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Colorimetric and fluorescent determination of sulfide and sulfite with kinetic discrimination†

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Two fluorescent probes, *m*-PSP and *p*-PSP, for sulfite and/or sulfide were constructed by connecting a pyridinium ion to a coumarin fluorophore through an α,β -unsaturated ketone. The presence of the pyridinium salt promoted the nucleophilic addition of sulfite and sulfide to the α,β -unsaturated ketone, which could be visualized by dramatic changes in the solution's color and fluorescence intensity. Both probes exhibit good selectivity (the selectivity coefficients toward major interferences are less than 0.07) and high sensitivity for sulfite and sulfide over biothiols and other potential analytes. The detection limits of *m*-PSP for the analysis of sulfite and sulfide are calculated to 8.5×10^{-7} M and 2.7×10^{-7} M, respectively. Living cell imaging results indicate that both probes can be applied in biological systems.

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Introduction

Sulfur-containing species such as sulfites, hydrogen sulfides and thiols are important in industrial and biological processes.¹ However, they play different roles in the areas of industry, environment and physiology.² Sulfites are widely used to preserve freshness and increase shelf life in many foods and beverages.³ Besides nitric oxide (NO) and carbon monoxide (CO), hydrogen sulfide has been regarded as the third gaseous messenger.⁴ Low-molecular-weight thiols are essential in maintaining the biological redox balance.⁵ Therefore, efficient methods for sensitive and selective determination of sulfur-containing species are urgently required.⁶ Several colorimetric/fluorescent probes for sulfite, sulfide, and thiols have been designed and synthesized based on their high binding affinity towards metal ions,⁷ nucleophilic reaction,⁸ reduction⁹ and the reaction with aldehydes.¹⁰ Sulfides, sulfites and thiols are well-known nucleophilic reagents and their nucleophilic reactivity decreases in the order: sulfite > sulfide > thiol.¹¹ Some of the fluorescent probes for these species are based on the Michael addition mechanism.¹² Probes for H₂S, sulfite and thiols have been constructed by conjugating an α,β -unsaturated ketone to a coumarin fluorophore.¹³ It has been found that a strong electron-withdrawing group at the β -position of an α,β -unsaturated ketone promotes the Michael addition reaction significantly,^{13d}

while a strong electron-withdrawing group at the 2-position enhances the reactivity slightly.^{13c} After carefully studying some reported fluorescent probes, we envisioned that the Michael addition mechanism could be exploited to kinetically discriminate between different sulfur-containing species by rationally designed probes.

In order to distinguish sulfites, sulfides and thiols, two novel probes *m*-PSP and *p*-PSP were designed and synthesized. Compound **m6** is the precursor of *m*-PSP and was used as a reference. Coumarin was selected as the fluorophore because of its ease of modification, photochemical stability and relatively high quantum yield.¹⁴ We designed these two fluorescent probes based on the following considerations: (1) the amino nitrogen was fused with two 6-membered rings in a julolidine structure to make both the absorption and emission wavelengths shift to longer wavelengths (compared to *m*-CP and **TSP2**); (2) electron-withdrawing groups were introduced to the probes to adjust the reaction rate. In this work, *m*- and *p*-pyridine units were incorporated instead of benzene in **TSP2** and were treated with MeI to form quaternary ammonium salts to further strengthen the electron-withdrawing property and to provide good solubility in water. We expected that the reactions between sulfur-containing species and the probes could be conducted in an aqueous solution. The reaction rates of both the probes would be much faster than those of **TSP2**, but lower than that of *m*-CP. Moreover, we envisaged that sulfite and sulfide might be distinguished at different time intervals based on their different reactivity (Scheme 1).

Experimental

Synthesis

Probes *m*-PSP and *p*-PSP were synthesized according to Scheme 2.

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† Electronic supplementary information (ESI) available: Experimental details of characterization and cell cultures; fluorescence titrations, and the details of the quantum-chemical calculations. See DOI: 10.1039/c4an01086h

Scheme 1 The chemical structures of the compounds studied.

Scheme 2 The synthetic procedures for *m*-PSP and *p*-PSP.

Synthesis of m1. 3-Methoxyaniline (0.04 mol, 4.9 g), 1-bromo-3-chloropropane (0.6 mol, 94 g), and anhydrous sodium bicarbonate (0.32 mol, 26.9 g) were added into a 500 mL three-necked round-bottomed flask equipped with an overhead mechanical stirrer and thermometer under N_2 atmosphere. The mixture was stirred at $\sim 70^\circ C$ for 1 h and then at $\sim 100^\circ C$ for 2 h, followed by reflux for 15 h. The reaction progress was traced by TLC. After cooling to room temperature, the reaction solution was filtered to obtain a solid, which was purified by column chromatography (PE-DCM = 2 : 1) to afford **m1** as a red oily solid (5.44 g, yield: 67.0%). 1H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.93 (m, 4H), 2.66 (m, 4H), 3.05 (m, 4H), 3.73 (s, 3H), 6.13 (d, 1H, $J = 8.0$ Hz), 6.72 (d, 1H, $J = 8.0$ Hz).

Synthesis of m2. **m1** (0.025 mol, 5.00 g) was dissolved in a mixed solution containing 25 mL of 47% HI, 40 mL of concentrated HCl, and 100 mL of H_2O . After refluxing for 15 h, another 25 mL of concentrated HCl was added to the solution. The solution was further refluxed for 40 h, and then cooled in an ice bath. 50% NaOH and phosphate buffer (6.9 g of $NaH_2PO_4 \cdot 2H_2O$ and 1.4 g of $Na_2HPO_4 \cdot 12H_2O$ in 100 mL of H_2O) were

employed to adjust the pH to ~ 6.0 . The product was extracted with dichloromethane, and the organic phase was washed with brine and dried over $MgSO_4$. The solvent was removed under reduced pressure to afford a brown residue, which was purified by column chromatography on silica gel (petroleum ether-ethyl acetate = 8 : 1) to yield compound **m2** as white crystals (3.87 g, 82%). 1H NMR ($CDCl_3$, 400 MHz) δ 1.97 (m, 4H), 2.67 (m, 4H), 3.09 (m, 4H), 4.44 (s, 1H), 6.06 (d, 1H, $J = 8.0$ Hz), 6.66 (d, 1H, $J = 8.0$ Hz).

Synthesis of m3. $POCl_3$ (5.0 mL) was added dropwise to freshly distilled DMF (5.0 mL) and stirred at $0-5^\circ C$ under N_2 atmosphere for 15 min. 3.8 g of **m2** (0.02 mol) in 10 mL of dry DMF was added dropwise to the $POCl_3$ -DMF solution. The solution was stirred at room temperature for 30 min, and at $60^\circ C$ for another 30 min. The mixture was slowly poured into ice water (100 mL), and aged for 2 h to afford blue-green solid. The precipitate was filtered and washed with water followed by purification using column chromatography on silica gel (petroleum ether- CH_2Cl_2 = 1 : 1) to afford **m3** as a yellow oil (3.72 g, yield: 86%). 1H NMR ($CDCl_3$, 400 MHz) 1.93 (m, 4H), 2.68 (t, 4H, $J = 6.2$ Hz), 3.28 (m, 4H), 6.84 (s, 1H), 9.37 (s, 1H), 11.8 (s, 1H).

Synthesis of m4. 10 drops of piperidine were added to the ethanol solution (50 mL) containing **m3** (3.5 g, 0.016 mol) and diethylmalonate (5.12 g, 0.032 mol). The above solution was refluxed for 24 hours. After removal of ethanol under reduced pressure, concentrated HCl (50 mL) and glacial acetic acid (50 mL) were added. After stirring at $\sim 80^\circ C$ for 6 hours, the resulting solution was cooled to room temperature and poured into ice water (250 mL). The solution's pH was adjusted to ~ 7.0 with NaOH solution (0.1 M) to obtain a yellow precipitate. The solid was washed with water and then purified by column chromatography on silica gel (petroleum ether-ethyl acetate = 3 : 1) to afford compound **m4** as an orange-yellow crystal (2.86 g, 74%). 1H NMR ($CDCl_3$, 400 MHz) δ 1.96 (m, 4H), 2.75 (t, 2H, $J = 6.5$ Hz), 2.88 (t, 2H, $J = 6.5$ Hz), 3.26 (m, 4H), 5.99 (d, 1H, $J = 9.2$ Hz), 6.84 (s, 1H), 7.46 (d, 1H, $J = 9.2$ Hz).

Preparation of m5. $POCl_3$ (5.0 mL) was added dropwise to freshly distilled DMF (5.0 mL). The solution was stirred at $0-5^\circ C$ under N_2 atmosphere for 30 min to yield a canary coloured solution. A solution of compound **m4** (2.5 g, 0.01 mol in 10 mL of DMF) was then added to the above solution. The mixed solution was first stirred at room temperature for 30 min, and at $\sim 60^\circ C$ for another 12 h. The reaction mixture was slowly added into ice water (100 mL) and aged for 2 h. NaOH solution (20%) was used to adjust the solution's pH to ~ 7.0 to yield a precipitate. The resulting solid was dried to afford compound **m5** as a crimson solid (2.18 g, 81%). 1H NMR ($CDCl_3$, 400 MHz) δ 1.91 (m, 4H), 2.69 (t, 2H, $J = 6.4$ Hz), 2.81 (t, 2H, $J = 6.4$ Hz), 3.30 (m, 4H), 6.91 (s, 1H), 8.06 (s, 1H), 10.03 (s, 1H).

Synthesis of m6 and m7. 30 drops of pyrrolidine were added to 50 mL of CH_2Cl_2 -EtOH (1 : 1, v/v) containing compound **m5** (1.0 g, 3.72 mmol) and 3-acetylpyridine (1.8 equiv.). The resulting clear red solution was stirred at room temperature for 12 h to afford reddish precipitate. The solid was purified by column chromatography on silica gel (CH_2Cl_2 -ethyl acetate = 2 : 1) to afford compound **m6** as red crystals (0.88 g, 64%). 1H

NMR (DMSO- d_6 , 400 MHz) δ (ppm): 9.28 (s, 1H), 8.78 (d, 1H, $J = 4.5$ Hz), 8.33 (d, 1H, $J = 7.9$ Hz), 8.15 (d, 1H, $J = 15.1$ Hz), 7.72 (s, 1H), 7.69 (d, 1H, $J = 15.4$ Hz), 7.44 (m, 1H), 6.93 (s, 1H), 3.34 (m, 4H), 2.90 (t, 2H, $J = 6.2$ Hz), 2.77 (t, 2H, $J = 6.2$ Hz), 1.99 (m, 4H). Compound **m7** was obtained by the same procedure with 4-acetylpyridine instead of 3-acetylpyridine (0.84 g, 61%). ^1H NMR (DMSO- d_6 , 400 MHz) δ (ppm): 8.81 (d, 2H, $J = 5.2$ Hz), 8.11 (d, 1H, $J = 15.2$ Hz), 7.83 (d, 2H, $J = 5.9$ Hz), 7.72 (s, 1H), 7.68 (d, 1H, $J = 15.2$ Hz), 6.93 (s, 1H), 3.34 (m, 4H), 2.91 (t, 2H, $J = 6.4$ Hz), 2.77 (t, 2H, $J = 6.4$ Hz), 1.97 (m, 4H).

Synthesis of *m*-PSP and *p*-PSP. Compound **m6** (200.0 mg, 0.54 mmol) and an excess amount of iodomethane were dissolved in acetonitrile (25 mL). The above solution was stirred in dark at room temperature for 8 h and then refluxed for 10 h. A dark-green precipitate was obtained by addition of cold absolute ethanol (20 mL). The precipitate was collected by filtration and recrystallization from absolute ethanol to obtain ***m*-PSP** (175 mg, 84%). ^1H NMR (DMSO- d_6 , 400 MHz) δ (ppm): 9.55 (s, 1H), 9.15 (d, 1H, $J = 6.1$ Hz), 9.03 (d, 1H, $J = 8.1$ Hz), 8.40 (s, 1H), 8.28 (m, 1H), 7.92 (d, 1H, $J = 15.3$ Hz), 7.83 (d, 1H, $J = 15.3$ Hz), 7.11 (s, 1H), 4.45 (s, 3H), 2.75 (m, 4H), 2.51 (m, 4H), 1.89 (m, 4H). ^{13}C NMR (400 MHz, DMSO- d_6) δ (ppm): 185.2, 160.4, 152.2, 148.8, 148.3, 147.5, 146.4, 144.0, 143.7, 137.0, 128.3, 127.3, 120.2, 118.8, 111.4, 108.7, 105.6, 50.2, 49.6, 48.7, 27.2, 21.0, 20.0; HR-MS m/z : 387.1702 ($M - \text{I}$) $^+$; calculated molecular weight of $\text{C}_{24}\text{H}_{23}\text{N}_2\text{O}_3^+$: 387.1703 for ($M - \text{I}$) $^+$. ***p*-PSP** was obtained by a similar procedure with **m7** instead of **m6** (180 mg, 86%). ^1H NMR (DMSO- d_6 , 400 MHz) δ (ppm): 9.18 (d, 2H, $J = 6.6$ Hz), 8.50 (d, 2H, $J = 6.7$ Hz), 8.40 (s, 1H), 7.87 (d, 1H, $J = 15.4$ Hz), 7.77 (d, 1H, $J = 15.4$ Hz), 7.11 (s, 1H), 4.42 (s, 3H), 2.75 (m, 4H), 2.51 (m, 4H), 1.89 (m, 4H). ^{13}C NMR (400 MHz, DMSO- d_6) δ (ppm): 186.8, 160.4, 152.2, 151.0, 149.0, 147.7, 147.3, 144.5, 127.4, 126.0, 120.2, 119.1, 111.3, 108.8, 105.6, 50.2, 49.7, 48.6, 27.2, 21.0, 20.0; HR-MS m/z : 387.1707 ($M - \text{I}$) $^+$; calculated molecular weight of $\text{C}_{24}\text{H}_{23}\text{N}_2\text{O}_3^+$: 387.1703 for ($M - \text{I}$) $^+$. Further experimental and computational details are given in the ESI. †

Results and discussion

The photophysical responses of *m*-PSP/*p*-PSP toward sulfite and sulfide

As designed, compounds ***m*-PSP**/***p*-PSP** (Table 1) display a strong absorption band in the visible region with a peak at 529 nm and 546 nm, respectively, which is 60–80 nm red-shifted relative to that of a *N,N*-diethyl amino coumarin compound. 13c,d About 18 nm red shift in the absorption of ***m*-PSP** relative to that of the intermediate **m6** indicates that the *N*-methylpyridinium ion is a stronger electron-withdrawing group than pyridine. The trend towards lower excitation energies going from coumarin to **m6** to ***p*-PSP** is nicely reproduced by quantum-chemical calculations (see ESI † for details). Compounds ***m*-PSP**/***p*-PSP** are virtually nonfluorescent, possibly because the strong charge-transfer character accompanied by a large structural reorganization leads to rapid nonradiative decay.

Initially, the time-dependent absorption and emission spectra of ***m*-PSP** in the presence of sulfite or sulfide were investigated in phosphate buffer (PBS; 20 mM, pH 7.4). With the

addition of sulfite, a new absorption peak at 418 nm rose dramatically and essentially reached the maximum within 10 min. The original peak at 529 nm reduced its intensity at the same time (Fig. 1a). The solution's color changed from purple to yellow (shown in the inset of Fig. 1a). The well-defined isosbestic point demonstrates a clear formation of a new compound. The fluorogenic signaling behavior of ***m*-PSP** toward sulfite was also measured. ***m*-PSP** itself is non-fluorescent because of the presence of a low-energy charge-transfer state in which an electron is transferred from the coumarin to the pyridinium unit (the weak emission at 515 nm is presumably due to an impurity containing a coumarin chromophore without the extended conjugation). Upon treatment with sodium sulfite, the addition of the reagent to the double bond induced an enhancement (about 2-fold, Fig. 1b, $\Phi_f = 0.0018$) in emission accompanied by a color change from dark-green to faint-green under illumination with a UV lamp. Sulfide induced a similar absorption spectral change of ***m*-PSP** (Fig. 1c), but it triggered much stronger fluorescence enhancement (about 40-fold, Fig. 1d, $\Phi_f = 0.024$) of ***m*-PSP** and a bright green fluorescence appeared. Importantly, the reaction of ***m*-PSP** with sulfide was much slower than that with sulfite; however, the reaction of ***m*-PSP** with Na_2SO_3 was very fast and could be completed within 10 min, whereas the reaction of ***m*-PSP** with Na_2S required more than 2 h. The kinetic curves of ***m*-PSP** with different reactants are shown in Fig. S3. † From these curves, it is clear that the reaction rate decreases in the order of sulfite > sulfide $\gg$ thiols ($t_{1/2} \approx 0.9$ min for sulfite vs. $t_{1/2} \approx 19$ min for sulfide, only about 15% of ***m*-PSP** was converted to the thiol product after 3 h). Therefore, sulfite and sulfide could be kinetically discriminated using ***m*-PSP** as the probe. The assay times of 10 min and 2 h

Fig. 1 Time-dependent absorption (a and c) and emission spectra (b and d) of ***m*-PSP** (10 μM) in the presence of 0.5 mM of sulfite (a and b, slit 10, 10 nm) and sulfide (c and d) in PBS (20 mM). The insets show the visible color (a and c) and visual fluorescence color (b and d, slit 5, 5 nm) changes of the probe with 0.5 mM of sulfite for 10 min (or sulfide for 2 h) in PBS, pH 7.4, 25 $^\circ\text{C}$, excited at the isosbestic point ($\lambda_{\text{ex}} = 450$ nm).

were used for the evaluation of the selectivity and sensitivity of **m-PSP** toward sulfite and sulfide, respectively.

The marked blue-shifts (~ 111 nm) in the absorption spectrum caused by sulfite and sulfide are in agreement with the addition of sulfite/sulfide to the electrophilic C=C double bond in **m-PSP**, leading to a shorter conjugation structure of the reaction products. The absorption band can be attributed to the coumarin chromophore. The emission, although strongly enhanced compared to that of the precursor **m-PSP**, is still quite weak. Computational evidence (ESI) indicates that low-energy charge transfer states are present in which the coumarin acts as the donor and the pyridinium ion acts as the acceptor. Thus, electron transfer is a significant decay channel for the excited coumarin chromophore in the adducts.

To gain more information of the reaction between sulfite and **m-PSP**, the reaction product of **m-PSP** treated with Na_2SO_3 was isolated (m/z 469.1429, calculated 469.1433). The partial ^1H NMR of **m-PSP** and the reaction product **m-PSP-SO₃H** are shown in Fig. 2 (Fig. S5† is the NMR titration spectrum of **m-PSP** and sodium sulfide). The resonance signals corresponding to the alkene protons H_β and H_α at 7.95 ppm and 7.84 ppm disappeared, and new peaks at 6.07, 5.32 and 4.49 ppm emerged. The addition of sulfite to the C=C resulted in the formation of a chiral center of $\text{H}_{\alpha'}$, and the two protons of the methylene group at $\text{H}_{\alpha'}$ are not equivalent. In addition, the resonance signal of H_δ (8.47 ppm) shifted to 7.78 ppm due to the shielding effect from the adjacent alkyl group.

HPLC monitoring of the reaction process (Fig. 3) confirmed the formation of a signal of major Michael addition product. Fig. 3 demonstrates that the peaks of **m-PSP** and its addition product (**m-PSP-SO₃H**) occur at about 13.1 min and 9.5 min, respectively. These peaks were already observed in the injection of the mixture of **m-PSP** and sulfite after 1 min, which verified that the Michael addition reaction between sulfite and **m-PSP** was very fast.

Fig. 2 Partial ^1H NMR spectrum of **m-PSP** and **m-PSP-SO₃H** in DMSO- d_6 .

Fig. 3 HPLC traces of **m-PSP** (A), mixture of **m-PSP** and sulfite at 1 min (B), **m-PSP-SO₃H** (C), mixture of **m-PSP** and sulfide at 1 h (D) and 12 h (E). [**m-PSP**] = 10 μM , [sulfite] = [sulfide] = 500 μM , detected using the absorption at 450 nm.

The spectral responses of **p-PSP** toward sulfite/sulfide are similar to those of **m-PSP** (Fig. S6,† the quantum yields of the addition products **p-PSP-SO₃H** and **p-PSP-SH** are 0.0016 and 0.015, respectively). However, **p-PSP** reacted with sulfite/sulfide at a faster rate, and the reactions between **p-PSP** and sulfite and sulfide were completed within 5 min and 2 h, respectively. The strong electron-withdrawing pyridinium group favors the nucleophilic addition reaction at the double bond more effectively when it is linked at the *para*-position. Therefore, the reaction rate of **p-PSP** was faster than that of **m-PSP**. Because compounds **TSP2** hardly reacted with sulfite/sulfide in PBS^{13c} (Fig. S7†), the strategy in the present work is proven to be reasonable.

The selectivity and competition of **m-PSP** toward sulfite and sulfide over various analytes in phosphate buffer solution

We next tested the selectivity of **m-PSP** for sulfide and sulfite by screening its photophysical responses to relevant analytes,

Table 1 Photophysical data of the dyes in PBS

Compound	$\lambda_{\text{abs}}/\text{nm}$	$\epsilon/\text{L mol}^{-1} \text{cm}^{-1}$	$\lambda_{\text{em}}/\text{nm}$	Φ_{f}
TSP2	465	26 300	605	0.054 ^{13c}
m6	511	52 800	625	0.007
m-PSP	529	30 100	513 ^a	0.00036 ^b
p-PSP	546	33 300	514 ^a	0.00029 ^b

^a The weak emission at 515 nm is presumably due to an impurity. ^b the quantum yields of **m-PSP** and **p-PSP** are probably not precise because of the impurity.

including F^- , Cl^- , Br^- , AcO^- , HCO_3^- , CN^- , SCN^- , NO_2^- , NO_3^- , PO_4^{3-} , SO_4^{2-} , $S_2O_3^{2-}$, S^{2-} , SO_3^{2-} , cysteine (Cys), homocysteine (Hcy) and glutathione (GSH), under the same experimental conditions. As shown in Fig. 4a, **m-PSP** was highly selective to SO_3^{2-} with a remarkable blue shift in the absorption spectrum at the assay time of 10 min. Thiols and sulfide induced minor absorption spectral changes (the selectivity coefficients towards thiols and sulfide are less than 0.03, Table 2), whereas other anions did not trigger noticeable changes in the absorption and emission spectra (Fig. S9a and S10a†). When the assay time was set at 2 h, both sulfite and sulfide caused dramatic blue shifts in the absorption spectra (Fig. S9b†), but they elicited different fluorescence changes (Fig. S10b†). Fig. 4b is the emission spectra of **m-PSP** in the presence of various species after equilibrating for 2 h. Sulfide caused a large fluorescence enhancement of **m-PSP**, whereas other species did not cause distinct changes in the emission spectrum (the selectivity coefficients toward thiols and sulfite are less than 0.07, Table 2). The above results indicate that **m-PSP** is highly specific toward sulfite and sulfide.

The competition experiments with commonly encountered anions and thiols were conducted to examine the potential of **m-PSP** as a selective probe for sulfites and sulfides. The absorption spectrum of **m-PSP** toward sulfite was not affected by other anions and thiols (Fig. 5a). Fig. 5b indicated that most of the species did not interfere with the detection of sulfide evidently, whereas the presence of sulfite greatly reduced the fluorescence intensity of **m-PSP** toward sulfide. Thus, **m-PSP** exhibits excellent selectivity and competition for sulfite/sulfide over other ions and reducing agents encountered in biological samples.

Fig. 4 The absorption (a, recorded 10 min after addition of the reagent) and emission (b, monitored 2 h after addition of the reagent) spectra of **m-PSP** (10 μ M) in the presence of 0.5 mM of various additives, including F^- , Cl^- , Br^- , AcO^- , HCO_3^- , CN^- , SCN^- , NO_2^- , NO_3^- , PO_4^{3-} , SO_4^{2-} , $S_2O_3^{2-}$, Cys, Hcy, GSH, Na_2S and Na_2SO_3 in PBS, pH 7.4, 20 mM, λ_{ex} = 450 nm.

Table 2 Selectivity coefficients of **m-PSP** toward major interferences

Interference	S^{2-}	SO_3^{2-}	Cys	Hcy	GSH
$K_{SO_3^{2-}, X^-}$ ^a	0.024	1	0.017	0.022	0.022
K_{S^{2-}, X^-} ^b	1	0.052	0.053	0.068	0.059

^a $K_{SO_3^{2-}, X^-}$ was obtained from the data of A_{418}/A_{529} . ^b K_{S^{2-}, X^-} was from the data of I_{515} .

Fig. 5 The absorption (a, tested 10 min after addition of the reagent) and emission (b, recorded 2 h after addition of the reagent) spectra of **m-PSP** (10 μ M) mixed with sulfite (a, 0.5 mM) and sulfide (b, 50 equiv.) in the presence of different additives (0.5 mM) in PBS, pH 7.4, 20 mM, λ_{ex} = 450 nm.

Determination of sulfite and sulfide in PBS

Quantitative analysis of sulfite and sulfide was investigated by UV-vis and fluorescence techniques. The fluorescence intensity change of **m-PSP** toward sulfite is relatively small; moreover, the fluorescence detection of sulfite is greatly interfered by the presence of sulfide. On the other hand, the absorption detection of sulfide is interfered by the presence of sulfite. Therefore, the ratio of absorbances at 418 nm to 529 nm (A_{418}/A_{529}) and the fluorescence intensity at 515 nm were used to quantify sulfite and sulfide, respectively. The assay time for sulfite was 10 min and that for sulfide was 2 h. The A_{418}/A_{529} ratio was linearly proportional to sulfite concentration in the range of 0–200 μ M (Fig. 6a), and the detection limit¹⁵ was determined to be 8.5×10^{-7} M, demonstrating the suitability of **m-PSP** for the quantitative measurement of sulfite. Fig. 6b illustrates that with increasing sulfide concentration, the fluorescence intensity at 515 nm increased steadily to about 10 equiv. of sulfide. From

Fig. 6 Concentration-dependent chromogenic signaling of sulfite (a, recorded 10 min after addition of the reagent), concentration-dependent fluorogenic signaling of sulfide (c, tested 2 h after addition of the reagent) by **m-PSP**, the absorbance ratio at 418 nm and 529 nm (A_{418}/A_{529}) as a function of sulfite concentration (b), and the fluorescence intensity at 515 nm vs. sulfide concentration (d), 20 mM PBS, pH 7.4, 25 $^{\circ}$ C, $[m-PSP]$ = 10 μ M, λ_{ex} = 450 nm.

Fig. 7 Bright-field (a, c and e) and fluorescence (b, d and f) images of living L929 cells incubated with 10 μM *m*-PSP. (a and b) for 30 min without any other additives, (c and d) co-incubation of 500 μM Na_2SO_3 for 30 min and (e and f) co-treated with 500 μM Na_2S for 2 h.

the concentration-dependent fluorescence intensity at 515 nm, the detection limit¹⁵ of *m*-PSP for the analysis of sulfide was estimated as 2.7×10^{-7} M.

Fluorescent imaging of sulfide and sulfite in living cells

Finally, the capacity of *m*-PSP for the fluorescent imaging of sulfite and sulfide in living cells was evaluated. L929 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C and under 5% CO_2 in a CO_2 incubator. *m*-PSP was found to be cell-permeable and can react with intracellular sulfite and sulfide. After incubation with 10 μM of *m*-PSP for 30 min, no fluorescence could be observed from the cells (Fig. 7b). Pretreatment of the cells with 500 μM sulfite, followed by the addition of *m*-PSP, resulted in bright green fluorescent images (Fig. 7d). Similar results were obtained when sulfide was used instead of sulfite (Fig. 7f). These results indicate that *m*-PSP could be an ideal probe for subcellular imaging of active sulfite and sulfide.

Conclusions

In summary, we have designed and synthesized two fluorescent turn-on probes for discrimination between sulfite and sulfide based on the mechanism of Michael addition reaction. Both the probes exhibit excellent chromogenic responses toward sulfite within 10 min; moreover, sulfide hardly interferes the detection of sulfite at this assay time (10 min). Sulfide, on the other hand,

caused significant fluorescence enhancement (about 40-fold) and dramatic color change after 2 h. Therefore, sulfites and sulfides could be distinguished by the spectral changes at different time intervals. The probes were also applied for the biological imaging of sulfite or sulfide inside living cells. Preliminary experiments indicate their potentials to probe sulfite and sulfide in biological systems.

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respectively. Hence, the nucleophile reactivity is expected to decrease in the order of sulfite > sulfide > thiol.

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