C NMR spectra were carried out on a Bruker AVANCE III spectrometer (Germany) with TMS as internal standard. The MS spectra were acquired on a Bruker micro TOF-QII mass spectrometer (Germany). were determined by using a Perkin-ElmerLS55 fluorimeter (U.S.). Optical density (OD) was detected by Thermo Fisher Multiskan FC microplate reader (U.S.). The fluorescence images of cells were determined by using a Leica confocal laser scanning microscope (Germany).

The solvents and chemicals for synthesis were used as received without further purification. Double distilled water was used throughout.

2.2 Synthesis of probe T1

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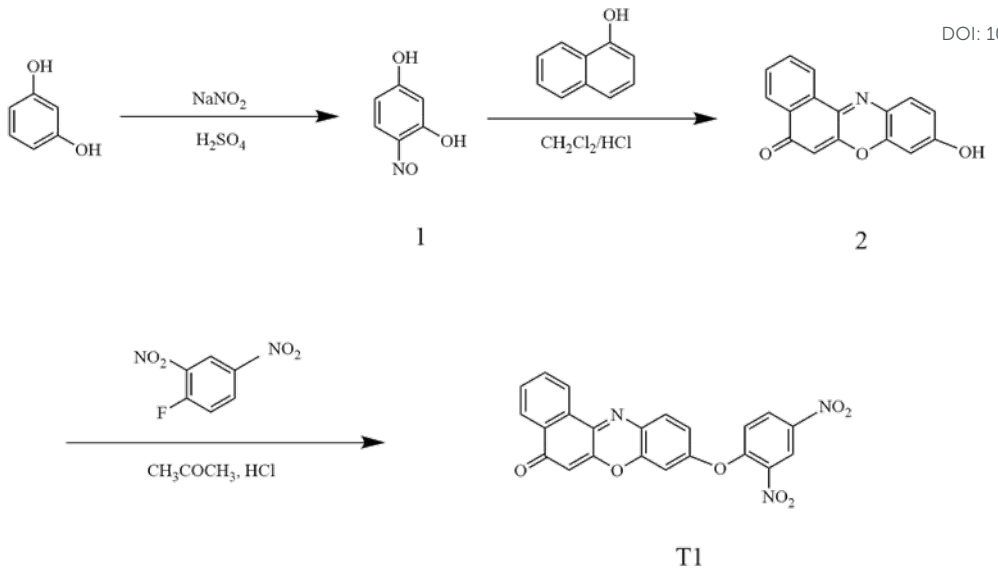

Scheme 1 Synthetic route for probe T1.

Compound 1 and 2 were readily prepared according to literatures[32,33]. Compound 2 (0.066 g, 0.25 mmol) was added to a round-bottom flask and dissolved in 5 mL of dichloromethane, followed by the addition of 0.5 mL of triethylamine. Then, 1-fluoro-2,4-dinitrobenzene (0.05 g, 0.25 mmol) was dissolved in 5 mL of dichloromethane and added to the above mixture. The reaction was stirred and refluxed at 65°C for 30 min. After rotary evaporation, 10 mL of 5% hydrochloric acid was added, and the mixture was washed three times with distilled water and dried. Purification was performed using petroleum ether and ethyl acetate (5:1) as the mobile phase through silica gel column chromatography, yielding the brown solid T1 (0.083 g, yield 75%). ^{1}H NMR (400 MHz, CD_3Cl) δ 8.92 (d, J = 2.7 Hz, 1H), 8.71 (d, J = 9.5 Hz, 1H), 8.48-8.40 (m, 1H), 8.30 (d, J = 9.3 Hz, 1H), 7.91 (d, J = 8.6 Hz, 1H), 7.83-7.78 (m, 2H), 7.21-6.99 (m, 3H), 6.45 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 132.70, 132.28, 132.09, 131.54, 130.89, 129.07, 128.81, 127.55, 125.97, 124.01, 120.51, 115.03, 107.97, 107.12, 66.95, 35.60, 29.85, 28.53, 24.15, 20.41, 14.05, 10.96. HRMS m/z: $C_{22}H_{12}N_3O_7$ $[M+H]^+$ calcd for 430.0670, found: 430.0686.

2.3 The general procedures for spectra detection

Dissolving probe T1 in dimethyl sulfoxide (DMSO) to prepare T1 stock solution (2.0×10^{-4} M), then diluted to 2.0×10^{-5} M with distilled water. H_2S stock solution (2.0×10^{-3} M) was acquired by dissolving NaHS in distilled water, and then diluting to 2.0×10^{-5} M. Taking 12.5 mL of the H_2S stock solution and dilute to 25 mL to obtain a 2.0 M H_2S donor solution. Before spectroscopic measurements, the solutions of F^-、Cl^-、CO_3^{2-}、HPO_4^{2-}、SO_4^{2-}、SO_3^{2-}、$S_2O_3^{2-}$、HSO_3^-、L-Glu、Hcy、Gly、GSH、Cys、L-Lys、H_2O_2、OCl^-、$ONOO^-$ and NO_2^- were freshly prepared by diluting stock solutions to 2.0×10^{-5} M. All samples were shaken for 10 s and waited for 20 min at room temperature before determination. The excitation and emission wavelength were set at 560.0 nm and 615.0 nm, respectively.

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2.4 Fluorescence imaging in living cells and animals

HCT116 cells were obtained from the National Collection of Authenticated Cell Cultures. CCK8 assays were performed to assess the cytotoxicity of T1 (0-100 μM). For imaging exogenous H_2S, after adding probe T1 (20 μM) to AC16 cells and incubating for 30 min in confocal culture dishes, different concentrations of HS^- (100 μM, 200 μM) were added. After 30 min, the cells were slowly washed three times with PBS for confocal imaging. For imaging endogenous H_2S, Sodium nitroprusside (SNP) is commonly used to stimulate cells to produce endogenous H_2S. Therefore, after adding probe T1 to AC16 cells, SNP (100 μM) was added for further incubation. After 30 min, cell was washed with PBS and imaged. Lipopolysaccharide (LPS) was used to induce cell inflammation. Firstly, the most suitable LPS concentration will be screened using the CCK8 method to establish a model of LPS-induced inflammation in AC16 cells. Next, the cell suspension at this concentration will be collected, and the expression levels of three inflammatory factors, IL-6, IL-1β, and TNF-α, will be detected using the ELISA method to verify the success of the inflammation model. Then, cell fluorescence images were analyzed. Subsequently, we separately investigated the ability of T1 to recognize H_2S in AOAA and ADT-OH treated HCT116 cells (further details can be found in the ESI†).

All animal procedures for this study were followed in accordance with the requirements of the National Experimental Animal Use Act and were approved by the Ethnics Committee of Xizang Minzu University (NO: 2025-037).

3. Results and discussions

3.1 Synthesis and optimized structure

As shown in Scheme 1, probe T1 can be obtained by three steps. Compound 1 and Compound 2 were acquired easily according to literatures. The structure of T1 was confirmed by ^1H NMR, ^{13}C NMR, and ESI-mass spectrometry (ESI†).

3.2 Spectral response of T1 to H_2S

To investigate the sensitivity of probe T1 in recognizing H_2S, different concentrations of H_2S were added to the probe solution. The fluorescence titration concentrations of H_2S were: 0, 5, 10, 15, 20, 30, 40, 50, 60, 80, and 100 μM, As shown in Fig. 2A, the fluorescence intensity at 615 nm gradually increased with the addition of H_2S. The H_2S concentration versus fluorescence intensity showed a good linear relationship between in the range of 0-50 μM. the limit of detection (3σ/k) for H_2S is 0.42 μM through experiments and calculations (Fig. 2B), indicating that the probe T1 has good sensitivity.

The fluorescence spectra of the probe T1 (20 μM) in response to various analytes including H_2S, F^-, Cl^-, CO_3^{2-}, HPO_4^{2-}, SO_4^{2-}, SO_3^{2-}, $S_2O_3^{2-}$, HSO_3^-, L-Glu, Hcy, Gly, GSH, Cys, L-Lys, H_2O_2, OCl^-, $ONOO^-$ and NO_2^- were studied. As shown in (Fig. 2C), the probe solution only produced a strong fluorescence signal in the presence of H_2S, indicating that the probe T1 has good selectivity

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for H_2S. As shown in (Fig. 2D), when 2 equivalents of coexisting substances were added to the probe T1-H_2S solution, fluorescence changes little, revealing that the probe T1 showed strong anti-interference capability and could still efficiently detect H_2S in the presence of interfering substances.

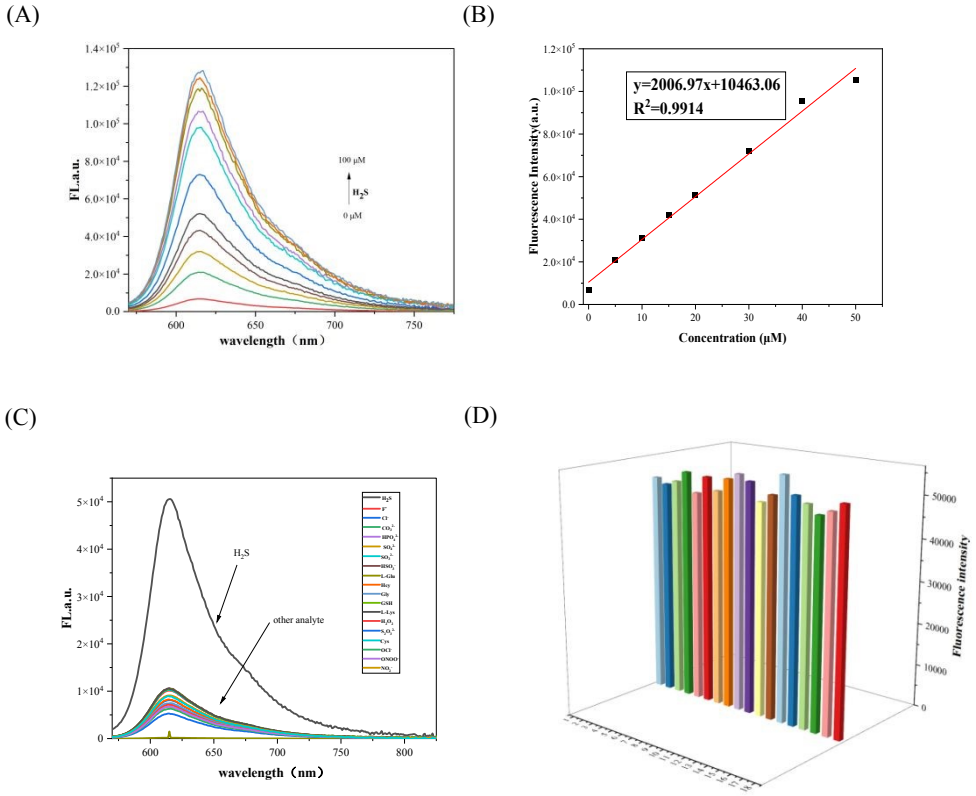

Fig. 2 (A) Fluorescence spectrum of probe T1 (20 μM) solution upon addition different amounts of H_2S. (B) The linear relationship between H_2S concentration and fluorescence intensity probe T1 (λ_{em}=615 nm). (C) Spectrum of probe T1 after addition of different targets (including ions or amino acids). (D) The fluorescence intensity of T1-H_2S with other interfering substances at 615 nm, 1-18: F^-, Cl^-, CO_3^{2-}, HPO_4^{2-}, SO_4^{2-}, SO_3^{2-}, $S_2O_3^{2-}$, HSO_3^-, L-Glu, Hcy, Gly, GSH, Cys, L-Lys, H_2O_2, OCl^-, $ONOO^-$ and NO_2^-.

To broaden the application of the probe T1 in organisms or the environment, its pH sensitivity must be considered as a prerequisite. As shown in Fig. S1†, the probe T1 itself exhibited almost no fluorescence when the pH is between 3 and 6. When pH > 6, a slight but insignificant increase in fluorescence was observed. After the addition of H_2S, a distinct fluorescence emerged at 615 nm within the pH range of 6-9. The study demonstrates the probe's potential practical value for H_2S detection.

3.3 Response of T1 to H_2S in living cells

3.3.1 The recognition ability of probe T1 for exogenous and endogenous H_2S in cells

As shown in Fig. S2†, the cell livability was above 90% when the probe concentration was 20 μM, indicating a good biocompatibility of the sequential response probe. Based on the probe's low toxicity, the recognition capability of probe T1 for exogenous and endogenous H_2S in AC16 cells

was studied. There was almost no fluorescence observed in cells after incubating with probe for 30 min (Fig. 3A). However, after adding H_2S (100 μM) and incubating for 30 min, a strong fluorescence signal could be detected (Fig. 3B); when the H_2S concentration increased to 200 μM, the fluorescence intensity also increased (Fig. 3C). These results indicate that probe T1 can recognize exogenous H_2S in AC16 cells. Subsequently, the recognition capability of probe T1 for endogenous H_2S in cells was explored. Sodium nitroprusside (SNP) is commonly used to stimulate cells to produce endogenous H_2S. As shown in Fig. 3(D), fluorescence appeared in the red channel, indicating that SNP stimulated the cells to produce H_2S, and probe T1 was able to recognize endogenous H_2S in cells.

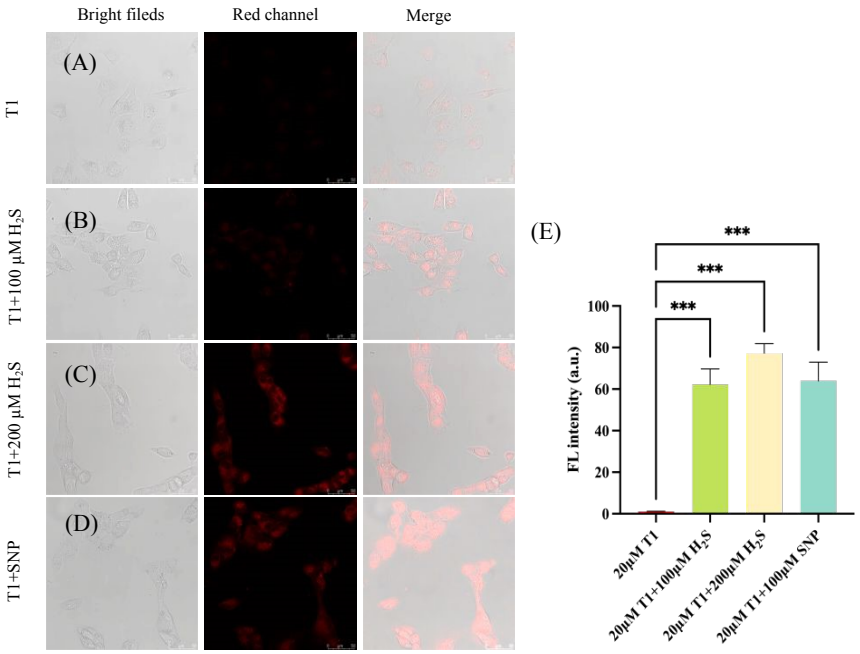

Fig. 3 (A-C) Confocal laser imaging of different concentrations of H_2S (0、100、200 μM) added to cells after pretreatment with T1 for 30 min; (D) Confocal laser imaging of SNP and T1 was added to cells after 30 min. (E) Comparison of fluorescence intensity of AC16 cells under different treatments. Red channel: 610-710 nm; λ_{ex}=522 nm; Scale bar=50 μm. ***P<0.001.

Human colon cancer cells (HCT116) express endogenous H_2S-synthesizing enzymes (CBS and CSE), resulting in high intracellular H_2S levels. Confocal imaging without probe T1 showed no intrinsic fluorescence in HCT116 cells (Fig. 4A). However, upon treatment with T1 (10, 20, 30 μM) for 20 min, a dose-dependent increase in fluorescence intensity was observed (Fig. 4B-D), indicating probe's capability to specifically recognize intracellular H_2S. This demonstrates its potential for early detection of H_2S-related pathologies.

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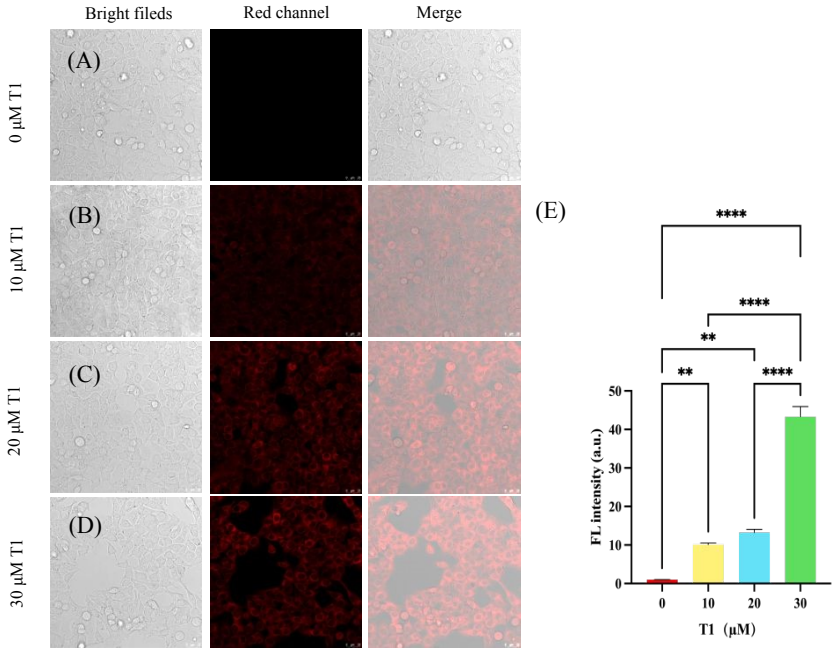

Fig. 4 Confocal imaging of probe T1 recognizing endogenous H_2S in HCT116 cells. (A) Imaging of HCT116 cells without probe; (B-D) Confocal imaging of HCT116 cells after the addition of probe T1 (10、20、30 μM) for 20 min. (E) Comparison of fluorescence intensity of HCT116 cells under different treatments. Red channel: 610-710 nm; λex=522 nm; Scale bar=25 μm. $^{**}P<0.01$, $^{****}P<0.0001$.

3.3.2 Probe T1 recognizes H_2S in LPS-induced cellular inflammation

The inflammation response activated by LPS can lead to an increase in H_2S levels[34,35]. Firstly, the LPS concentration of 8 μg/mL was selected as the modeling concentration (Fig. S3†). Subsequently, the expression levels of three inflammatory factors: IL-6, IL-1β, and TNF-α were verified using the ELISA method at an LPS concentration of 8 μg/mL (Fig. S4†). Then, AC16 cells were treated with 8 μg/mL LPS for 24 h to induce inflammation. Confocal imaging of AC16 cells treated with probe T1 revealed distinct fluorescence patterns: untreated cells showed no signal (Fig. 5A), whereas LPS-stimulated cells exhibited pronounced fluorescence (Fig. 5B). This demonstrates T1's specificity in detecting LPS-induced H_2S during inflammatory responses.

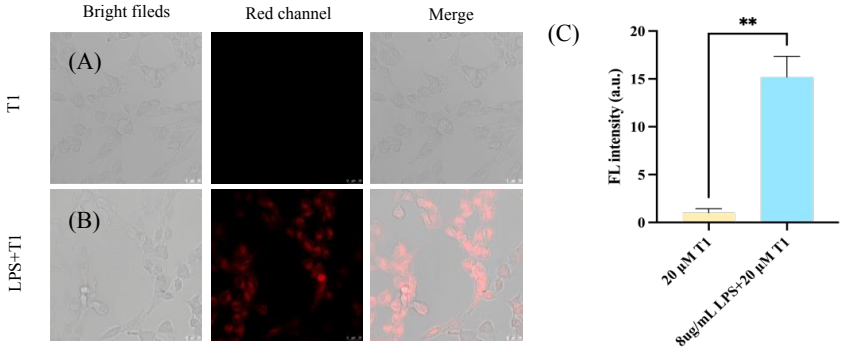

Fig. 5 (A) Confocal laser imaging of T1 (20 μM) added to AC16 cells; (B) Confocal laser imaging of T1 (20 μM) added to AC16 cells after pretreatment with 8 μg/mL LPS for 24 h; (C) Comparison of fluorescence intensity of AC16 cells under different treatments . Red channel: 610-710 nm； λex=522 nm; Scale bar= 25 μm. $^{**}P<0.01$.

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3.3.3 AOAA inhibits H_2S cell imaging

After adding the CBS inhibitor (AOAA) (1 mmol/L) to HCT116 cells and incubating for (0, 10, 20, 30, 40 min), the probe T1 (20 μM) was added and incubated for 20 min. Fluorescence imaging was conducted using a laser confocal microscope. As shown in Fig. 6, with the increase in inhibition time, the red channel fluorescence gradually dimmed, indicating that probe T1 can successfully monitor the level of H_2S inhibited by AOAA.

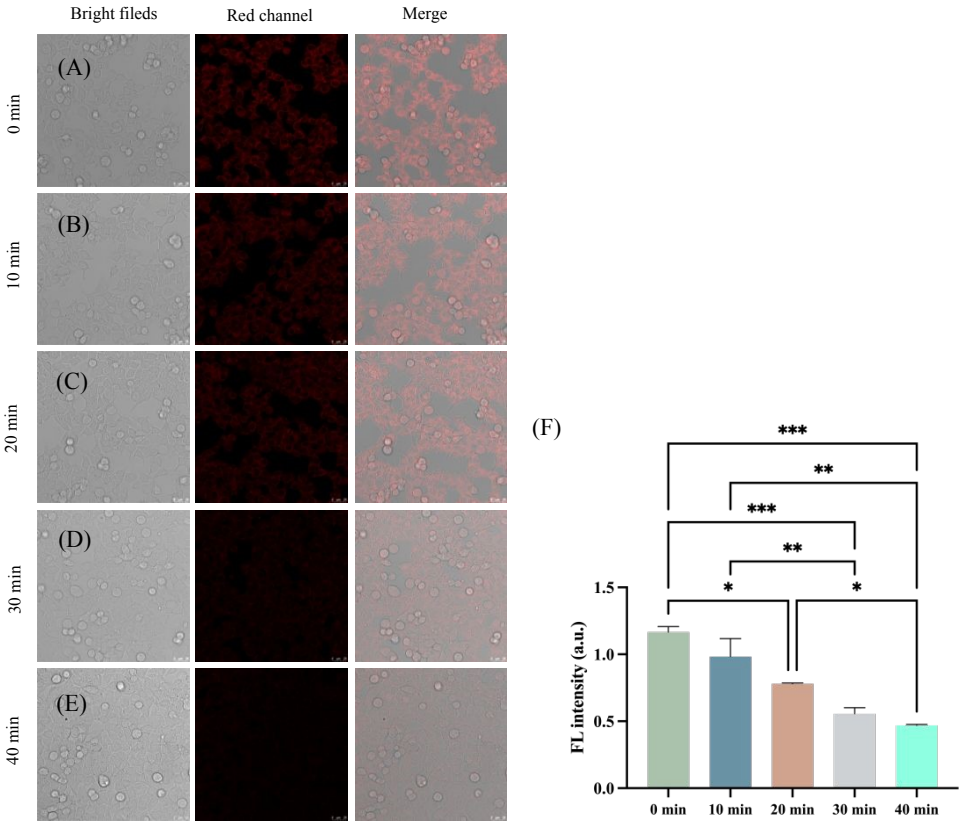

Fig. 6 (A-E) Confocal laser imaging of T1 (20 μM) added to HCT116 cells after pretreatment with AOAA (1 mmol/L) for (0、10、20、30、40) min；(F) Comparison of fluorescence intensity of HCT116 cells at different time treatments. Red channel: 610-710 nm；λex=522 nm; Scale bar= 25 μm. **P*<0.05 , ***P*<0.01, ****P*<0.001.

3.3.4 H_2S prodrug ADT-OH release time response

ADT-OH, as a donor of H_2S, cleaves and releases H_2S intracellularly and is widely used to monitor the H_2S release efficiency of prodrugs. As shown in Fig. 7, with the extension of the incubation time with ADT-OH, the fluorescence intensity increased accordingly. This indicates that the probe T1 can monitor the release of H_2S from the ADT-OH prodrug in real-time, demonstrating a significant advantage in evaluating the release rate of prodrug H_2S through fluorescence imaging. This can be applied to study the release rates of H_2S prodrugs and promote the progress of novel drug development.

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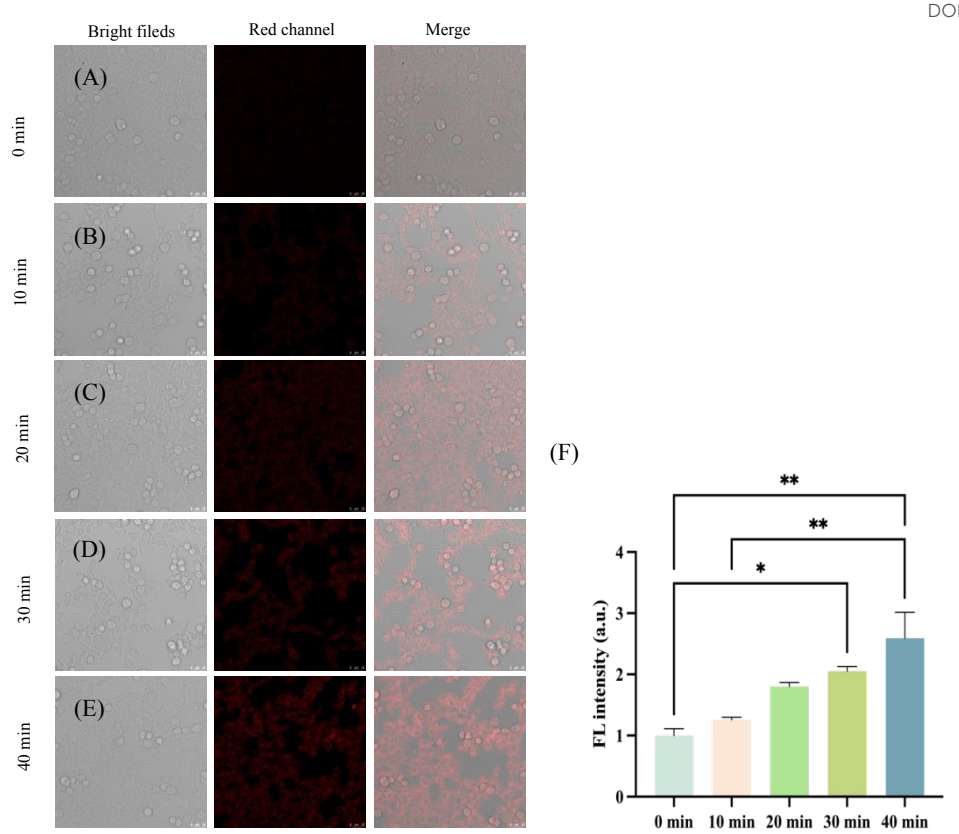

Fig. 7 (A-E) Confocal laser imaging of T1 (20 μM) added to HCT116 cells after pretreatment with ADT-OH (2 mmol/L) for (0、10、20、30、40) min；(F) Comparison of fluorescence intensity of HCT116 cells at different time treatments. Red channel: 610-710 nm；λex=522 nm; Scale bar= 25 μm. *P<0.05 , **P<0.01.

Encouraged by the successful intracellular detection of H_2S, we next explored whether the probe could be extended to in vivo fluorescence imaging. To evaluate this, Kunming mice (20-25 g) were selected as the model organism, and the experimental results are presented in Fig. S10†. After intraperitoneal injection of the probe into the mice, no significant fluorescence was observed (Fig. S10A†). However, upon subsequent injection of HS^- at the same abdominal site, distinct fluorescence emission was detected (Fig. S10B†). The result indicate that the probe exhibits excellent biocompatibility and holds promising potential for applications in biological labeling, detection, and staining.

3.4 Potential applications of T1

In addition, T1 displayed excellent biological tracking applications shown in (Fig. 8A-D), the probes T1 (200 μM) and T1+H_2S (200 μM) were photographed under sunlight and UV light (365 nm) at temperatures of 4、25、37、45°C, with no significant differences observed. This indicates that probe T1's ability to detect H_2S in vitro is not affected by temperature. Subsequently, the probe T1+H_2S solution was used as an ink dye to mark filter paper, plastic, and walls, and then

photographed. As shown in Fig. 8E-G, there is no fluorescence under sunlight, but distinct fluorescent markings can be observed under UV light. Therefore, probe T1+H_2S has shown excellent prospects for biological tracking applications.

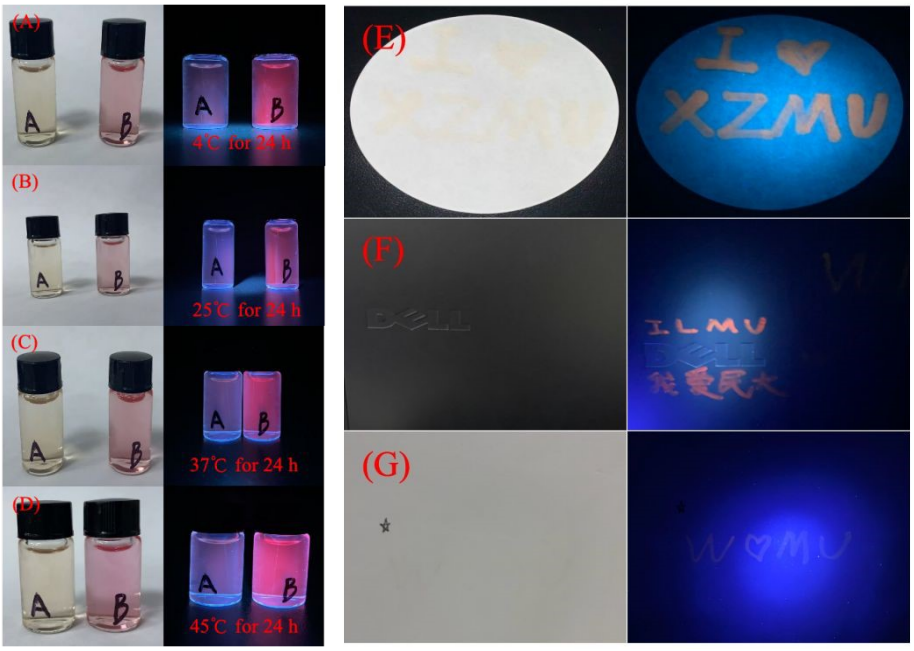

Fig. 8 (A-D)Bright and UV light plots of probe T1: A, T1+H_2S: B at different temperatures; (E-G)Bright and UV light images of probe T1+H_2S as fluorescent ink for handwriting on filter paper, plastic, walls.

4. Conclusion

In summary, a novel fluorescent probe (T1) based on the resorufin dye is designed and synthesized for detecting H_2S. The probe shows high sensitivity and rapid response to H_2S with a low detection limit and excellent selectivity and interference resistance. Moreover, the probe displays low cytotoxicity, which is successfully implemented to visualize the changes of exogenous and endogenous H_2S in cells. T1 can monitor the H_2S produced from LPS-induced cell inflammation and the reduction of H_2S levels due to the inhibition of CBS in HCT116 cells by AOAA. It can also monitor the release of H_2S from the H_2S prodrug ADT-OH in HCT116 cells, demonstrating broad application value, and exhibits excellent biocompatibility. In addition, the probe T1 displays excellent security printing applications in solution which displays outstanding fluorescence stability to ensure its real-time applications. Moreover, probe T1 can recognize H_2S at different temperatures making it suitable as a fluorescent dye for ink printing technology with good printing and anti-counterfeiting performance. We expect that synthesized probe will find more interesting applications as sensing and labeling agents in biology and medicine. In addition, there are some limitations of this study， for example, when validating the inhibitory effect of AOAA on H_2S biosynthesis, Western blot analysis of CBS expression should be complemented. These investigations will be systematically supplemented and refined in our future experimental studies.

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Author contributions

Yangmei Hou: methodology, experimentation, formal analysis, writing-original draft preparation. Jiaxuan Li, Yingqi Ding, Ji Zhuoma, Yimeng Zhao, Zhuoga Langjie: experimentation. Meipan Yang: review & editing, supervision, conceptualization, funding acquisition, writing-review & editing. All authors read and contributed to the manuscript.

Data availability

The authors confirm that the data supporting the findings of this study are available within the article and the ESI.†

Conflicts of interest

There are no conflicts to declare.

Acknowledgments

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Data Availability Statement

Development of an effective H_2S-activatable fluorescent probe for imaging in drug-induced living cells

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The authors confirm that the data supporting the findings of this study are available within the article and the ESI.