A Novel Colorimetric Fluorescent Probe for SO\textsubscript{2} and Its Application in Living Cells Imaging

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Abstract: A novel chromenylium-based fluorescent probe was exploited for sulphur dioxide (SO\textsubscript{2}) detecting. The probe displayed a remarkable fluorescence turn-on response towards SO\textsubscript{2} based on the nucleophilic addition reaction to the carbon-carbon double bond with 105 nm Stock shift. The probe was successfully applied for the quantification of SO\textsubscript{2}. The linear detection range was from 0–160 µM with the detection limit as low as 99.27 nM. It also exhibited high selectivity for SO\textsubscript{2} than other reactive species and amino acids. Furthermore, cell staining experiments indicated that the probe was cell membrane permeable and could be used for high-performance imaging of SO\textsubscript{2} in living cells. The superior properties of the probe made it highly promising for use in chemical and biological applications.

Keywords: fluorescent probe; SO\textsubscript{2}; colorimetric; living cell imaging

1. Introduction

As one of well-known air pollutants, sulphur dioxide (SO\textsubscript{2}) has been studied extensively in toxicology [1]. However, recent works show that SO\textsubscript{2} is generated endogenously and mainly from sulphur-containing amino acids through biosynthetic pathways, such as transamination by aspartate aminotransferase (AAT) [2,3]. Moreover, SO\textsubscript{2} play significant roles in physiological processes especially in the regulation of cardiovascular function in synergy with NO and the lowering of blood pressure [4,5]. The abnormal endogenous levels of SO\textsubscript{2} are believed to be linked with lung cancer, cardiovascular disease, and a number of neurological disorders [6–10]. Toxicological studies further suggested that SO\textsubscript{2} and its derivatives could change the characteristics of voltage-gated sodium channels and potassium channels in rat hippocampal neurons [11], affect thiol levels and, hence, affect redox balance in cells [12] and produce a neuronal insult [13]. These observations have led to speculation that SO\textsubscript{2} in the fourth gasotransmitter [14]. Thus, it is of great importance to develop novel analytical methods for the study of the biological and pathological roles of SO\textsubscript{2} in physiological systems [15,16].

In recent years, fluorescence probes attract a great deal of attention due to the superiorities of simple operation, good selectivity, and high sensitivity as well as noninvasive imaging of biological molecules and processes with high spatial and temporal resolution in real-time [17–20]. For SO\textsubscript{2} detection, it was mainly based on the nucleophilic addition reaction to the aldehydes, ketones, or carbon-carbon double bonds [21–37]. Some of them had been applied for detection of SO\textsubscript{2} in organelles or in tumors [24–26,36]. However, some drawbacks of those probes are difficult to avoid, such as the small Stokes shift, high detection limit, poor selectivity, remarkable interference from biothiols, or reactive oxygen species, and so
on. Therefore, it is necessary to exploit more efficient fluorescent probes for SO\(_2\) and its derivatives with larger Stokes shifts and stronger intensities, in particular, with high sensitivity and selectivity. In recent years, Yuan and Lin developed a series of chromenylium-based fluorescent probes with larger Stokes shifts, substantial quantum yields, and photostability under physiological conditions which are widely used to detection and imaging of bio-active species in vivo, such as H\(_2\)O\(_2\), HClO, biothiols, H\(_2\)S, Sec, and so on \[38–41\]. Intrigued by their findings, we designed one novel fluorescent probe for SO\(_2\) by modifying chromenylium with an electron-withdrawing group (substituted pyridine) to synthesize BPO-Py-Cl, which could be a selective probe to detect SO\(_2\) in HeLa cells based on the nucleophilic addition reaction to the carbon-carbon double bond (Scheme 1).

![Scheme 1. The detection process of BPO-Py-Cl with SO\(_2\).](image1.png)

2. Results

2.1. Synthesis and Characterization of BPO-Py-Cl

The synthesis route of the target molecule is illustrated in Scheme 2. It takes four steps to complete its composition, as follows: BPOH was synthesized according to the literature \[40,42\]. First, the Friedel-Crafts acylation reaction of 3-(diethylamino)phenol with phthalic anhydride can obtain compound 3 which could further react with cyclohexanone by a two-step cascade reaction to acquire oxonium 5. Then, a condensation reaction between 4-hydroxybenzaldehyde with compound 5 will produce BPOH. Finally, BPO-Py-Cl could be obtained by a nucleophilic substitution reaction of BPOH with the 2-chloropyridine derivative. The probe has been characterized by \(^1\)H-NMR, \(^{13}\)C-NMR, and HRMS. Detailed synthetic procedures and structure characterizations were given in the experimental section and supporting information.

![Scheme 2. Synthesis route of fluorescent probe BPO-Py-Cl.](image2.png)
2.2. Optical Response of BPO-Py-Cl toward SO$_2$

The optical property of BPO-Py-Cl (10 µM) was explored and the maxima absorption at 552 and 391 nm were observed in 10 mmol PBS (pH 7.4) buffer solution containing 20% DMSO as co-solvent (Figure 1). After interacting with 500 µM sulphite, the colour of the solution changed from purple to pale yellow under visible light and green fluorescence increased prominently under a 365 nm ultraviolet lamp. The absorption peaks of BPO-Py-Cl were blue-shifted to 493 nm and 379 nm, respectively. These results demonstrated that BPO-Py-Cl could detect SO$_2$ efficiently.

![Figure 1](image1.png)

**Figure 1.** The absorption spectra and colorimetric changes of BPO-Py-Cl (10 µM) interacting with SO$_2$ (500 µM) under visible and 365 nm ultraviolet lamps.

2.3. pH-Dependent Fluorescence Response of the Probe toward SO$_2$

The effect of pH on the fluorescent properties was examined. As shown in Figure 2, the fluorescent intensity of the BPO-Py-Cl had almost no remarkable changes in 10 mmol PBS buffer solution containing 20% DMSO when the pH increased from 4.0 to 10.0. However, upon addition of 500 µM Na$_2$SO$_3$, the fluorescent intensity at 495 nm exhibited conspicuous enhancement when pH increased from 4.0 to 6.0. When the pH continuously increased to 10.0, the fluorescent intensity decreased dramatically. Therefore, pH 6.0 was chosen as the optimal condition for BPO-Py-Cl.

![Figure 2](image2.png)

**Figure 2.** The pH dependent fluorescent changes of BPO-Py-Cl (10 µM) in PBS or after addition of SO$_2$ (500 µM). The experiment was repeated three times (±S.D.).
2.4. Quantitative Determination of SO₂

The fluorescent titrations were conducted in 10 mmol pH 6.0 PBS buffer containing 20% DMSO as a co-solution. As expected, the fluorescence intensity of BPO-Py-Cl at 495 nm increased gradually along with the increment of Na₂SO₃. To our delight, 400 µM Na₂SO₃ can lead to an intensity increase as much as 11.7 times greater (Figure 3a and Figure S1). The quantum yields increased from 0.043 to 0.673 after interacting with 400 µM Na₂SO₃. In the given concentration range from 0 to 160.0 µM, the fluorescent signal intensity was linearly related to the concentration of Na₂SO₃ (Figure 3b), with the detection limit of 99.27 nM.

![Figure 3.](image)

**Figure 3.** (a) Fluorescence titration of BPO-Py-Cl (10 µM) with SO₃²⁻ (0, 20, 40, 60, 80, 100, 150, 200, 250, 300, 350, 400, 450, 500 µM) (λₑₓ = 390 nm, slit: 2.5 nm/5 nm) in a 10 mM PBS:DMSO = 8:2 pH 6.0 buffer solution; and (b) linear fit of fluorescence intensity changes with Na₂SO₃. λₑₓ = 390 nm. Slit: 2.5 nm/5 nm.

2.5. Selectivity Experiments

Based on the excellent fluorescent properties observed with Na₂SO₃, the selectivity of the probe for SO₂ over other reactive species and amino acids were examined. As shown in Figure 4 and Figure S2, 500 µM sulphite could lead to noteworthy fluorescent spectrum changes of BPO-Py-Cl. Meanwhile, 500 µM of representative anions (F⁻, Cl⁻, Br⁻, I⁻, CO₃²⁻, AcO⁻, PO₄³⁻, SCN⁻, S₂O₃²⁻), biologically-abundant metal ions (Li⁺, Na⁺, K⁺, Mg²⁺, Ca²⁺, Cu²⁺, Zn²⁺, Ni²⁺, Fe²⁺, Fe³⁺, Pb²⁺), reactive oxygen species (NaClO, H₂O₂, TBHP), reactive nitrogen species (NO₂⁻ and NO₃⁻), other reactive sulphur species (SO₄²⁻, H₂S, Cys, Hcy, and GSH) and amino acids (Ala, Arg, Asp, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Val) hardly led to any change. Figure 5 shows the fluorescent intensity and colour changes of the BPO-Py-Cl before and after the addition of sulphite or other species under visible light and 365 nm UV lamp. Obviously, only sulphite caused a remarkable colour change under the UV lamp.

![Figure 4.](image)

**Figure 4.** Fluorescence spectrum changes of BPO-Py-Cl (10 µM) interacting with 500 µM amino acids (Arg, Met, Ser, Asp, Gly, Ala, His, Val, Lys, Leu, Glu, Pro, Ile, Phe), reactive oxygen species (H₂O₂, NaClO, TBHP), reactive nitrogen species (NO₂⁻ and NO₃⁻), other reactive sulphur species (SO₄²⁻, H₂S, Cys, Hcy, and GSH) and amino acids (Ala, Arg, Asp, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Val) in 10 mM pH 6.0 PBS buffer solution containing 20% DMSO, λₑₓ = 390 nm. Slit: 2.5 nm/5 nm.

2.6. Possible Mechanism

As reported by Lin, the carbon–carbon double bond linked with oxonium can provide the latent nucleophilic addition site for SO$_2$ [37]. From the absorption spectrum (Figure 1), it can be speculated that Na$_2$SO$_3$ broke the conjugate system of BPO-Py-Cl. To verify the nucleophilic addition reaction of sulphite to the carbon–carbon double bond, the spectrum of compound 5 was investigated in pH 6.0 PBS buffer solution containing 20% DMSO. As we can see in Figure 6 and Figure S3, compared with the BPO-Py-Cl interacting with SO$_2$, compound 5 had a similar excitation and emission wavelength, which demonstrated that both of them have similar conjugated systems. Therefore, nucleophilic addition reactions of sulphite to the carbon–carbon double bond was considered as the possible mechanism.

Figure 6. The fluorescent spectrum of compound 5 and BPO-Py-Cl interacting with SO$_2$. 
2.7. Living Cell Imaging

Encouraged by the aforementioned results, we further performed the capacity of probe for the fluorescent imaging in living cells. First, a standard CCK-8 assay in HeLa cells was investigated at different concentrations of BPO-Py-Cl. Figure 7 shows the changes of the cell survival rate with the concentration of BPO-Py-Cl increasing, and the cell survival rate was about 85% after incubation with 2 µM BPO-Py-Cl for 24 h. With 10 µM BPO-Py-Cl, it decreased to 40%.

![Figure 7. Cell viability by a standard CCK-8 assay. The experiment was repeated three times (±S.D.).](image)

Finally, the utility of BPO-Py-Cl to image SO$_2$ was next carried out for living cells (Figure 8). The HeLa cells were incubated with BPO-Py-Cl (10 µM) in culture medium for 30 min at 37 °C, and exhibited weak fluorescence in the green channel. In a control experiment, the cells were pre-treated with BPO-Py-Cl for 30 min and further incubated with Na$_2$SO$_3$ (500 µM) for another 90 min. Obvious fluorescence increases in the green channel were observed compared with BPO-Py-Cl alone. These results demonstrated that BPO-Py-Cl could be used for the detection and imaging of SO$_2$ in living cells.

![Figure 8. Cell image experiments of BPO-Py-Cl. HeLa cells were incubated with 10 µM BPO-Py-Cl for 30 min, (a–c) green channel, bright field, and the merged channel of green and bright field channels. HeLa cells were incubated with 10 µM BPO-Py-Cl for 30 min, then washed with PBS and incubated with SO$_3^{2−}$ (500 µM) for another 90 min; (d–f) are the green channel, bright field, and the merged channel of green and bright field channels.](image)
3. Materials and Methods

3.1. Materials and General Instruments

All chemicals and reagents were purchased from commercial suppliers Aladdin-Reagent (Shanghai, China), Sigma-Aldrich (St. Louis, MO, USA), and TCI (Shanghai, China) and used without any further purification if not declared. All the solvents for optical spectroscopic studies were either HPLC or spectroscopic grade. TLC analyses were performed on GF 254 silica gel. Silica gel (HG/T2354-92, 200–300 mesh) used for column chromatographic purifications were obtained from Qingdao Haiyang chemical Co., Ltd (Shandong, China). High-performance mass spectra were performed on a Bruker Daltonics Bio TOF (Karlsruhe, Germany) mass spectrometer in electrospray ionization mode. NMR spectra were recorded on a Bruker AMX-400 instrument using tetramethyl silane as the internal reference for $^1$H-NMR (400 MHz) or CDCl$_3$ as the internal standard for $^{13}$C-NMR (100 MHz). UV–VIS absorption spectra and fluorescent spectra were recorded by a UV-1900 UV–VIS spectrophotometer and F4600 spectrofluorimeter with a 10 mm quartz cuvette from Hitachi PharmaSpec (Tokyo, Japan).

3.2. Synthesis and Characterization

2-(4-(Diethylamino)-2-hydroxybenzoyl)benzoic acid (3): 16.5 g (100 mmol) 3-diethylamino phenol, 19.0 g (128 mmol) phthalic anhydride, and 70 mL toluene were added to a 250-mL round round-bottom flask which was heated to 80 °C for 10 h, 90 °C for 5 h, 100 °C for 2 h, and 110 °C for 1 h, successively under N$_2$. After cooling to RT, the precipitate was filtered off and washed with PhMe to obtain 28 g crude purple solid (compound 3) (89.5% yield) which was used for the next reaction without purification.

9-(2-Carboxyphenyl)-6-(diethylamino)-1,2,3,4-tetrahydroxanthylium perchlorate (4): concentrated H$_2$SO$_4$ (70 mL) was cooled to 0 °C. Then, 6.6 mL of cyclohexanone (63.7 mmol) and 9.5 g of compound 3 (32 mmol) were added dropwise to the cooled H$_2$SO$_4$, respectively, with vigorous stirring. The mixture was further stirred at 90 °C for 1.5 h, cooled down to room temperature, and poured onto ice (300 g). The resulting precipitate was filtered off and washed with cold water (100 mL) after 7 mL 70% perchloric acid was added to the ice mixture. The precipitate was further purified by silica gel column chromatography with DCM to DCM/MeOH (10/1, $v/v$). About 9.4 g of purple solid (compound 4) was obtained (62% yield).

(E)-9-(2-Carboxyphenyl)-6-(diethylamino)-4-(4-hydroxybenzylidene)-1,2,3,4-tetrahydroxanthylium perchlorate (BPOH): 4-hydroxybenzaldehyde (0.53 g, 4.32 mmol) and 1.72 g (3.60 mmol) of compound 5 were mixed in 40 mL AcOH. Then the mixture was heated to 90 °C overnight under N$_2$. Subsequently, the solvent was removed under reduced pressure. After that, the crude product was dissolved in 50 mL dichloromethane, washed with water (50 mL) three times, and dried by sodium sulphate. The crude product was concentrated and purified with chromatography on a silica gel column with DCM to DCM/MeOH (10/1, $v/v$) to get 1.35 g BPOH as dark purple solid (65% yield).

(E)-9-(2-Carboxyphenyl)-4-(4-((4-chloropyridin-2-yl)oxy)benzylidene)-6-(diethylamino)-1,2,3,4-tetrahydroxanthylium (BPO-Py-Cl): About 174.0 mg (0.3 mmol) of BPOH was dissolved in 10 mL of DMF. Then, 441.0 mg (3.0 mmol) of 2,4-dichloropyridine and 207 mg (1.5 mmol) of K$_2$CO$_3$ were added into the above solution. After reacting for 3–5 h at 50 °C under supervision by TLC, the solvent was removed under reduced pressure to obtain the crude product which was purified on a silica gel column chromatography with DCM to DCM/MeOH (20/1, $v/v$) to obtain an aubergine solid of 70 mg BPO-Py-Cl (33.3% yield). $^1$H-NMR (400 MHz, CDCl$_3$), 8.28 (d, 1H, $J = 5.6$ Hz), 7.99 (d, 1H, $J = 7.6$ Hz), 7.68 (t, 1H, $J = 7.6$ Hz), 7.58 (t, 1H, $J = 7.2$ Hz), 7.49 (d, 2H, $J = 8.4$ Hz), 7.41 (s, 1H), 7.27 (d, 1H, $J = 7.6$ Hz), 7.13 (d, 2H, $J = 8.4$ Hz), 6.89–6.85 (m, 2H), 6.53 (d, 1H, $J = 8.8$ Hz), 6.45 (d, 1H, $J = 2.4$ Hz), 6.39 (dd, 1H, $J = 2.8$ Hz, $J = 8.8$ Hz), 3.39 (q, 4H, $J = 6.8$ Hz), 2.86–2.82 (m, 1H), 2.69–2.65 (m, 1H), 2.11–2.07 (m, 1H), 1.75–1.63 (m, 3H), 1.20 (t, 4H, $J = 6.8$ Hz). $^{13}$C-NMR (100 MHz, CDCl$_3$), 170.1, 166.4, 152.8, 152.3, 152.2, 150.8, 149.4, 146.8, 134.5, 131.5, 131.2, 129.3, 128.6, 123.5, 120.6, 112.0, 111.5,
Na$_2$SO$_3$ was used as the source of SO$_2$. A probe stock solution of the BPO-Py-Cl (2.0 mM) was prepared in DMSO. Other stock solutions of representative anions (F$^-$, Cl$^-$, Br$^-$, I$^-$, CO$_3^{2-}$, AcO$^-$, PO$_4^{3-}$, SCN$^-$, S$_2$O$_3^{2-}$), biologically-abundant metal ions (Li$^+$, Na$^+$, K$^+$, Mg$^{2+}$, Ca$^{2+}$, Cu$^{2+}$, Zn$^{2+}$, Ni$^{2+}$, Fe$^{2+}$, Fe$^{3+}$, Pb$^{2+}$), reactive oxygen species (NaClO, H$_2$O$_2$, TBHP), reactive nitrogen species (NO$_2^-$ and NO$_3^-$), other reactive sulphur species (SO$_4^{2-}$, H$_2$S, Cys, HCy, and GSH), and amino acids (Lys, Glu, Leu, Met, Arg, His, Phe, Ser, Ile, Val, Ala, Asp, Pro,) were prepared in deionized water at a concentration of 50 mM. All the test solutions were prepared by mixing 75 µL of each species stock solution and 15 µL of the probe stock solution in 3.0 mL with PBS buffer/DMSO (8:2, v/v) solution. The absorption and emission spectra were recorded after the test solution was incubated at room temperature for 2 h.

3.4. Quantum Yields

The fluorescence quantum yield ($\Phi$) was calculated using the following formula:

$$\Phi_x = \Phi_s \times \left(\frac{A_s}{A_x}\right) \times \left(\frac{D_s}{D_x}\right) \times \left(\frac{n_s^2}{n_x^2}\right)$$

where the subscripts s and x refer to the standard material and test sample, respectively. $\Phi_s$ and $\Phi_x$ represent the fluorescence quantum yield of the standard and test sample; ns and nx represent the refractive index of the solvents used; As and Ax represent the absorption intensity at the excitation wavelength of the standard and test sample; Ds and Dx represent the integral of the fluorescence intensity of the standard and test sample. Fluorescein in 0.1 M NaOH ($\Phi_s = 0.85$) was used as the standard [43].

3.5. CCK-8 Assay for the Cell Cytotoxicity

The cytotoxicity of the probe was determined by CCK-8 assays. HeLa cells were first seeded in 96-well plates (about 7000 cells per well) and cultured overnight for 70–80% cell confluence. After the cells were incubated with BPO-Py-Cl at different concentrations (2, 4, 6, 8, 10 µM in DMSO/cell culture medium = 1:49) for twenty-four hours, 10 µL of CCK-8 mixed in 90 µL of PBS was added to each well of the 96-well assay plate for additional 1 h incubation at 37 °C. The absorbance was measured at a wavelength of 450 nm using an ELISA plate reader (Model 550, BioRad, Hercules, CA, USA). All the samples were repeated three times.

3.6. Determination of the Detection Limit

The detection limit was calculated based on the fluorescence titration [44] with the following equation:

$${\text{detection limit}} = 3\sigma_{bi} / m$$

where $\sigma_{bi}$ is the standard deviation of blank measurements, m is the slope between intensity versus sample concentration (signal-to-noise ratio of 3:1). The standard deviation of blank measurements was determined when the emission intensity of probe (10 µM) without Na$_2$SO$_3$ was measured 10 times. Then, fluorescent titrations were conducted under the present conditions. A good linear relationship between the fluorescence intensity and the concentration of Na$_2$SO$_3$ was obtained, the slope between intensity versus Na$_2$SO$_3$ concentration was m in the above-mentioned formula.
3.7. Fluorescence Imaging of SO\textsubscript{2} in Living Cells

HeLa cells were incubated in DMEM medium (which contained 10% foetal bovine serum and 1% antibiotic-antimycotic) at 37 °C in an incubator (5% CO\textsubscript{2}/95% air). Then cells were seeded in confocal dishes (4 × 10\textsuperscript{3} per well) and incubated for 24 h. Before staining, the cells were washed three times with physiological PBS and incubated with BPO-Py-Cl (10 µM) for 30 min at 37 °C. After being washed with physiological PBS three times to remove free probes, cell imaging was carried out after the cells were further incubated with Na\textsubscript{2}SO\textsubscript{3} (500 µM) for another 90 min. Meanwhile, the control experiment was performed. The cells were incubated with BPO-Py-Cl (10 µM) for 30 min and then washed with PBS three times. Then, cell imaging was performed. The confocal fluorescent images were recorded for the green channel, corresponding to the wavelength range 460–530 nm, which was monitored for excitation at 405 nm.

4. Conclusions

In summary, a novel chromenyl derivative bearing the electron-withdrawing group BPO-Py-Cl was synthesized, and the probe exhibited a remarkable turn-on fluorescence response toward SO\textsubscript{2} with a 105 nm Stock shift. It exhibited high sensitivity and selectivity with a low detection limit (99.27 nM) for SO\textsubscript{2}. In addition, the probe was cell membrane permeable and could be successfully applied to the imaging of SO\textsubscript{2} in living cells.

**Supplementary Materials:** The supplementary materials are available online.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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Sample Availability: Samples of the compounds BPOH and BPO-Py-Cl are available from the authors.