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ARTICLE

A novel FRET-based fluorescent probe for the selective detection of hydrogen sulfide (H_2S) and its application for bioimaging

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Hydrogen sulfide (H_2S) is an endogenously produced gaseous signaling molecule with multiple functions in many physiological and pathological processes. Herein, our group developed a novel fluorescent probe (**Flu-N_3**) for H_2S on the basis of 7-amino-4-methylcoumarin and fluorescein FRET system. Adding H_2S to the solution of **Flu-N_3** resulted in a clearly observable fluorescence enhancement (50-fold). This probe showed high sensitivity and selectivity towards H_2S over other reactive species with a detection limit (0.031 μM), fast response time (within 8 s). Moreover, **Flu-N_3** displayed little toxicity to HepG-2 cells and were successfully applied to image exogenous and endogenous H_2S in living cells and nude mouse.

Introduction

Hydrogen sulfide (H_2S) is proved to be third most attractive gas signaling molecules in biology, with extensive biological effects. Endogenous H_2S mainly generated by some enzymes including cystathionine *γ*-lyase (CSE), cystathionine *β*-synthase (CBS), and 3-mercaptopyruvate sulphur-transferase (MST) from sulfur-containing substrates, then H_2S as a final product have negative feedback regulation on these enzymes.[1-5] The scope of physiologically relevant H_2S concentration is from nanomolar to millimolar in human body, a third of it exists in the presence of gas molecules and two-thirds exists in the form of NaHS, which not only ensures the stability of the H_2S in the body, but also maintains the pH level of the internal environment. Along with nitric oxide (NO) and carbon monoxide (CO), endogenous H_2S acts as a gaseous mediator for regulating endocrine, neuronal, cardiovascular, immune and gastrointestinal systems.[6-10] The change in concentration of H_2S also has been influenced many diseases including Alzherimer's disease, Down's syndrome, diabetes, and liver cirFlusis.[11-15] In short, H_2S and NO, CO may together form a unique network of gas signals, which are independent of each other and regulate the way the network to participate in the regulation of the disease.

With the development of molecular tools, to use responsive fluorescent probes to detect H_2S sensitively and selectively in living systems precedes other traditional methods including colorimetric, electrochemical and gas chromatographic (GC) techniques.[16-19] To date, fluorescence method has attracted our interesting in their non-destructive, non-invasion, simple operation, as well as the capacity of rapid detection target analyte with high spatial and temporal resolution, it is an effective tool for detecting and imaging H_2S in biological systems.[20-29] Although the research of fluorescent probes for H_2S has achieved great success, most of them showed a delayed response with H_2S.[30-37] Thus, we intended to develop fast response H_2S probes based on the reduction of aromatic azides by H_2S. Based on our interesting in developing fluorescent probes that were more effective and fast detection of H_2S in a solution or cell, we also undertook the effort of constructing a fluorescence resonance energy transfer (FRET) system. The FRET system is a common way since fluorescent probes on the basis of FRET strategy are capable of reducing auto-fluorescence and scattering within biological samples.[38-39] However, the prerequisite for an efficient FRET process is substantial overlap between acceptor absorption and the donor emission, which would limit the development of FRET-based probes. Therefore, it is of great significance to explore the new fabrication of H_2S fluorescence probes based on the FRET mechanism. That is, the introduction of fluorescein based on commercial dyes 7-amino-4-methylcoumarin to construct a large conjugated structure and an FRET system. The emission of the coumarin fluorophore is centered in the blue region. We planted to use FRET mechanism to achieve longer-wavelength emission of novel H_2S probe. As shown in Scheme 2, a traditional strategy for H_2S probes could be based on a two fluorophore cassette comprised of a reaction-site-containing FRET acceptor and a FRET donor (here azido-containing coumarin) linked by a rigid piperazidine unit. After removing the reaction site in the FRET

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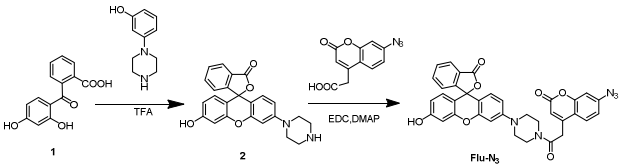
donor then FRET occurs, which results in a significant fluorescence enhancement of the FRET acceptor.

Herein, our group developed a new fluorescent probe named **Flu-N_3** (Scheme 1) for H_2S on the basis of 7-amino-4-methylcoumarin (AMC) and fluorescein (termed 'Fludol') FRET system. In this design, four factors were considered: (1) coumarin and Fludol were selected as two fluorophores due to their excellent solubility, larger molar extinction coefficient, high quantum yields, and well-separated maximum emission wavelengths (ca. 445 nm for coumarin and ca. 542 nm for Fludol). (2) In addition, this single part 7-Azido-4-methylcoumarin (AzMC) is also a fluorescent probe useful for the detection of H_2S. Thus, the azide part of the probe provide selective reaction sites for H_2S. (3) Two fluorophores were linked by a rigid piperazine and provided an advantage for Förster resonance energy transfer (FRET) in the coumarin-Fludol scaffold, which reducing the π-π stacking between dyes. The piperazidine linker also can improve the cell permeability of the probe. (4) Azidation of coumarin and Fludol could effectively quench the fluorescence of the probe via the intramolecular spirocyclization and intramolecular charge transfer (ICT) effect, respectively.[40-44] The probe should bear very low background fluorescence that is favorable for high sensitivity. Therefore, the aromatic azide moiety is selectively reduced in the presence of H_2S, amine-group compounds would also be formed in this process, which should exhibit green fluorescence of Fludol because of FRET. **Flu-N_3** exhibited desirable properties such as high selectivity, fast response for H_2S as well as low cytotoxicity. The probe was also applied for fluorescence imaging of H_2S in HepG-2 cells and live mice model.



Scheme 1 Synthesis of the probe.

Experimental

Materials and Instruments.

All reagents and solvents were purchased from commercial suppliers and used without further purification. Deionized water was used throughout all experiments. The solutions of anions were prepared from their sodium salts. The stock solutions of probe **Flu-N_3** were prepared in DMSO. SNP solution (20 mM) was prepared by dissolving 8.8 g SNP in 2 mL DMSO. Chromatography was carried out on silica gel using silica gel GF254 plates with a thickness of 0.20-0.25 mm. A pH meter (Mettler Toledo, Switzerland) was used to determine the pH. Ultraviolet-visible (UV-vis) spectra were recorded on a Cary 50 Bio UV-visible spectrophotometer. Fluorescence spectra were measured on Hitachi F-7000 fluorescence spectrophotometer. All fluorescence and UV-vis spectra data were recorded at 2 min after the analytes addition. A PO-120 quartz cuvette (1 cm) was purchased from Shanhai Huamei Experiment Instrument Plants, China. ^{1}H NMR and ^{13}C NMR experiments were performed with a Bruker AVANCE-600 MHz NMR spectrometer, respectively (Bruker, Billerica, MA). Coupling constants (J values) are reported in hertz. ESI determinations were carried out on AB Triple TOF 5600plus System (AB SCIEX, Framingham, USA). Imaging of cells was measured by a Zeiss LSM880 Airyscan confocal laser scanning microscope. ELX808 atomatic enzymatic marker was used to measure cytotoxicity experiments.

Solution preparation and optical measurement.

Stock solutions of probe **Flu-N_3** were prepared in DMSO. The stock solution of H_2S (2 mM) was prepared in deionized water, sodium hydrosulfide solid was added to aqueous solution to prepare a H_2S solution. Reagents with analytical grades and demineralized water were used for preparing the solutions. Stock solutions (2 mM) of Cys, Hcy, GSH, F^-, Cl^-, Br^-, I^-, SCN^-, SO_3^{2-}, SO_4^{2-}, $S_2O_3^{2-}$, ClO_4^-, PO_4^{3-}, NO_3^-, NO_2^-, AcO^-, HCO_3^-, CO_3^{2-}, were prepared by direct dissolution of proper amounts of sodium salts. All other chemicals used were of analytical grade. UV-vis and fluorescence spectra were detected in MeCN: PBS (9:1 v/v, PBS buffer, pH 7.4) solutions. Fluorescence measurements were carried out with a slit width of 5 nm/2.5 nm (λ_{ex} = 470 nm).

Cell culture and Imaging.

HepG-2 cells were cultured at 37°C incubator with 5% CO_2 and 95% air and grown in Dulbecco's Modified Eagle's medium supplemented with 12% Fetal Bovine Serum and 1% antibiotics at in humidified environment. The cells were stained with **Flu-N_3** (5 μM) for 15 min. Then the stained cells were incubated with H_2S for another 15 min at 37°C. Before imaging, the stained cells were washed one time with PBS. Fluorescence imaging studies were performed on Zeiss LSM880 Airyscan confocal laser scanning microscope. To evaluate the cytotoxicity of **Flu-N_3**, the cell viability was determined by Cell Counting Kit-8 (CCK-8), and the absorbance at 450 nm was measured to explicate the cells viability. HepG-2 cells were seeded on a 96-well microtiter to a total volume of 100 μL/well, then the cells were incubated at 37℃ in a 5% CO_2 incubator for 24 h. Different concentrations of **Flu-N_3** (0, 1, 2.5, 5, 10, 20, 30 and 50 μM) were then added to the wells. After incubation for 5 or 10 h, CCK-8 (10% in serum free culture medium) was added to each well, and then the cells continue to be incubated for another 1 h. The absorbance of each well was measured at 450 nm by a microplate reader.

In vivo imaging.

Balb/c nude mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. Experiments were conducted according to the National Institute of Health Guide for the Care and Use of Laboratory Animals. The experimental procedures were approved by the Institute of Pharmacology and Toxicology Academy of Military Medical Sciences PLA, Peop. Rep. China ethical committee on animal care and use, and all efforts were made to minimize animal suffering and reduce the number of animals used for the experiments. Imaging procedures were conducted with adult nude mice under general anesthesia by injection of sodium pentobarbital (0.5 mL/0.03 %). Then the mice will be injected in the abdomen with 50 μM of Flu-N_3, H_2S was carefully injected to the same location. Then, the fluorescence

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images were recorded at different period of time (0, 5, 15, 30, 45, 60 min).Images was taken using an excitation laser of 475 nm and emission was collected 520±5 nm filter.

Preparation and characterization of compounds.

Compound **1**: Fluorescein (1.7 g, 5.12 mmol) was dissolved in 12 mL of 50 % NaOH solution (w/v) and heated at 160 ℃ in an oil bath for 1 h. After cooling to room temperature, the mixture was poured into 80 mL of ice water, acidified with concentrated HCl, and allowed to stand at room temperature for 2 h. The precipitate was filtered and dried to afford the desired product as a beige powder (0.98 g, 74.1 % yield). m.p. 201.3-202.6℃. Elemental Analysis: Found C, 64.9; H, 3.4%. Molecular formula: $C_{14}H_{10}O_5$, requires: C, 65.2; H, 3.9%. ^{1}H NMR (600 MHz, MeOD): δ 8.11 (dt, J_1 = 13.7, J_2 = 6.9 Hz, 1H), 7.75-7.70 (m, 1H), 7.67-7.61 (m, 1H), 7.39 (dt, J_1 = 14.4, J_2 =7.2 Hz, 1H), 6.98-6.92 (m, 1H), 6.34 (d, J = 2.3 Hz, 1H), 6.27- 6.22 (m, 1H) ^{13}C NMR (151 MHz, DMSO-d_6): δ 200.9, 167.1, 165.4, 164.8, 140.4, 135.1, 132.7, 130.4, 130.1, 127.8, 113.7, 108.7, 102.9 (Fig. S1).

Compound **2**: To compound **1** (0.87 g, 3.37 mM) in 20 mL of TFA was added 1-(3-hydroxyphenyl)-piperazine (0.5 g, 2.80 mM), then heated to reflux and stirred for 36 h. The solvent was removed by rotavapor, and the left residue was dissolved in 10 mL of H_2O. The crude product was extracted with EtOAc (25 mL × 3), and dried with Na_2SO_4. The product was further purified by column chromatograph, and obtained as a red solid (0.8 g, 71%). m.p. 210.7-212.0℃. Elemental Analysis: Found C, 71.9; H, 4.8; N, 6.8%. Molecular formula: $C_{24}H_{20}N_2O_4$, requires: C, 72.0; H, 5.0; N, 7.0%. ^{1}H NMR (600 MHz, DMSO-d_6) : δ 8.88 (s, 1H), 8.00 (d, J = 7.6 Hz, 1H), 7.80 (t, J = 7.5 Hz, 1H), 7.73 (t, J = 7.5 Hz, 1H), 7.26 (d, J = 7.6 Hz, 1H), 6.78 (d, J = 8.9 Hz, 1H), 6.69 (s, 1H), 6.60-6.54 (m, 1H), 3.52-3.39 (m, 2H), 3.23 (s, 2H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 169.1, 162.8, 160.1, 158.9, 158.7, 158.5, 152.7, 152.4, 152.3, 152.0, 136.0, 130.5, 129.5, 129.0, 126.6, 125.1, 124.4, 118.2, 116.2, 113.1, 112.5, 109.9, 109.8,102.6,102.4, 45.0, 42.8 (Fig. S2).

Compound **Flu-N$_3$:** To a mixture of 7-azido-4-carbamoylmethylcoumarin[45] (49 mg, 0.2 mmol), compound **2** (0.2 mmol), EDC (38 mg, 0.2 mmol) and DMAP (2.44 mg, 0.02 mmol) was added CH_2Cl_2/DMF (v:v 5:1, 15 mL) at room temperature. The mixture was stirred for 24 hours. Then solvent was evaporated under reduced pressure and resulted residue was subjected to falsh column chromatography for purification. Compound **Flu-N$_3$** was obtained as a red solid in 50% yield. m.p. 116.7-118.5℃. Elemental Analysis: Found C, 66.8; H, 4.6; N, 11.1%. Molecular formula: $C_{35}H_{25}N_5O_7$, requires: C, 66.9; H, 4.1; N, 11.2%. ^{1}H NMR (600 MHz, DMSO-d_6): δ 10.12 (s, 1H), 8.00 (d, J = 7.2 Hz, 1H), 7.82-7.67 (m, 3H), 7.22 (dd, J_1 = 60.9, J_2=23.6 Hz, 3H), 6.85 (s, 1H), 6.77 (s, 1H), 6.69 (s, 1H), 6.55 (d, J = 16.8 Hz, 3H), 6.36 (s, 1H), 4.05 (d, J = 49.9 Hz, 2H), 3.72 (s, 2H), 3.62 (s, 2H), 3.40 (s, 2H), 3.30-3.23 (m, 2H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 169.2, 167.3, 160.0，159.9, 152.9, 152.6, 152.4, 152.3, 151.9, 143.6, 136.1, 130.6, 129.6, 128.9, 128.1, 125.1, 124.5, 117.1, 116.0, 115.2, 113.1, 112.4, 112.3, 110.1, 109.4, 107.3, 102.7, 101.8, 83.7, 47.8, 47.5, 45.2, 41.3, 40.9, 36.53 (Fig. S3). MS (ESI): Calcd for $[C_{35}H_{25}N_5O_7+H]^+$: 628.1754, found: m/z 628.1851 (Fig. S4).

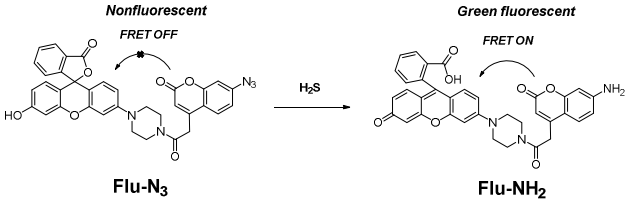
Results and Discussion

The absorption and fluorescence spectra of **Flu-N$_3$** were investigated in MeCN: PBS (9:1 v/v, PBS buffer, pH 7.4). Fig. S1 shows the change of **Flu-N$_3$** (5 μM) in the UV-vis spectrum upon addition of H_2S (0-110 μM), the absorption peak of the probe at 508 nm is increased gradually. With the increase of H_2S concentration, the absorption peak at 508 nm was almost unchanged, and it was speculated that the reduced product was stable in the presence of H_2S.

Subsequently, the fluorescence spectra response of control probe **Flu-N$_3$** (5 μM) toward H_2S (0-140 μM) was examined under the same condition. On treatment of **Flu-N$_3$** with H_2S (Fig. 1a), about 50-fold remarkable fluorescence enhancement was obtained, which implies that azide moieties of the probe were reduced by H_2S to produce strongly fluorescent. The obvious green fluorescence change of **Flu-N$_3$** before and after reaction would facilitate sensitive detection. Furthermore, a plot of fluorescence intensity at 538 nm versus the concentrations of H_2S displayed a good linearity (Fig. 1b). The detection limit was calculated to be 0.031 μM with the definition: Detection limit = 3σ/k, which is highly sensitive among the existing H_2S fluorescent probes.

In order to determine the effect of different pH on probe function, the influence of pH on the fluorescence behavior of **Flu-N$_3$** and its response to H_2S were investigated. In the absence of H_2S, the part of fluorescein in the probe showed an open-loop state with the enhancement of alkalinity and be accompanied by an increase in auto-fluorescence. Therefore, under the alkaline conditions, the fluorescent enhancement effect of the probe is weaker as the hydrogen sulfide increases. The results (Fig. 1c) indicated that the probe can work effectively under the reaction conditions of pH 7.4. Next, we further examined the time courses of the fluorescence intensities of **Flu-N$_3$** in the presence of 10 equiv. HS^- in MeCN: PBS (9:1 v/v, PBS buffer, pH 7.4). As Fig. 1d depicted, upon the addition of H_2S, the fluorescence intensity increased rapidly and reached a plateau within 8 s.

To explore selectivity and special recognition ability of **Flu-N$_3$** toward H_2S, the influence of various analytes was investigated in MeCN: PBS (9:1 v/v, PBS buffer, pH 7.4) including $S_2O_3^{2-}$, SO_3^{2-}, ClO^-, ClO^{2-}, ClO^{4-}, F^-, HSO^{3-}, NO^{2-}, $S_2O_4^{2-}$, $S_2O_5^{2-}$, SCN^-, Cys, GSH, Hcy. As shown in Fig. S2, obvious change to the fluorescence intensity of **Flu-N$_3$** solution was only observed upon reaction with H_2S, the addition of other relevant analytes just caused slight fluorescence change to the **Flu-N$_3$** solution. It is a bonus that the probe **Flu-N$_3$** exhibits excellent selectivity toward H_2S (140 μM) over Hcy (1.0 mM), Cys (1.0 mM), and GSH (1.0 mM). All these experimental results indicated that **Flu-N$_3$** was highly selective for H_2S among the various analytes in abiotic systems.



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Scheme 2 Possible reactions of **Flu-N_3** with H_2S

Proposed mechanism

The fluorescence turn-on mechanism of **Flu-N_3** is also described in Scheme 2. **Flu-N_3** is non-fluorescent due to an effective intramolecular spirocyclization and intramolecular charge transfer (ICT) effect between Azidation of coumarin and Fludol. Meanwhile, two fluorophores were linked by a rigid piperazine and provided an advantage for Förster resonance energy transfer (FRET) in the coumarin-Fludol scaffold, which reducing the π-π stacking between dyes. When the probe is treated with H_2S, as we expected **Flu-N_3** would be converted into **Flu-NH_2**, H_2S would exactly react with the azide moiety to produce amino group and release green fluorescence of fluorescein. The results above also agreed with the mass spectral peak of the reaction product at m/z 602.1936 correspond to $[M+H]^+$ for compound **Flu-NH_2** (Fig. S6)

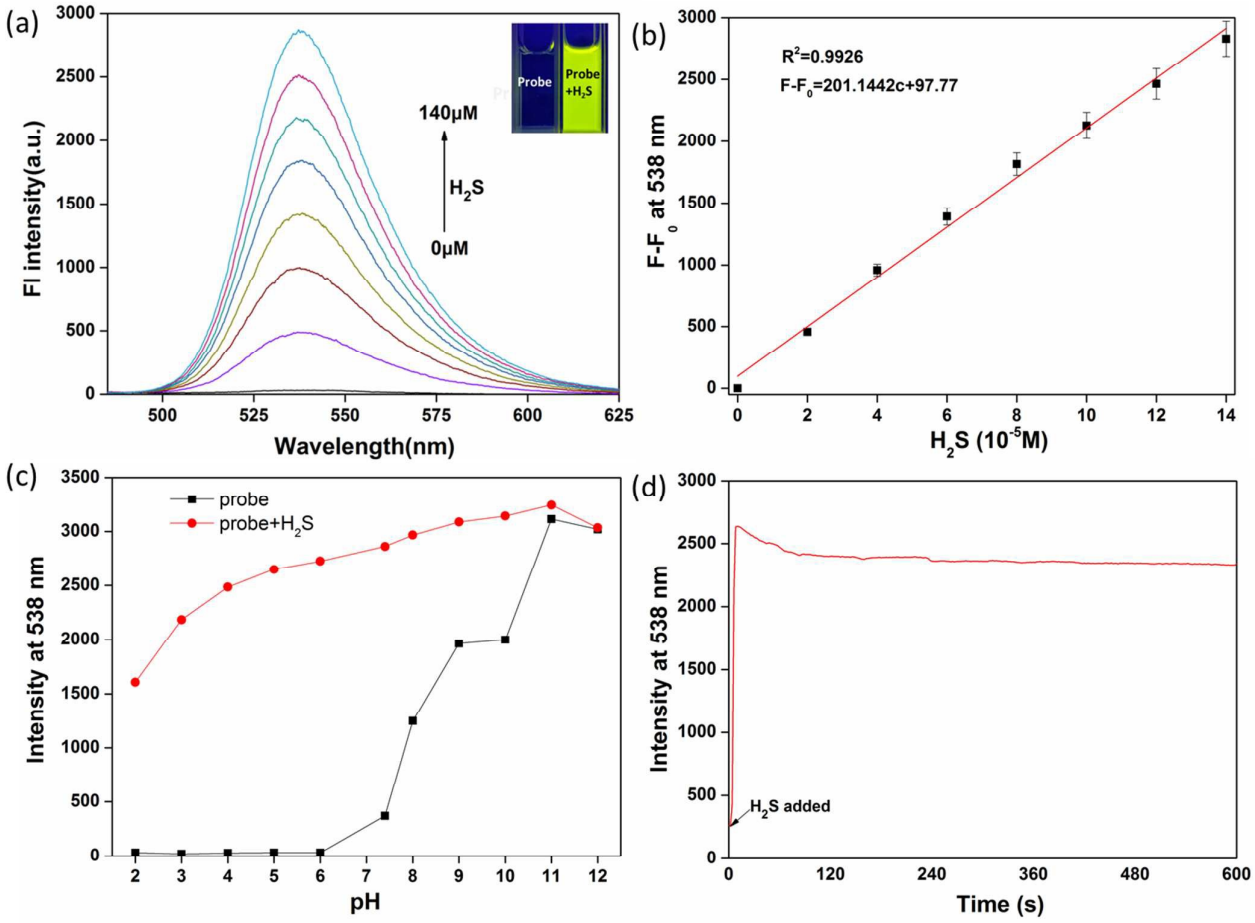

Fig. 1 (a) Fluorescence response of **Flu-N_3** (5 μM) upon the addition of H_2S (0-140 μM) in MeCN: PBS (9:1 v/v, PBS buffer, pH 7.4) solution. Inset: The color changes of **Flu-N_3** without and with addition of H_2S under a 365 nm lamp illumination; (b) Dependence of fluorescence intensity at 538 nm on H_2S concentration; (c) Effect of pH on the fluorescence intensity of **Flu-N_3** (5 μM) at 538 nm in MeCN: PBS (9:1 v/v, PBS buffer, pH 7.4) solution with or without H_2S (140 μM). λ_{ex} =470 nm. Slit width=5 nm/2.5 nm; (d) Time profile of **Flu-N_3** (5 μM) in presence of H_2S (10.0 equiv.) at 538 nm in MeCN: PBS (9:1 v/v, PBS buffer, pH 7.4) solution.

Cell experiments

We proceeded to perform the cellular imaging experiments to further explore whether the probe could detect endogenous and exogenous H_2S. Here, HepG-2 cells were chosen for intracellular fluorescence imaging experiments. As shown in Fig. 2b, we incubated HepG-2 cells with **Flu-N_3** (5.0 μM) for 30 min at 37℃ and observed a weak fluorescence signal. Furthermore, the cells were treated with 100 μM H_2S for 30 min and incubated with probe **Flu-N_3** (5.0 μM) for another 15 min, an obvious fluorescence increase in the green channel (Fig. 2c) were observed. To detect endogenously generated H_2S in living cells, the cells were stimulated with sodium nitroprusside (SNP) for 30 min. SNP stimulated cells were then incubated with **Flu -N_3** for 20 min and cell imaging was performed (Fig. 2a). The cell imaging results showed an enhancement in fluorescence intensity is due to the fact that SNP treatment

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stimulated cells to generate H_2S. In short, endogenous H_2S concentration was not large enough to enhance fluorescence when untreated with SNP. From the above results, we propose that **Flu-N_3** could be a good candidate for detecting H_2S in living cells.

Moreover, the CCK-8 method with **Flu-N_3** was carried out to evaluate its cytotoxicity. As illustrated in Fig. S6, the result of the experiments showed **Flu-N_3** exhibited good biocompatibility and low cytotoxicity of **Flu-N_3** towards HepG-2 cells at a concentration of 50 μM (81.5 % viability).

Fluorescence imaging of H_2S in live animal

Given the results that obtained on fluorescence detection of H_2S in solution and intercellular environment, we investigated the fluorescence imaging applicability of the probe **Flu-N_3** for visualizing H_2S in mice *in vivo*. The balb/c mice were fed commercial mice chow in individual stainless steel cages and left freely wandering in their housing for two weeks with 12 h dark/light cycles for acclimatization before the experiment. Living animal imaging system is used in mice imaging. After all the preparatory work has been completed, the mice were in anesthesia by 100 μL pentobarbital intraperitoneal injection. As the Fig. 3 shows, the mice will be injected in the abdomen with 50 μM of **Flu-N_3**, H_2S was carefully injected to the same location. Then, the fluorescence images were recorded at different period of time (0, 5, 15, 30, 45, 60 min). Comparing with the fluorescence of **Flu-N_3**, the fluorescent intensity increased steadily in a time range of 30 min was observed When H_2S was added. Though the probe can be used to in vivo imaging of exogenous H_2S, near-infrared probes should be more favorable for endogenous H_2S imaging in vivo[45].

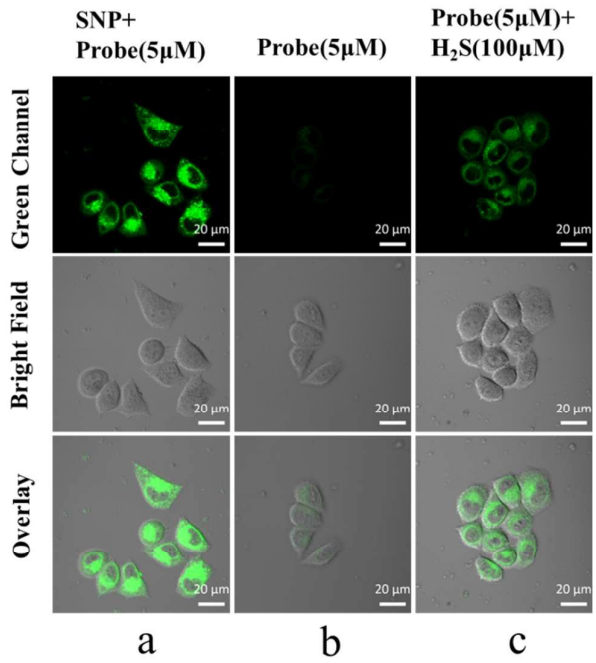

Fig. 2 Cell images of HepG-2 cell lines. (a): cells were stimulated with SNP for 30 min at 37℃ and then treated with **Flu-N_3** (5.0 μM) for 20 min. (b)cells were treated with **Flu-N_3** (5.0 μM) for 20 min at 37℃. (c): cells were treated with **Flu-N_3** (5.0 μM) for 20 min and then incubated with H_2S (100 μM) for 20 min. Images were taken λ_{ex}= 488 nm and λ_{em} range 500-550 nm.

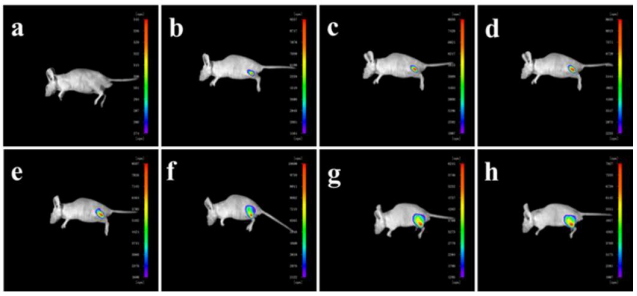

Fig. 3 Fluorescence images of **Flu-N_3** responding to exogenous H_2S in nude mice. (a) Nude mice without any treatment, (b) 50 μM of **Flu-N_3** was subcutaneously injected only, (c-h) 50 μM H_2S were subcutaneous injected for 0, 5, 15, 30, 45, 60 min respectively. Images were taken using an excitation laser of 475 nm and an emission filter of 520±5 nm.

Conclusions

In summary, we have developed an FRET-based turn on probe **Flu-N_3** for sensing H_2S, which was composed of 7-amino-4-methylcoumarin (energy donor) and fluorescein (energy acceptor). The addition of H_2S to the solution of **Flu-N_3** resulted in a clearly observable enhancement (50-fold) of emission intensity at 538 nm whereas the addition of Cys, Hcy and GSH induced weak fluorescence enhancement. **Flu-N_3** exhibited outstanding properties, such as high sensitivity and selectivity (detection limit of 0.031 μM), fast response time (within 8 s) and low toxicity. Notably, **Flu-N_3** was further used to image H_2S in living cells and mice successfully. The excellent properties of **Flu-N_3** make it have great potential in biological applications.

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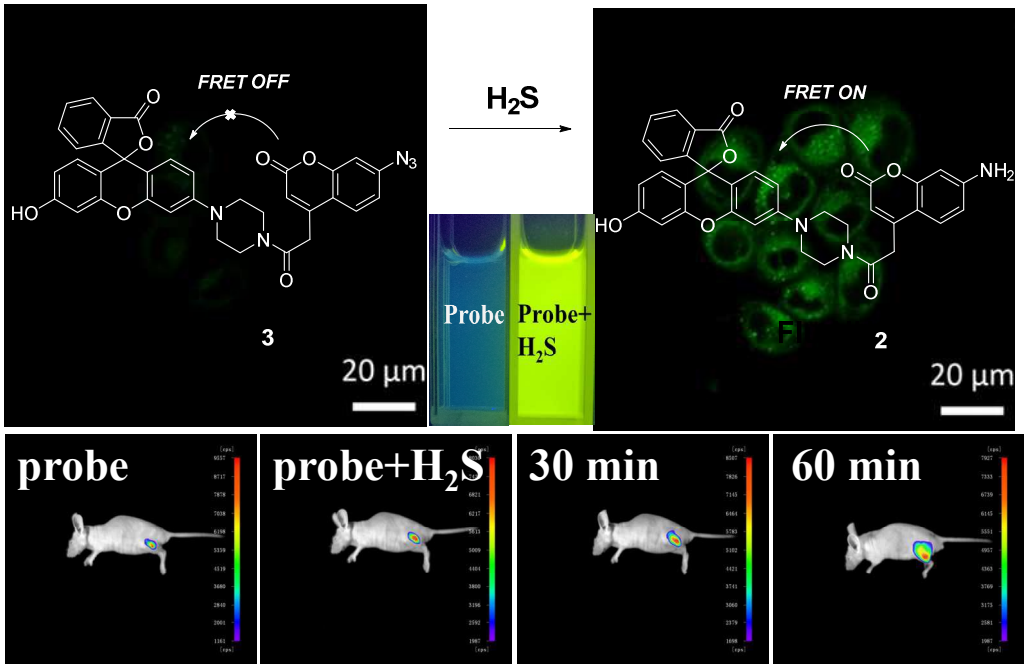
A novel FRET-based fluorescent probe for the selective detection of hydrogen sulfide (H_2S) and its application for bioimaging



ABSTRACT: Hydrogen sulfide (H_2S) is an endogenously produced gaseous signaling molecule with multiple functions in many physiological and pathological processes. Herein, our group developed a novel fluorescent probe (**Flu-N_3**) for H_2S on the basis of 7-amino-4-methylcoumarin and fluorescein FRET system. Adding H_2S to the solution of **Flu-N_3** resulted in a clearly observable fluorescence enhancement (50-fold). This probe showed high sensitivity and selectivity towards H_2S over other reactive species and exhibits detection limit (0.031 μM), fast response time (within 8 s). Moreover, **Flu-N_3** displayed little toxicity to HepG-2 cells and were successfully applied to image exogenous and endogenous H_2S in living cells and nude mouse.