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PAPER

A dual functional probe: sensitive fluorescence response to H_2S and colorimetric detection for SO_3^{2-}†

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Sheng Chen,[a] Chao Ma,[b] Mao-Sen Yuan,[a] Wenji Wang,[a] Dong-En Wang,[a] Shu-Wei Chen[a] and Jinyi Wang[a,b]*

Hydrogen sulfide (H_2S) and sulfite (SO_3^{2-}) are two important members of sulfur-containing species, which play different and important roles in industrial and biological processes. Accordingly, the development of efficient methods for simple, rapid, sensitive and selective monitoring of H_2S and SO_3^{2-} is of utmost importance in both environmental and biological science. In this study, we developed a new dual functional probe **NIR-DNP** for discriminative detection of H_2S and SO_3^{2-}. This probe could sense H_2S and SO_3^{2-} by two different approaches, a significant near-infrared fluorescence enhancement and color change from purple to cyan induced by H_2S, as well as a visible color change from purple to colorless caused by SO_3^{2-}. The detection limits of the probe **NIR-DNP** for H_2S and SO_3^{2-} in aqueous solutions were 36.53 nM and 33.33 nM, respectively. Competitive experiments demonstrated that the probe **NIR-DNP** had high fluorescence selectivity for H_2S and excellent colorimetric selectivity for SO_3^{2-} over other analytes. The sensing mechanism of the probe toward H_2S and SO_3^{2-} were respectively based on H_2S-induced thiolysis of dinitrophenyl ether and SO_3^{2-}-induced nucleophilic addition. Further investigation showed that the probe **NIR-DNP** could be used to develop an easy-to-prepare and easy-to-detect paper-based test strip for cheap and effective detection of SO_3^{2-}. Also, the probe **NIR-DNP** has the potential to image exogenous and endogenous H_2S in living cells.

Introduction

Hydrogen sulfide (H_2S) and sulfite (SO_3^{2-}) are two important members of sulfur-containing species, which play vital roles in industrial, environmental and biological processes.[1-3] H_2S has been the third endogenous gaseous signaling molecule following nitric oxide and carbon monoxide.[4] Physiological levels of endogenous H_2S have been known to be involved in a variety of physiological processes such as anti-inflammation,[5] neuromodulation[6] and apoptosis.[7] Abnormal levels of H_2S are closely linked with various diseases such as liver cirrhosis[8] and Alzheimer's disease.[9] Sulfite (SO_3^{2-}), because of its antioxidant and preservative properties, is widely used to preserve foods, beverages and pharmaceutical products from oxidation and microbial reactions.[10] However, long-term and frequent exposure to high doses of sulfite can cause adverse reaction and acute symptom, including dermatitis, urticaria, flushing, hypotension, abdominal pain and diarrhea.[11] The development of efficient methods for simple, rapid, sensitive and selective monitoring of H_2S and SO_3^{2-} is very important in both environmental and biological science.

Currently, several techniques such as electrochemical assay,[12] gas chromatography[13] and sulfide precipitation[14] have been developed for the detection of H_2S and SO_3^{2-}. However, these methods often require tedious sample and reagent preparation or complicated instruments, and therefore are not suitable for routine laboratory and on-site analysis. Fluorescent and colorimetric means provide good alternatives due to their low cost, easy-operation, simple instrumental implementation, fast response and excellent selectivity. Much effort has been devoted to the development of fluorescent and colorimetric probes for H_2S and SO_3^{2-}. To date, several probes have been designed for H_2S detection by taking the advantages of some specific chemical reactions, including reduction of azides/nitro groups,[15,16] demetallation of macrocyclic Cu(II) complexes,[17] nucleophilic reaction[18] and thiolysis of dinitrophenyl ether.[19] Similarly, many probes for sulfite have been also designed on the basis of selective deprotection of levulinate groups,[20] reaction with aldehyde groups,[21] Michael-type additions,[22] as well as complexation with amines[23] in the presence of SO_3^{2-}. However, most of these probes could only sense one of SO_3^{2-} and H_2S. One probe that could simultaneously detect for SO_3^{2-} and H_2S remains relatively few due to some of their common features such as nucleophilic and reducing properties.[24] In addition, most of the existed probes for H_2S have emissions in the ultraviolet or visible region, in which the fluorescence imaging is easily interfered by cell auto-fluorescence.[25] As we known, near-infrared (NIR, 650-900 nm) fluorescent probes

[a] College of Science, Northwest A&F University, Yangling, Shaanxi 712100, P. R. China..
[b] College of Veterinary Medicine, Northwest A&F University, Yangling, Shaanxi 712100, P. R. China.

† Electronic Supplementary Information (ESI) available: Synthesis, additional absorption and fluorescence spectral details, calculation of the detection limit, data for investigation of sensing mechanisms, effect of pH, cytotoxicity of various concentrations of the probe to cell, and ^{1}H- NMR, ^{13}C- NMR, HRMS, ESI-MS, FT-IR spectra. See DOI: 10.1039/x0xx00000x

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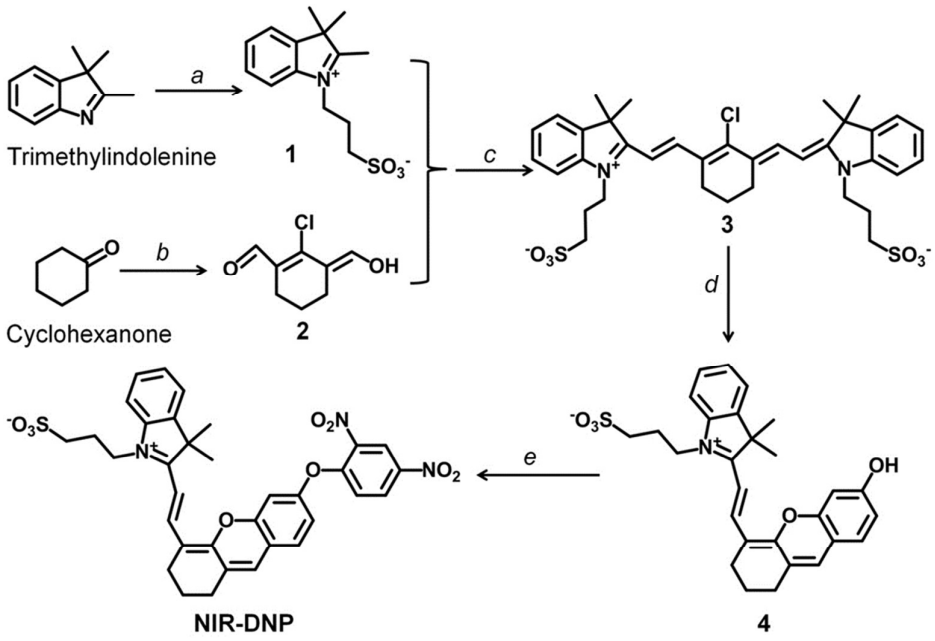

Scheme 1 Synthesis of probe **NIR-DNP**. Reagents and conditions: (a) 1,3-propane sultone, toluene, reflux, 24 h, 89%; (b) DMF, CH_2Cl_2, $POCl_3$, reflux, 3 h, 73.9%; (c) sodium acetate, acetic anhydride, 70 °C, 1 h, 80.8%; (d) resorcinol, triethylamine, DMF, N_2 atmosphere, 50 °C, 4 h, 46.6%; (e) 1-fluoro-2,4-dinitrobenzene, K_2CO_3, DMF, N_2 atmosphere, 50 °C, 5 h, 67%.

have unique advantages for tracing molecules *in vitro* and *in vivo*, such as minimum photo-damage to biological samples, deep tissue penetration, and minimum interference from background auto-fluorescence by bio-molecules in living systems.[25-27] Therefore, it is necessary to develop new types of probes that can be used for simultaneous detection of H_2S and SO_3^{2-}, preferably with emission located in the NIR region.

In this work, we design and synthesize a new NIR fluorescence probe **NIR-DNP** (Scheme 1) which can distinguish H_2S and SO_3^{2-} by naked eyes. The probe was constructed by connecting 2,4-dinitrophenyl to hemicyanine skeleton, in which a propanesulfonate residue was introduced to indolium group to improve its water solubility.[28] The probe displayed a selective recognition of H_2S via a visible color change from purple to cyan and a significant fluorescence enhancement in NIR region over other relevant species, and also exhibited a sensitive response to SO_3^{2-} through a visible color change from purple to colorless. In addition, the further applications of **NIR-DNP** in paper-based test strip for SO_3^{2-}, as well as imaging of exogenous and endogenous H_2S in living cells were studied.

Experimental section

Materials

2,3,3-Trimethylindolenine was purchased from J&K Chemical Technology (Shanghai, China). Cyclohexanone, resorcinol and 1,3-propanesulfonate were obtained from Aladdin (Shanghai, China). 1-Fluoro-2,4-dinitrobenzene was purchased from Xiya Reagent (Shandong, China). Whatman No. 1 filter paper was purchased from Fisher Scientific (Pittsburgh, PA). Dry solvents used in the synthesis were purified using standard procedures. All other chemicals were obtained from qualified reagent suppliers with analytical reagent grade.

Instruments

^{1}H-nuclear magnetic resonance (NMR) and ^{13}C-NMR spectra of the products were recorded on a Bruker 500 MHz/125 MHz NMR spectrometer using tetramethylsilane (TMS) as the internal standard (chemical shifts in ppm). Electrospray ionization mass spectroscopy (ESI-MS) data were obtained using a Thermo Scientific LCQ FLEET mass spectrometer. High-resolution mass spectra (HRMS) data were recorded on a Waters Xevo® G2-XS QTOF mass spectroscopy (Waters, Manchester, UK) equipped with a Zpray™ ESI source operating in the negative ion mode. Fourier-transform infrared spectroscopy (FT-IR) was recorded on a Nicolet 5700 spectrometer at wavelengths of 400 cm^{-1} to 4000 cm^{-1} and a resolution of 3 cm^{-1} over 32 scans. The pH values were measured using a digital pH meter (PHS-3C, Lei-ci, Shanghai, China). The UV-Vis absorption spectra were measured by using Shimadzu UV1780 spectrometer (Shimadzu, Japan). The fluorescence spectra measurements were performed using Shimadzu RF-5301 fluorescence spectrometer (Shimadzu, Japan). The fluorescence images of cells were taken using an inverted microscope (Olympus CKX41, Japan).

Synthesis of the probe NIR-DNP

The probe **NIR-DNP** was prepared as depicted in Scheme 1. For the detailed information on the synthesis of the intermediates **1**–**4**, see the electronic supplementary

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information (ESI†). The probe **NIR-DNP** was synthesized by the nucleophilic substitution reaction between compound **4** and 1-fluoro-2,4-dinitrobenzene. To a solution of **4** (24.58 mg, 0.05 mmol) in N,N-dimethylformamide (DMF, 2 mL) was added potassium carbonate (13.8 mg, 0.1 mmol) and the mixture was stirred for 30 min under N_2 atmosphere. Then, 1-fluoro-2,4-dinitrobenzene (18.6 mg, 0.1 mmol) dissolved in DMF (2 mL) was add to the solution. The resulting mixture was stirred for 5 h at 50 °C. The solvent was evaporated and the crude product was purified by column chromatography on silica gel (CH_2Cl_2: CH_3OH = 20:1, *v/v*) to afford the probe **NIR-DNP** as a blue-purple solid (22 mg, 67% yield). ^{1}H NMR (500 MHz, $CDCl_3/CD_3OD$): δ 8.93 (d, J = 2.7 Hz, 1H), 8.74 (d, J = 15.1 Hz, 1H), 8.49 (dd, J = 9.2, 2.8 Hz, 1H), 7.65 (d, J = 5.8 Hz, 1H), 7.59 – 7.53 (m, 4H), 7.48 (t, J = 7.4 Hz, 1H), 7.33 (d, J = 9.2 Hz, 1H), 7.26 – 7.24 (m, 2H), 7.06 (dd, J = 8.5, 2.2 Hz, 1H), 6.87 (d, J = 15.1 Hz, 1H), 4.65 (t, 2H), 3.02 – 2.99 (m, 2H), 2.82-2.79 (m, 4H), 2.37 – 2.31 (m, 2H), 2.00 – 1.93 (m, 2H), 1.81 (s, 6H) ppm; ^{13}C NMR (125 MHz, $CDCl_3/CD_3OD$): δ 179.00, 159.80, 156.18, 154.38, 153.91, 147.83, 146.89, 142.67, 142.28, 141.02, 140.24, 130.73, 130.55, 129.33, 129.12, 128.01, 122.46, 121.96, 120.16, 120.07, 116.59, 115.92, 113.21, 107.65, 106.17, 51.06, 47.27, 44.16, 29.26, 27.46, 23.91, 23.57, 20.12 ppm; IR (KBr) ν 3040.96, 2923.43, 2854.68, 1603.70, 1578.15, 1534.07, 1499.74, 1455.64, 1346.55, 1319.87, 1260.96, 1207.56, 1152.62, 1115.21, 1061.72, 1034.94, 966.35, 922.66, 833.94, 761.06 cm^{-1}; HRMS (ESI, *m/z*): Calcd. for $[C_{34}H_{31}N_3O_9S + H]^+$, 658.1815; Found, 658.1951.

General procedure for spectra measurement

The stock solution of the probe **NIR-DNP** was prepared at 1.0 mM in DMSO. Stock solutions of various testing species in water or phosphate buffer saline (PBS) were prepared from Na_2S, Na_2SO_3, $CaCl_2$, KCl, NaCl, NaF, NaBr, $NaNO_3$, $NaNO_2$, NaN_3, Na_2SO_4, $Na_2S_2O_3$•$5H_2O$, CH_3COONa, Na_2CO_3, H_2O_2, $NaHCO_3$, NaH_2PO_3, Cysteine and glutathione. Na_2S (50 mM) aqueous solution was used as a H_2S source in all experiments, and Na_2SO_3 (50 mM) aqueous solution was used as a SO_3^{2-} source.[17,29] A typical test solution was prepared by placing 20.0 μL **NIR-DNP** stock solution (1.0 mM), 180.0 μL DMSO and appropriate aliquot of each analyte stock solution into a 5.0-mL centrifugal tube, and diluting the solution to 2.0 mL with PBS (20 mM, pH 7.4). For the detection of H_2S, the resulting solution was incubated with appropriate testing species at ambient temperature for 45 min before recording the UV-Vis absorption and fluorescence spectra. For the detection of SO_3^{2-}, the resulting solution was incubated with appropriate testing species for 3 min before recording UV-Vis absorption spectra. The 650-nm excitation wavelength was used for all the measurements.[26] The excitation and emission slit widths were all 5 nm.

Cell culture and fluorescence imaging

Human glioblastoma (U-251) cells were obtained from the Chinese Academy of Sciences (Shanghai, China). U-251 cells routinely cultured with Dulbecco's modified Eagle's medium (DMEM, pH 7.4) supplemented with 10% fetal bovine serum (FBS, Gibco Invitrogen, CA, USA), penicillin (100 U/mL), and streptomycin (100 U/mL) at 37°C in 5% CO_2 atmosphere. To maintain cells in the exponential growth phase, they were normally passaged at a ratio of 1:3 every 72 h. Before using, U-251 cells in the exponential growth phase were collected and re-seeded on 24-well plates at a density of 5 ×10^4 cells/mL and cultured for 24 h. For exogenous H_2S imaging, the culture medium (the DMEM supplemented with 10% FBS) was removed and the cells were washed twice with PBS (0.01 mol/L, pH 7.4). Then, the cells were treated with **NIR-DNP** (50 μM) at 37 ºC for 60 min. After washing with PBS, the cells were incubated for another 60 min in the presence of Na_2S (500 μM). The controls were simultaneously performed by treating the cells only with the probe **NIR-DNP** for 60 min at 37 ºC. After washed twice with PBS, the cells were incubated with 4% (m/v) paraformaldehyde aqueous solution for 20 min at room temperature. Then, Hoechst 33258 (0.5 μg/mL) in PBS was added to stain the nuclei for 10 min. Fluorescence imaging was taken after washing the cells with PBS twice. For endogenous H_2S imaging, cells were first incubated with thiols (Cysteine, 500 μM) for 60 min at 37 ºC. After washing, the cells were then incubated with **NIR-DNP** (50 μM) for another 60 min. The medium was replaced by PBS and cells were imaged immediately.

Results and discussion

Synthesis of probe NIR-DNP

The synthesis of the probe **NIR-DNP** is outlined in Scheme 1. The intermediates **1** and **2** were first prepared, commencing from 2,3,3-trimethylindolenin and cyclohexanone according to the literature procedures.[30,31] The intermediate **3** was obtained by condensation reaction of intermediates **1** and **2** in acetic anhydride. The intermediate **4** was subsequently synthesized by treating **3** with resorcinol in the presence of triethylamine at 50 °C for 4 h. Finally, under basic condition, intermediate **4** was treated with 1-fluoro-2,4-dinitrobenzene to give the target probe **NIR-DNP** through nucleophilic substitution reaction. The chemical structures of the compounds were well characterized by ^{1}H NMR, ^{13}C NMR, HRMS and FT-IR. The detailed synthetic procedures and relevant spectral data are given in the experimental section and electronic supplementary information (ESI†).

Sensing property of probe NIR-DNP for H_2S

The sensing ability of probe **NIR-DNP** for H_2S was investigated in PBS (20 mM, pH 7.4) containing 10% DMSO (v/v). **NIR-DNP** (10 μM) showed a maximum absorption at 595 nm (ε = 2.96 ×10^4 M^{-1} cm^{-1}). Upon the addition of Na_2S (50.0 equiv) to **NIR-DNP** solution, the absorbance at 595 nm decreased notably, whereas a new absorption peaked at 685 nm appeared and gradually increased against time, as well as a distinct color change from purple to cyan (Fig. 1A and inset). And the UV spectrum was close to the characteristic peak of compound **4** (ε = 2.95 ×10^4 M^{-1} cm^{-1}) (Fig. S1A, ESI†). The

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results showed that the probe allowed colorimetric detection of H_2S by 'naked eyes'. Fluorescence analysis (Fig. 1B) exhibited that **NIR-DNP** solution was almost non-fluorescence (Φ= 0.007, Table S1, ESI†). However, upon the addition of Na_2S, a large fluorescence enhancement at 707 nm was observed, and the resulting fluorescence spectrum was consistent with compound **4** (Φ= 0.031) (Fig. S1B and Table S1, ESI†). Kinetic studies of probe **NIR-DNP** (10 μM) with the addition of Na_2S (50.0 equiv) showed that the emission intensity at 707 nm gradually increased against time until it reached a plateau at about 45 min ($t_{1/2} \approx 7$ min) under the test conditions (Fig. 1B, inset). The observed first order rate constant k_{obs} was determined to be about 0.0718 min^{-1} (Fig. S2, ESI†) and the emission intensity was found to increase about 53-fold at 707 nm. The relatively fast and distinct fluorescence signal change in NIR region indicated that probe **NIR-DNP** can be used as a sensitive NIR fluorescent sensor for 'turn-on' detection of H_2S in aqueous solutions.

To further evaluate the capability of **NIR-DNP** for highly sensitive detection of H_2S, the changes of the fluorescence spectra of **NIR-DNP** in the presence of different concentrations of Na_2S were also investigated (Fig. 1C and S3, ESI†). Upon treatment with increasing concentrations of Na_2S, a characteristic fluorescence emission at 707 nm was observed and enhanced gradually. The fluorescent intensity reached stability when the amount of Na_2S was more than 200 μM (Fig. S3, ESI†). Further analysis showed that the fluorescent intensity at 707 nm increased linearly with the concentration of Na_2S ranging from 0 to 7.5 μM (R = 0.9993) (Fig. 1C, inset). The detection limit of **NIR-DNP** for H_2S was experimentally determined to be 36.53 nM based on signal-to-noise ratio (S/N) = 3 under the test conditions, which was much lower than those obtained by most of the existing small-molecule fluorescent probes for H_2S (Table S2, ESI†). This result further confirmed that **NIR-DNP** is highly sensitive to H_2S. Also, the result indicated that the probe **NIR-DNP** was favorable for imaging intracellular H_2S whose physiological relevant concentrations are estimated to be in the range from nanomolar to millimolar levels.[32]

To investigate the specificity of the probe to H_2S, the effects

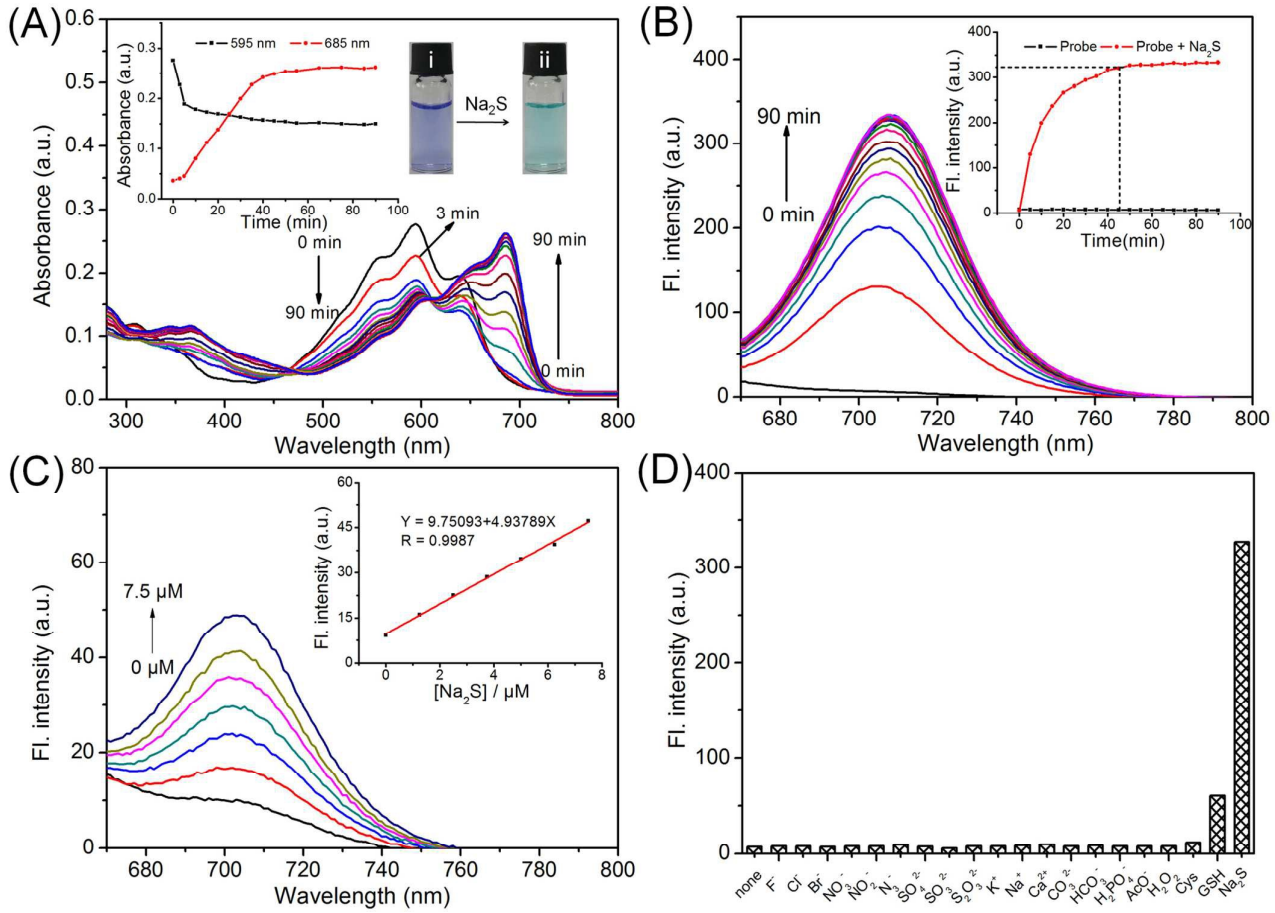

Fig. 1 (A) Time-dependent absorbance spectra of probe **NIR-DNP** (10 μM) upon the addition of Na_2S (500 μM) in PBS (20 mM, pH 7.4) containing 10% DMSO (v/v). Inset: plot of the absorbance at 595 nm and 685 nm as a function of time; the color change of **NIR-DNP** (10 μM) before (i) and after (ii) the addition of Na_2S (500 μM) under visible light. (B) Time-dependent fluorescent spectra of **NIR-DNP** (10 μM) upon addition of Na_2S (500 μM) in PBS (20 mM, pH 7.4) with 10% DMSO (v/v). Inset: plot of the emission intensity at 707 nm as a function of time. (C) Fluorescence spectra of **NIR-DNP** (10 μM) upon addition of Na_2S (0-7.5 μM). Inset: plot of the emission intensity at 707 nm as a function of the concentrations of Na_2S. (D) Fluorescent intensity of **NIR-DNP** (10 μM) at 707 nm in the presence of various species (1 mM Cys, 5 mM GSH and 500 μM others).

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of various analytes including typical anions, cations and other biologically relevant species on the fluorescent intensity of **NIR-DNP** were tested (Fig. 1D and S4, ESI†). The fluorescent intensity of the probe **NIR-DNP** did not show observable change upon the addition of various analytes including anions (F^-, Cl^-, Br^-, N_3^-, CO_3^{2-}, HCO_3^-, CH_3COO^-, SO_4^{2-}, $H_2PO_4^-$), cations (Na^+, K^+, Ca^{2+}), as well as reactive oxygen species (H_2O_2), nitrogen species (NO_3^-, NO_2^-) and sulfur species (SO_3^{2-}, $S_2O_3^{2-}$). However, after reacted with Na_2S, the fluorescent intensity of the probe **NIR-DNP** enhanced remarkably (~ 47-fold). It is worth mentioning that although the addition of millimolar concentration of the biothiols (i.e., 1 mM for Cys or 5 mM for GSH) to the probe gave a limited increase in the fluorescence intensity, it was far weaker than that caused by Na_2S. The high selectivity of the probe **NIR-DNP** to H_2S could be attributed to the obvious distinction in terms of size and pK_a values between H_2S and biothiols.[33,34] To check the practical ability of **NIR-DNP** as a H_2S selective fluorescent probe, competition experiments were also carried out by adding Na_2S to **NIR-DNP** solution in the presence of above analytes. As shown in Fig. S5A (ESI†), most of the analytes did not produce an obvious change in the fluorescence intensity except for SO_3^{2-}. When competing with SO_3^{2-}, the fluorescence intensity was about 60% lower than that in its absence. Moreover, biothiol (Cys or GSH) did not show significant influence on the detection of H_2S, even its concentration was in millimolar range (Fig. S5B, ESI†). All these results indicated that probe **NIR-DNP** had high selectivity to H_2S.

Sensing property of probe NIR-DNP for SO_3^{2-}

In the selectivity experiments of probe **NIR-DNP** to H_2S, we found that the color of the probe solution in the presence of SO_3^{2-} changed from purple to colorless. This phenomenon inspired us that probe **NIR-DNP** may have the ability to sense H_2S and SO_3^{2-} by different approaches. As we know, single probe that could simultaneously detect for SO_3^{2-} and H_2S remains relatively few (Table S2, ESI†). Therefore, the sensing ability of the probe **NIR-DNP** for SO_3^{2-} was also investigated in PBS (20 mM, pH 7.4) containing 10% DMSO (v/v). Firstly, the time-dependent absorption spectra of **NIR-DNP** (10 μM) in

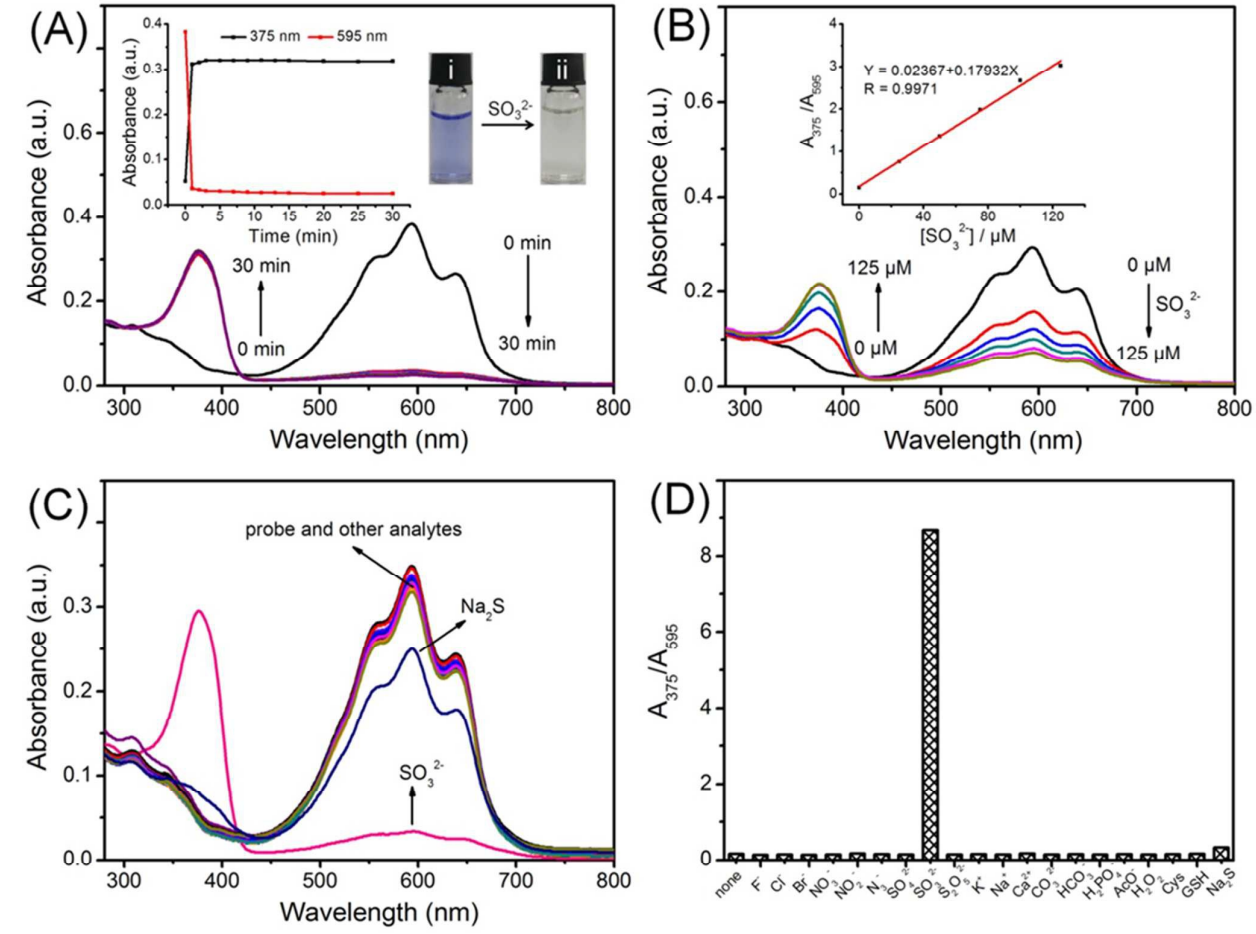

Fig. 2 (A) Time-dependent absorption spectra of **NIR-DNP** (10 μM) upon addition of SO_3^{2-} (500 μM) in PBS (20 mM, pH 7.4) with 10% DMSO (v/v). Inset: plot of the absorbance at 595 nm and 375 nm as a function of time; the color change of **NIR-DNP** (10 μM) before (i) and after (ii) the addition of Na_2SO_3 (500 μM) under visible light. (B) Absorption spectra of **NIR-DNP** (10 μM) upon addition of Na_2SO_3 (0-125 μM). Inset: plot of the A_{375}/A_{595} ratio as a function of the concentrations of SO_3^{2-}. (C) Absorption spectra of **NIR-DNP** (10 μM) upon addition of various species (1 mM Cys, 5 mM GSH and 500 μM others). (D) Absorption ratio A_{375}/A_{595} of **NIR-DNP** (10 μM) in the presence of various species.

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the presence of SO_3^{2-} (50.0 equiv) were studied (Fig. 2A). The result showed that upon the addition of SO_3^{2-} (50.0 equiv), the absorption band at 595 nm decreased rapidly. Meanwhile, a new absorption band centered at 375 nm appeared, accompanied with the solution color changing from purple to colorless (Fig. 2A, inset). The isosbestic point at 419 nm indicated the formation of a new compound. Further study indicated that the absorption peak at 595 nm decreased rapidly to the baseline over 0 to 180 seconds, and a new peak at 375 nm increased to the top of the wavelength range (Fig. 2A, inset). The A_{595}/A_{375} ratio varied from 7.37 to 0.078 after complete conversion, which was about 95-fold decrease (Fig. S6, ESI†). Such a rapid and significant change in the signal ratios at the two wavelengths indicated that probe **NIR-DNP** could be used as a colorimetric sensor for high sensitive detection of SO_3^{2-}.

The changes of the absorption spectra of **NIR-DNP** with SO_3^{2-} concentration increasing were further studied (Fig. 2B and S7, ESI†). Upon the addition of increasing concentrations of SO_3^{2-}, the absorption intensity at 595 nm gradually decreased, while the absorption intensity at 375 nm gradually increased. The A_{375}/A_{595} ratio was linearly proportional to SO_3^{2-} concentration in the range of 0-125 μM (R = 0.9971) (Fig. 2B, inset). The detection limit was determined to be about 33.33 nM based on S/N = 3, suggesting that the probe was potentially useful for quantitative determination of SO_3^{2-} in a large dynamic range and has high sensitivity.

Considering coexistence of other competing species in real samples, the selectivity of **NIR-DNP** toward SO_3^{2-} was also studied in the presence of other analytes including F^-, Cl^-, Br^-, N_3^-, CO_3^{2-}, HCO_3^-, NO_3^-, NO_2^-, $S_2O_3^{2-}$, CH_3COO^-, SO_4^{2-}, $H_2PO_4^-$, Na^+, K^+, Ca^{2+}, Na_2S, Cys, GSH and H_2O_2. The results

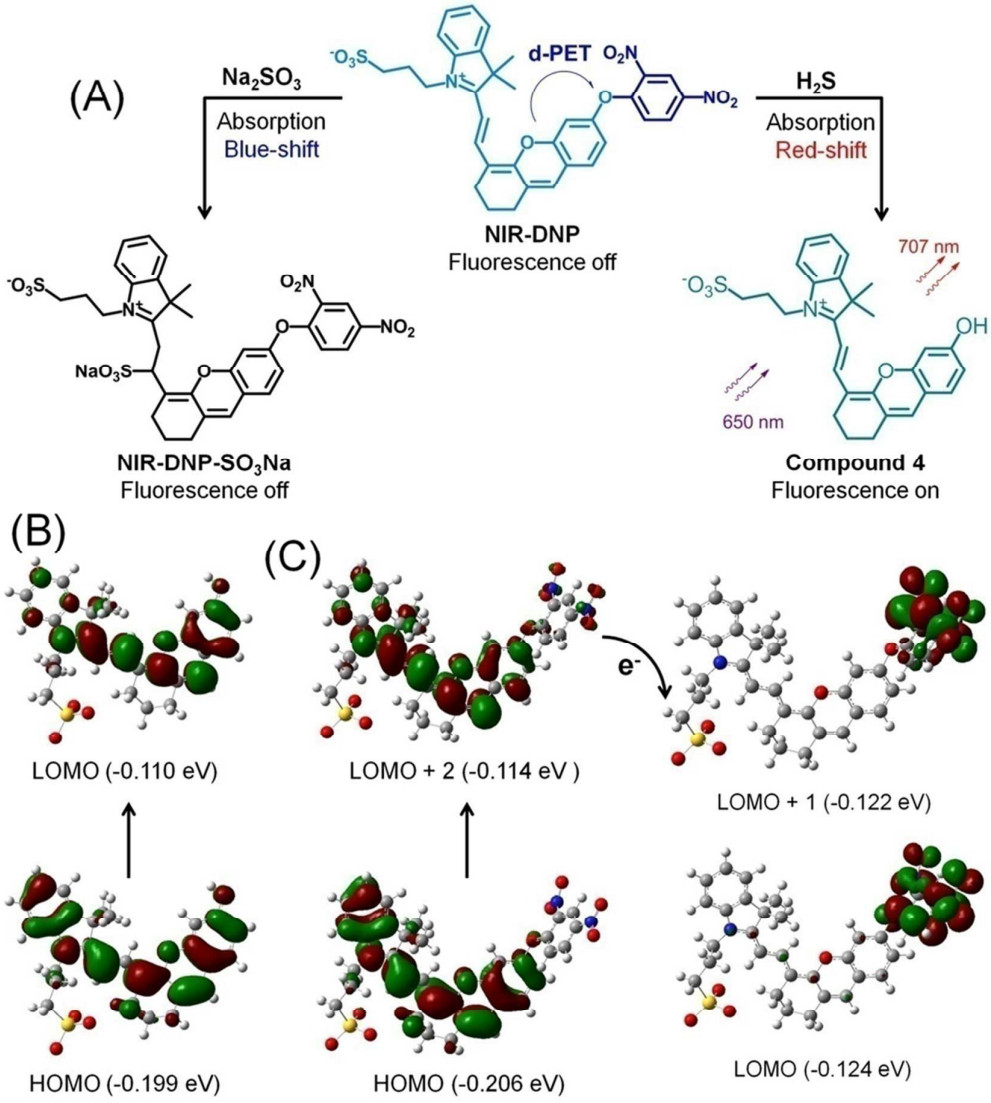

Fig. 3 (A) The mechanism of probe **NIR-DNP** for discriminative sensing sulfite and sulfide. (B) Frontier molecular orbital plots of compound **4** in water. (C) Frontier molecular orbital plots of probe **NIR-DNP** in water. The result showed that the fluorescence emission of compound **4** is quenched by d-PET process.

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(Fig. 2C) showed that no obvious responses were observed in the UV-vis spectra of **NIR-DNP** upon the addition of the above species. However, the addition of SO_3^{2-} induced a new absorption band centered at 375 nm, accompanied by decrease of the absorption band at 595 nm. Observation of the color changes of the probe **NIR-DNP** before and after the addition of sulfite and other species under visible light showed that only SO_3^{2-} caused a marked change from purple to colorless (Fig. S8A, ESI†). Quantitative analysis (Fig. 2D) exhibited that the A_{375}/A_{595} ratio of the probe was remarkably enhanced when **NIR-DNP** was treated with SO_3^{2-}. It is worth noting that during the period (3 min) of SO_3^{2-} detection, Na_2S could only induce a small decrease in the absorbance at 595 nm (Fig. 1A and 2C) and a neglectable change of the A_{375}/A_{595} ratio (Fig. 2D). However, when the probe was treated with Na_2S for 45 min, the color of the probe solutions changed from purple to cyan (Fig. S8B, ESI†). This result indicated that **NIR-DNP** could be used for distinguishing SO_3^{2-} and H_2S by naked eyes in time dimension. Moreover, the addition of biothiols (1 mM for Cys and 5 mM for GSH) to the probe could not cause the change of A_{375}/A_{595} ratio, which might be ascribed to their steric effects and high pK_a values.[34] This result imply that the probe was chemoselective for SO_3^{2-} over these biothiols at physiological pH. The detection of SO_3^{2-} using **NIR-DNP** in the presence of other analytes remain effective (Fig. S9, ESI†). All these investigations clearly indicated that the probe **NIR-DNP** possessed excellent selectivity for SO_3^{2-}.

Sensing mechanism of NIR-DNP toward H_2S

To confirm that the turn-on fluorescence response of **NIR-DNP** was triggered by H_2S, the thiolysis product of **NIR-DNP** was characterized by using NMR and EI-MS analysis, as well as theoretically calculated. ^{1}H NMR analysis showed that the product of **NIR-DNP** after treated with Na_2S was identical to the authentic compound **4** (Fig. S10, ESI†), which was further confirmed by EI-MS analysis. EI-MS showed a peak at *m/z* =490.32, which is the same with the theoretical molecular mass of compound **4** (calcd for $[M - 1]^-$ = 490.18) (Fig. S11, ESI†). To better understand the photophysical properties of the probe **NIR-DNP** and the thiolysis product **4**, theoretical calculations were also performed through the density functional theory (DFT) and time-dependent DFT (TDDFT) by using Gaussian 09 program (Fig. 3B, 3C, and Table S3, ESI†). For compound **4**, the π electrons on both the HOMO and LUMO were essentially distributed in the entire backbone of compound **4**. By contrast, for **NIR-DNP**, the π electrons on HOMO and LUMO+2 were primarily resided on the electron-donating moiety of compound **4**, whereas, those on LUMO and LUMO+1 were mainly located on the electron-withdrawing group of 2,4-dinitrobenzene. This result indicated that **NIR-DNP** could bear efficient photo-induced electron transfer (PET) from compound **4** to the 2,4-dinitrobenzene group, rendering the relatively weak fluorescence of the probe. By contrast, the nearly complete overlap of electrons on the transition orbitals may induce the strong fluorescence emission for compound **4**. All these investigations demonstrated that the mechanism of **NIR-DNP** for H_2S sensing was a thiolysis process of dinitrophenyl ether with the release of compound **4** (Fig. 3A).

Sensing mechanism of NIR-DNP toward SO_3^{2-}

The obvious blue-shifts in the absorbance spectra of **NIR-DNP** toward SO_3^{2-} are consistent with the addition of SO_3^{2-} to the electrophilic C=C bond in **NIR-DNP**, which lead to a short conjugation structure of the reaction product. To confirm the formation of the addition product **NIR-DNP**-SO_3Na, ^{1}H NMR titration experiment was carried out by addition of Na_2SO_3 into **NIR-DNP** in CD_3OD. The result (Fig. S12, ESI†) showed that the resonance signal corresponding to the alkene proton H_a at 7.54 ppm disappeared after treated with Na_2SO_3, and a new peak at 3.82 ppm assigned to the proton $H_{a'}$ appeared. The addition of Na_2SO_3 to C=C resulted in the formation of a chiral center of $C_{a'}$ with the nonequivalent protons of the methylene group at C_b. Therefore, the signal for H_b at 8.72 ppm shifted to a high field and appeared as two peaks at 2.48 ppm and 3.02 ppm, respectively, which was similar with those reported previously.[22,35] EI-MS analysis further confirmed this result. A peak appeared at *m/z* =760.24 in the mass spectrum, which is nearly identical to the theoretical molecular mass of **NIR-DNP**-

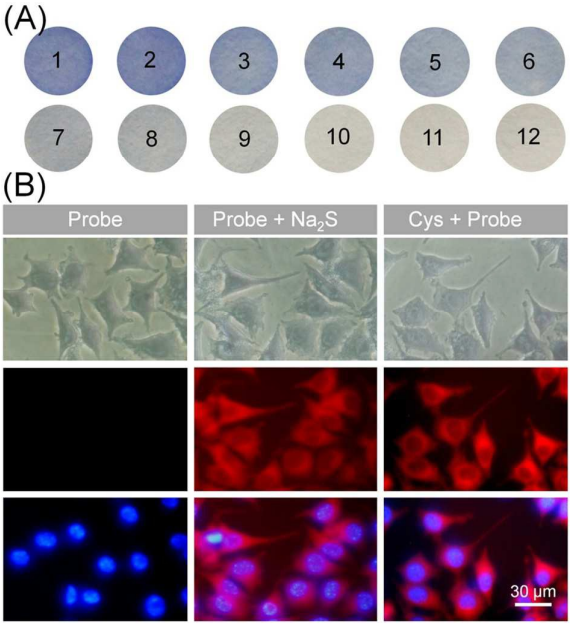

Fig. 4 (A) Detection of SO_3^{2-} in the absence and presence of other analytes by using paper-based test strips treated by probe **NIR-DNP** (100 μM): 1, 0 μM SO_3^{2-}; 2, other analytes mixture; 3, 10 μM SO_3^{2-}; 4, 50 μM SO_3^{2-}; 5, 100 μM SO_3^{2-}; 6, 200 μM SO_3^{2-}; 7, 500 μM SO_3^{2-}; 8, 500 μM SO_3^{2-} and other analytes; 9, 1 mM SO_3^{2-}; 10, 5 mM SO_3^{2-}; 11, 10 mM SO_3^{2-}; 12, 50 mM SO_3^{2-}. Other analytes mean a mixture of K^+, Na^+, F^-, Cl^-, Br^-, NO_3^-, NO_2^-, N_3^-, SO_4^{2-}, $S_2O_3^{2-}$, CO_3^{2-}, HCO_3^-, $H_2PO_4^-$, AcO^- (500 μM of each analyte in PBS, pH 7.4). (B) Fluorescence images of probe **NIR-DNP** in U-251 cells. The cell nuclei were stained with Hoechst 33258. From left to right, cells were incubated with the probe **NIR-DNP** (50 μM) for 60 min; cells were first incubated with the probe **NIR-DNP** (50 μM) for 60 min, and then incubated with Na_2S (500 μM) for another 60 min; cells were first incubated with Cys (500 μM) for 60 min, and then incubated with the probe **NIR-DNP** (50 μM) for another 60 min. From top to bottom, bright-field images; fluorescence images; merged image of fluorescence and Hoechst 33258-stained images.

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SO_3Na (calcd. for [**NIR-DNP**-SO_3Na–1]$^-$, 760.13) (Fig. S13, ESI†). These results suggested that the mechanism of **NIR-DNP** for SO_3^{2-} sensing was a SO_3^{2-}-induced nucleophilic addition, with generating a short conjugation structure of the addition product **NIR-DNP**-SO_3Na (Fig. 3A).

Effect of pH

The pH adaptability is very important for the practical applications of a new designed probe. In the current study, the pH adaptability of the probe **NIR-DNP** was also investigated (Figs. S14 and S15, ESI†). The result showed that the fluorescence intensity and the A_{375}/A_{595} ratio of **NIR-DNP** had no change in the pH range from 6.0 to 9.4, suggesting that the probe was stable and insensitive to pH changes in this range. However, upon treatment with Na_2S (50 equiv), strong fluorescence emission was observed in the pH range from 6.5 to 9.4 (Fig. S14, ESI†), indicated that the probe **NIR-DNP** could be used to recognize H_2S in physiological pH region. Additionally, upon the addition of SO_3^{2-} (50 equiv), significant enhancement of the A_{375}/A_{595} ratio were observed in the pH range of 6.75 to 8.5 (Fig. S15, ESI†), revealing that the probe **NIR-DNP** could be also used to detect SO_3^{2-} under physiological condition. The pH cross-talk of probe in the "on" state (compound **4**) was also studied. The results suggested compound **4** was a double NIR-emissive ratiometric fluorescent probe which exhibited pH-dependent optical responses (Fig. S16 and S17, ESI†), similar with the studies reported previousely.[36,37] The pK_a value was calculated to be 6.7 which was derived from the titration curve of emission ratio (I_{706}/I_{669}) (Fig. S18, ESI†).

Paper-based test strips.

To demonstrate the practical application of the probe **NIR-DNP** for the detection of SO_3^{2-}, a paper-based test strip was developed by wetting a neutral filter paper with **NIR-DNP** methanol solution (100 μM). After drying in air, a blue-purple test paper was ready for use. For the detection of SO_3^{2-}, SO_3^{2-} contained solution was dropped on the test paper using a glass capillary. As shown in Fig. 4A, a rapid color change occurred, even in the presence of other analytes. However, the mixture of other analytes without SO_3^{2-} could not cause any detectable changes. In addition, the test papers showed different color changes to different concentrations of SO_3^{2-} ranging from 10 μM to 50 mM. These results indicated that the probe **NIR-DNP** could be used to develop an easy-to-prepare and easy-to-detect paper-based test strip for a cheap and effective way to detect SO_3^{2-}.

Cell imaging

To demonstrate the potential application of the probe **NIR-DNP** in fluorescence imaging of cells, live cell imaging experiments were also performed using U-251 cells. Firstly, the cytotoxicity of the probe was evaluated by MTT assay.[38] The result (Fig. S19, ESI†) showed that more than 80% cells were viable when up to 80 μM **NIR-DNP** was used, indicated that the probe did not show significant cytotoxicity in the 0-80 μM range. As displayed in Fig. 4B, U-251 cells incubated with **NIR-DNP** (50 μM) for 60 min at 37 °C showed no intracellular fluorescence. Owing to the low level of H_2S in cells, an exogenous Na_2S was added to increase the intracellular H_2S level.[17,29] The cells were treated with **NIR-DNP** (50 μM) for 60 min, followed by Na_2S (500 μM) for another 60 min. As a result, red fluorescence was clearly observed in the cytoplasm, suggesting that **NIR-DNP** could be used for exogenous H_2S imaging in U-251 cells. To further test whether **NIR-DNP** could detect endogenous production of H_2S, cells were first treated with Cys (500 μM) for 60 min. The results of MTT assays indicated that Cys did not show obvious cytotoxicity at the experimental conditions (Fig. S20, ESI†). The cells were then treated with **NIR-DNP** (50 μM). As shown in Fig. 4B, Cys induced the production enhancement of endogenous H_2S,[39,40] and clear fluorescence was observed, implying that **NIR-DNP** could be also used for endogenous H_2S imaging in U-251 cells. Moreover, probe **NIR-DNP** can be also used for H_2S imaging in murine hepatocytes (Fig. S21, ESI†). All the results above suggested that **NIR-DNP** was cell-permeable and can react with intracellular H_2S efficiently, which can be used for H_2S imaging in cells with low cytotoxicity.

Conclusions

In this study, a dual functional probe **NIR-DNP** was synthesized by introducing 2,4-dinitrobenzene to hemicyanine skeleton. The probe **NIR-DNP** exhibited colorimetric response to SO_3^{2-} along with a color change from purple to colorless, and NIR fluorescence enhancement response to H_2S along with a color change from purple to cyan. The probe could be used to sense H_2S and SO_3^{2-} with a high selectivity, even in the presence of other biologically relevant species. The detection limit of the probe was 36.53 nM for H_2S and 33.33 nM for SO_3^{2-}. ^1H NMR and ESI-MS analysis, as well as DFT and TDFT theoretical calculation demonstrated that the sensing mechanism of the probe toward H_2S and SO_3^{2-} were respectively based on H_2S-induced thiolysis of dinitrophenyl ether and SO_3^{2-}-induced nucleophilic addition. Further investigation showed that the probe **NIR-DNP** could be used to develop an easy-to-prepare and easy-to-detect paper-based test strip for cheap and effective detection of SO_3^{2-}. Also, the probe **NIR-DNP** has the potential to image exogenous and endogenous H_2S in living cells. Based on these results, we are actively pursuing more sensitive and wide-ranging analogues for simultaneous detection of SO_3^{2-} and H_2S, as well as fluorescence imaging of H_2S in living cells, tissues and animals in our further works.

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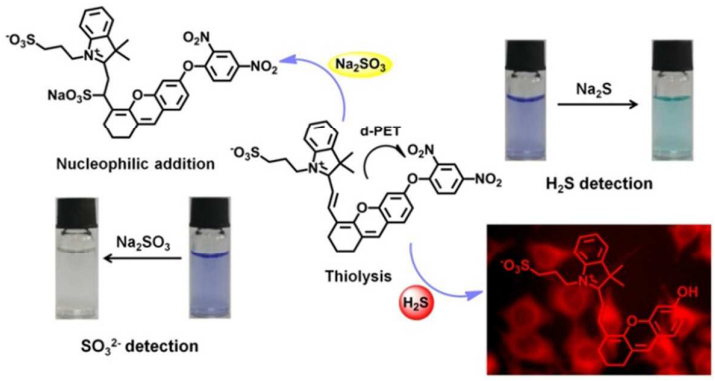
Graphic Abstract



In this study, we developed a new dual functional probe NIR-DNP for discriminative detection of H_2S and SO_3^{2-}.