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"Turn-on" fluorescent probe for detection of H_2S and its applications in bioimaging

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ABSTRACT

A novel fluorescent probe (named YQ-1) containing disulfide-bond coumarin derivative was developed for H_2S. In response to H_2S, YQ-1 showed remarkable fluorescent emission enhancement at 462 nm. Besides, YQ-1 exhibited higher selectivity, faster response rate, low cytotoxicity and low detection limit (0.052 μM). Further, YQ-1 was used to detect the presence of H_2S level in living A549 cells, indicating YQ-1 has good membrane permeability and fluorescence properties.

1. Introduction

Hydrogen sulfide (H_2S), an important endogenously produced gaseous signaling compound, plays an essential role in cellular physiology and pathology. It is also considered the third most important gasotransmitter for regulating cardiovascular, neuronal, immune, endocrine, and gastrointestinal systems, along with nitric oxide and carbon monoxide [1–3]. Endogenous H_2S is synthesized by mammalian tissues via two pyridoxal-5′-phosphate-dependent enzymes responsible for metabolism of L-Cysteine, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), and by a third pathway that involves the combined action of 3-mercaptopyruvate sulfurtransferase (3-MST) and cysteine aminotransferase (CAT) [4–8]. Some studies show that production of endogenous H_2S and the exogenous administration of H_2S can exert effects in various pathologies [9]. In addition, endogenous H_2S as a signal molecule, it modulates blood pressure and neuronal activity, relaxes vascular smooth muscle, inhibits insulin signaling, and possess antiinflammatory and anti-apoptotic properties [10–13]. A variety of disease phenotypes have been linked to endogenous levels of H_2S, including Alzheimer's disease, Down's syndrome, diabetes, and liver cirrhosis [14–17]. On the other hand, the excess H_2S in the environment will stimulate respiratory system and excessive inhalation will make people mental confusion, cardiac arrest and even death [18]. Single, short-term and medium-term inhalation exposures to H_2S have also resulted in respiratory, olfactory, cardiovascular, neurological, hepatic, and developmental neurochemical effects and abnormal growth in developing cells [19]. Therefore, efficient and reliable measurements of H_2S concentrations in living systems would be helpful to understand human health and disease.

The fluorescence based assays with advantages of high selectivity and sensitivity, real-time imaging, non-invasive detection and high spatiotemporal resolution [20–25], in contrast with the traditional detection method of H_2S such as colorimetric assays, gas chromatography, and sulfide precipitation [26–28]. Over the past few years, the synthesis and design strategies for the development of fluorescent probes was mainly based on unique reaction mechanisms, including H_2S-mediated reductions of azides or nitro groups, nucleophilic addition of H_2S, copper sulfide precipitation reaction, thiolysis of leaving groups [29–32]. Since M. Xian's group laboratory and several other groups reported the first reaction-based fluorescent probes for H_2S detection [33–37]. The strategy was based on a distinct H_2S-mediated nucleophilic addition followed by an intramolecular cyclization to turn on the fluorescence signals [38]. This approach is particularly specific for H_2S, thus all of these works have us to design new H_2S fluorescent probe.

In this work, we report a reactive disulfide-containing probe for the detection of H_2S based on the potential double nucleophilic attack of H_2S. This coumarin derivative was prepared from 2,2′-Dithiosalicylic acid in one step using the procedure shown in Scheme 1. Theoretically, the release of fluorophore involves two stages, that is, twice nucleophilic reaction of H_2S with disulfide-bond and ester group of YQ-1, whereas other thiols such as cysteine can only undergo nucleophilic reaction once. Therefore, the fluorescent signal should be selective only for H_2S.

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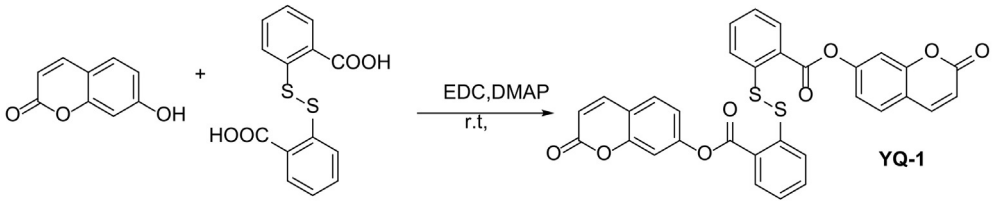


Scheme 1. The synthesis of YQ-1.

YQ-1 displayed a remarkable fluorescence "turn on" response to H_2S with low detection limit and high selectivity. Furthermore, YQ-1 was successfully applied in fluorescent imaging in living cells.

2. Materials and Methods

2.1. Materials and Instruments

All solvents and reagents for synthesis and analyses were of analytical grade and bought from Sigma-Aldrich (St. Louis, MO) without further purification. The solutions of cation were prepared from their chloride salts. A pH meter (Mettler Toledo, Switzerland) was used to determine the pH. TLC analysis was performed using precoated silica plates. HITACHI F-7000 fluorescence spectrophotometer was employed to measure fluorescence spectra. Shanghai Huamei Experiment Instrument Plants, China, provided a PO-120 quartz cuvette (10 mm). ^{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). The ability of YQ-1 reacting to H_2S in the living cells was also evaluated using Leica Dmi8 Microsystems.

2.2. Preparation and Characterization of the YQ-1

Synthesis of probe (YQ-1) is summarized in Scheme 1. 2,2′-Dithiosalicylic acid (210 mg, 1.0 mmol), 4-dimethylaminopyridine (40 mg, 0.3 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimidehydrochloride (EDC, 400 mg, 2.1 mmol) and 7-hydroxycoumarin (370 mg, 2.1 mmol) were mixed in 30 mL anhydrous dichloromethane. The mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure. The resulting residue was further purified by column chromatography on silica gel using petroleum: dichloromethane: ethyl acetate (5:1:1, v/v/v) as eluent to give the target as off-white solids (445 mg, 75%). ^{1}H NMR (DMSO d_6, 600 MHz): δ (ppm): 8.36 (d, J = 7.7 Hz, 2H), 8.13 (d, J = 9.4 Hz, 2H), 7.87 (d, J = 8.0 Hz, 2H), 7.77 (t, J = 8.9 Hz, 2H), 7.74 (d, J = 7.7 Hz, 2H), 7.56 (s, 2H), 7.51 (s, 2H), 7.41 (d, J = 8.3 Hz, 2H), 6.53 (d, J = 9.6 Hz, 2H). ^{13}C NMR (DMSO d_6, 150 MHz): δ (ppm): 163.2, 159.1, 153.5, 152.0, 143.2, 139.1, 134.0, 131.9, 128.9, 126.0, 125.2, 124.9, 118.2, 116.4, 115.2, 109.8 (Fig. S1). ESI-MS m/z: [probe + H]$^+$ Calcd. For 595.0443, Found 595.0542 (Fig. S2).

2.3. Solutions Preparation and Optical Measurements

The stock solutions 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 F^-, Cl^-, Br^-, I^-, NO_3^-, NO_2^-, AcO^-, HCO_3^-, CO_3^{2-}, SCN^-, SO_3^{2-}, SO_4^{2-}, $S_2O_3^{2-}$, ClO_4^-, PO_4^{3-}, Cys, Hcy, GSH, were prepared by direct dissolution of proper amounts of sodium salts. All other chemicals used were of analytical grade.

The optical properties of YQ-1 were measured by UV–visible absorption spectra and fluorescence emission spectra in an aqueous solution (PBS:MeCN = 7:3, pH = 7.4) using the following various analytes: F^-, Cl^-, Br^-, I^-, NO_3^-, NO_2^-, AcO^-, HCO_3^-,CO_3^{2-}, SCN^-, SO_3^{2-}, SO_4^{2-}, $S_2O_3^{2-}$, ClO_4^-, PO_4^{3-}, Cys, Hcy, GSH. And any changes of fluorescence intensity were detected using fluorescence instrument (λ_{ex} = 410 nm, slit: 5 nm/5 nm).

2.4. Cell Viability Assay

A549 cells were also used to study the cytotoxicity of YQ-1. The cell viability assay was assessed by Cell Counting Kit-8 (CCK-8), and the absorbance at 450 nm was measured to explicate the cells viability [39]. A549 cells were seeded on a 96-well microtiter to a total volume of 100 μL/well, then the cells were incubated at 37 °C in a 5% CO_2 incubator for 24 h. Different concentrations of YQ-1 (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 the plate was incubated for another 1 h. The absorbance of each well was measured at 450 nm on a microplate reader.

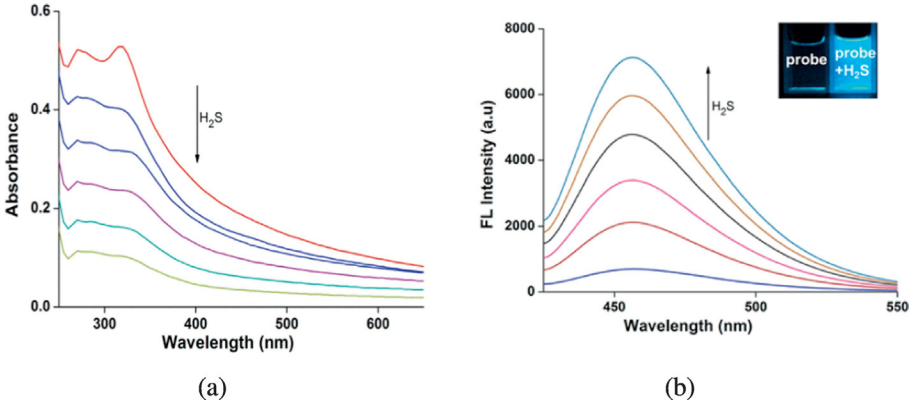


Fig. 1. UV–vis spectral change (a) and fluorescence spectral change (b) of YQ-1 (10 μM) on the addition of H_2S (0–100 μM) to the PBS: MeCN = 7:3 (v/v, pH = 7.4) solution. λ_{ex} = 410 nm, slit: 5 nm/5 nm. Inset: the color change of YQ-1 without and with addition of H_2S under UV irradiation.

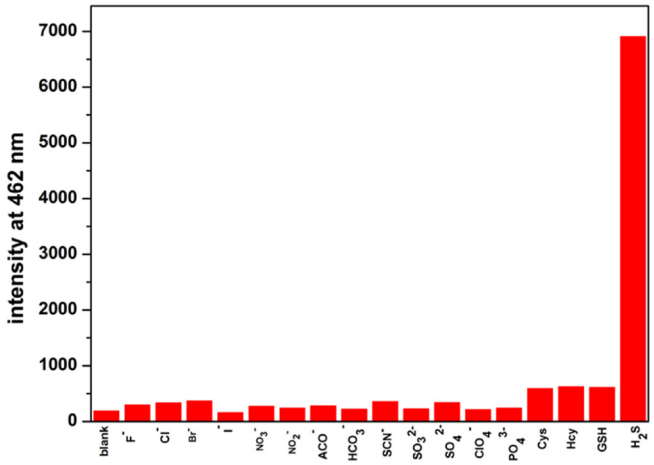


Fig. 2. The Fluorescence responses of YQ-1 (10 μM) solution in PBS: MeCN = 7:3 (10 mM, pH = 7.4, 3:7, v/v) solution with H_2S (0.1 mM) and other various relevant analytes (1 mM): F^-, Cl^-, Br^-, I^-, NO_3^-, NO_2^-, AcO^-, HCO_3^-, CO_3^{2-}, SCN^-, SO_3^{2-}, SO_4^{2-}, $S_2O_3^{2-}$, ClO_4^-, PO_4^{3-}, Cys, Hcy, GSH (λ_{ex} = 410 nm).

2.5. Cell Imaging Experiments

The A549 cells were grown in Dulbecco's Modified Eagle's medium supplemented with 12% Fetal Bovine Serum and 1% antibiotics at 37 °C in humidified environment of 5% CO_2. The cells were plated on 6-well plates and were incubated overnight. Before the experiments, cells were washed with PBS 3 times. YQ-1 dissolved in MeCN (10 μL, 10 μM) was added to the cell medium (2 mL) at 10 μM final concentration. After incubating for 30 min, excess YQ-1 was lightly washed with phosphate buffered saline (10 mM, pH = 7.4, 3:7, v/v) three times. Meanwhile, another portion of A549 cells pre-treated with YQ-1 was treated with H_2S (50 μM) and incubated for further 30 min at 37 °C. Cell imaging was then carried out after washing cells with PBS buffer three times. Besides, YQ-1 could achieve blue channel recognition and fluorescence images were recorded.

3. Results and Discussion

3.1. Optical Properties of YQ-1

The UV–vis absorbance and fluorescence emission spectra were investigated to evaluate the optical properties of YQ-1 in MeCN-PBS buffer (10 mM, pH = 7.4, 3:7, v/v). As the Fig. 1(a) shows, YQ-1 (10 μM) exhibited a major absorption band centered at 320 nm and the intensity decreased with the increasing concentration of H_2S (0–100 μM). Fig. 1(b) displayed the corresponding fluorescence spectra of YQ-1 (10 μM) upon the titration with different equivalents of H_2S in MeCN-PBS buffer (10 mM, pH = 7.4, 3:7, v/v), the fluorescence emission intensity at 462 nm gradually increased with the increase of H_2S concentration. Meanwhile, the fluorescence of the solution changed from colorless to blue under illumination with a 365 nm UV lamp.

3.2. The Selective Response of YQ-1 to H_2S

In order to evaluate the selectivity of probe to H_2S, YQ-1 (10 μM) was treated with various relevant analytes, including F^-, Cl^-, Br^-, I^-, NO_3^-, NO_2^-, AcO^-, HCO_3^-, CO_3^{2-}, SCN^-, SO_3^{2-}, SO_4^{2-}, $S_2O_3^{2-}$, ClO_4^-, PO_4^{3-}, Cys, Hcy, GSH, then their fluorescence emission was determinated by fluorescence spectrophotometer. As shown in Fig. 2, comparing with other analytes, fluorescence dramatic enhancement was observed upon the addition of H_2S (0.1 mM), even at high concentrations (1 mM), the response of YQ-1 to any of the tested thiols was very low, exhibiting at least 140- to 700-fold selectivity towards sulfide, which indicated that YQ-1 was an excellent turn-on sensor for H_2S. Furthermore, a visual fluorescence color change photograph for H_2S and other analytes under illumination with a 365 nm UV lamp was recorded in Fig. S4. These results distinctly demonstrated the outstanding selectivity of YQ-1.

3.3. pH Dependence of YQ-1

As the pH value of biological environment is often considered as an extremely influencing factor on interactions. To evaluate the effect of different pH values on the fluorescence response of probe towards H_2S, the fluorescence intensity changes of probe induced by H_2S were investigated at pH 2–9. As shown in Fig. 3, YQ-1 gave obvious fluorescence enhancement responses at pH 7–9 with the addition of H_2S, which indicated that the fluorescence probe to H_2S works fine in this pH range. In contrast, YQ-1 is no fluorescent and the fluorescence intensity is nearly unchanged in the pH range 2–6. YQ-1 did not show significant signals change at acidic pH (2–6) probably as a result of the weak nucleophilicity of H_2S under such a pH range. Accordingly, physiological pH 7.4 is selected for further research.

3.4. The Time-dependence in the Detection Process of H_2S

To further understand the reaction of YQ-1 with H_2S, time-dependence modulations in the fluorescence spectra of probe were monitored in the presence of 10 equiv. of H_2S (Fig. 4). From the fluorescence curve

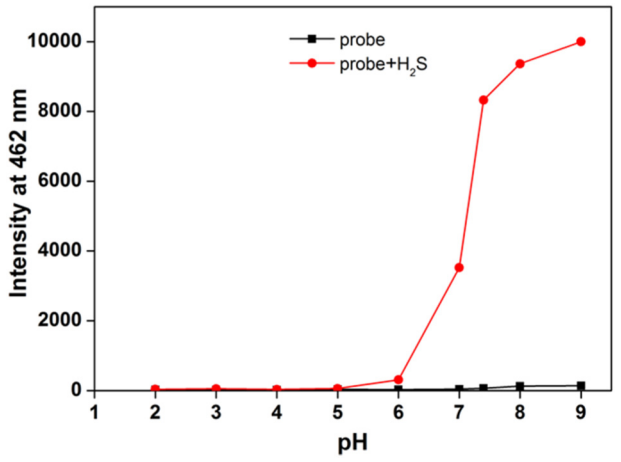


Fig. 3. Fluorescence intensity at 462 nm of probe (10 μM) at different pH values in the presence of H_2S (100 μM) and in the absence of H_2S.

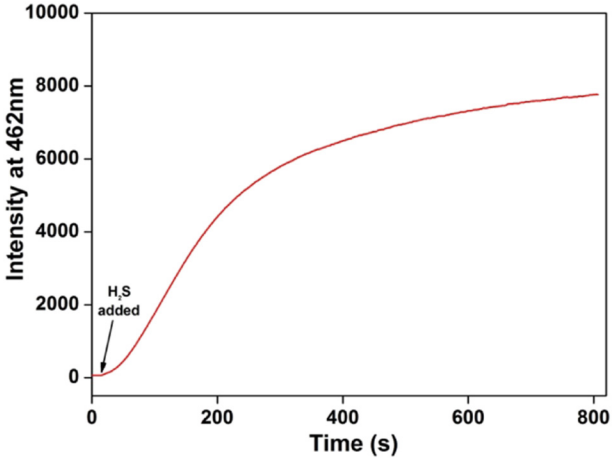


Fig. 4. Reaction time profiles of YQ-1 in the presence of H_2S in PBS: MeCN = 7:3 (10 mM, v/v, pH = 7.4) solution.

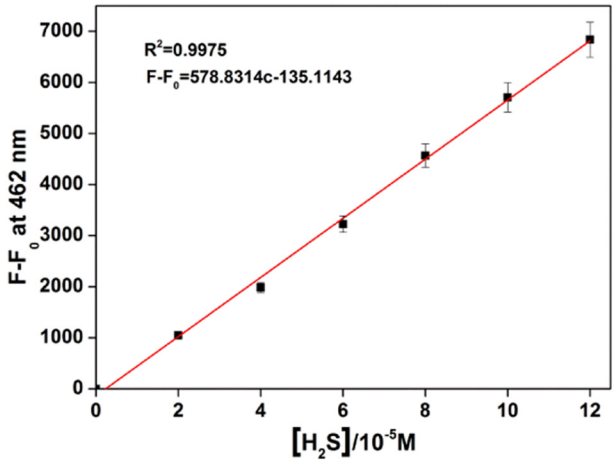


Fig. 5. The linearity of the relative fluorescence intensity versus H_2S concentration.

we can figure out that the maximal fluorescence signal was reached within 10 min, indicating the sensor can achieve real-time detection of H_2S. Therefore, 10 min reaction time was chosen for the following experiments.

3.5. The Detection Limit of YQ-1 for H_2S

The detection limit of YQ-1 for H_2S was investigated in the Fig. 5 and was found to be 0.052 μM based on the definition by IUPAC (CDL = 3Sb/m) [40–43]. The detection limit for H_2S was calculated by the equation: detection limit = 3σ/m, where σ = standard deviation of 10 blank measurements and m = slope obtained from the graph of fluorescence intensity versus concentration of H_2S. YQ-1 is able to detect low micromolar concentrations of H_2S.

3.6. Proposed Mechanism

The reaction mechanism of the present system was investigated and showed in Scheme 2. As expected, YQ-1 would specifically react with H_2S to release the fluorophore. H_2S (pK_a 6.9) as a nucleophile could react with —S—S— group of YQ-1 and generate an unstable intermediate A. If the electrophile presented at suitable position, such as the ester group shown in A, the —SH group should undergo a spontaneous cyclization to release the fluorophore 7-Hydroxycoumarin A2 and form product A1 [44]. When YQ-1 was treated with biological thiols, product B should not undergo the cyclization to release the fluorophore, thus fluorescence enhancement was not observed from the solution containing GSH, Cys, Hcy. These results clearly demonstrated the excellent selectivity of YQ-1 even when H_2S coexisted with biological thiols. We presumed that the color change and fluorescence enhancement could be attributed to the intramolecular cyclization that releases the fluorophore, which was confirmed by MS (Fig. S3), ^{1}H NMR (Fig. S1, S2). As shown in Scheme 2, the cyclization product 7-Hydroxycoumarin is the raw material for the synthetic probe and confirmed by MS (Fig. S3). The ESI-MS of the probe + NaHS, *m/z*: 163.09. Furthermore, ^{1}H NMR titrations experiment was performed with the aim of supporting our points. As shown in Fig. S5, with addition of NaHS (resolved in D_2O) to probe in DMSO d_6, the resonance of the original proton at 8.43(H_a), 8.15(H_d), 7.57(H_b), 6.55(H_c) ppm disappeared and new peaks at 8.03(H_a), 7.85(H_d), 6.56(H_b), 6.05(H_c) ppm appeared. These signal changes demonstrated how the nucleophilic addition-cyclization process occurred during the detection process of YQ-1 towards H_2S.

3.7. Cell Viability Assay

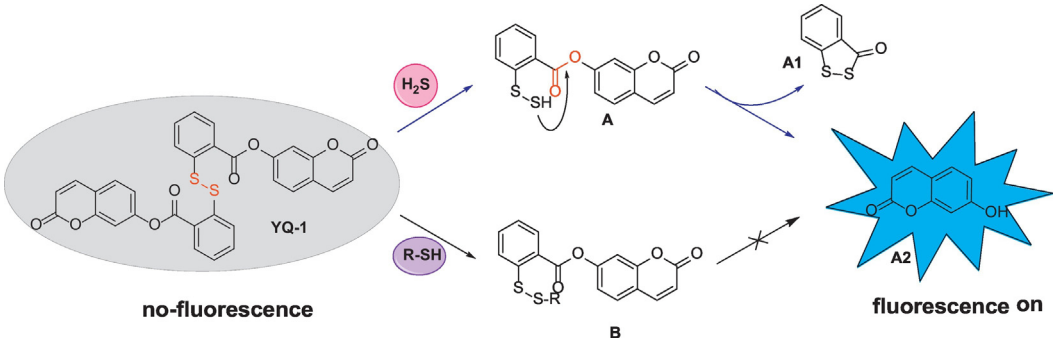
In terms of practical applications, biocompatibility is essential for bio-probes. The effect of bio-probes on the physiological behaviors of cells should be emphasized in the matter of live cell imaging applications [45]. Accordingly, cytotoxicity of YQ-1 for human A549 cells was studied by CKK-8 method (Fig. S6). Cytotoxicity experiments displayed minimal cytotoxicity of YQ-1 towards A549 cells at a concentration of 50 μM (92.3% viability). In general, at the staining concentration of YQ-1, no evident cytotoxic responses were observed for all of the tested cells after incubation for 10 h. These results showed that YQ-1 exhibited good biocompatibility and low cytotoxicity for luminescence cell imaging under the applied conditions.

3.8. Imaging of Living Cell

To evaluate the capability of YQ-1 to detect H_2S in cells, A549 cells stain with YQ-1 (10 μM) were treated with 50 μM NaHS for 30 min. As show in Fig. 6b, A549 cells only incubated with YQ-1 (10 μM) for 30 min, fluorescence could not observed in the blue channel. In a further experiment it was found that the fluorescence intensities from the blue channel clearly increased after the cells were incubated with NaHS (Fig. 6d). These cell experiments showed the good cell-membrane permeability of YQ-1, thus it could be used to detect the presence of H_2S in living cells.

4. Conclusions

In summary, a novel probe YQ-1 for highly selective and sensitive detection of H_2S based on coumarin derivatives was synthesized and characterized, which can be used as a fluorescent response probe for H_2S in PBS: MeCN (10 mM, 7:3, v/v) solution. This reaction proved to be selective for H_2S and it did not proceed with other biological thiols such as cysteine and glutathione. Furthermore, YQ-1 displayed a convenient detection process (within 10 min), a good linearity range, low cytotoxicity and a low detection limit of H_2S (0.052 μM). The fluorescence scanning microscopic experiments demonstrated that YQ-1 can be used to detect intracellular H_2S in living A549 cells.



Scheme 2. Proposed sensing process of YQ-1for H_2S.

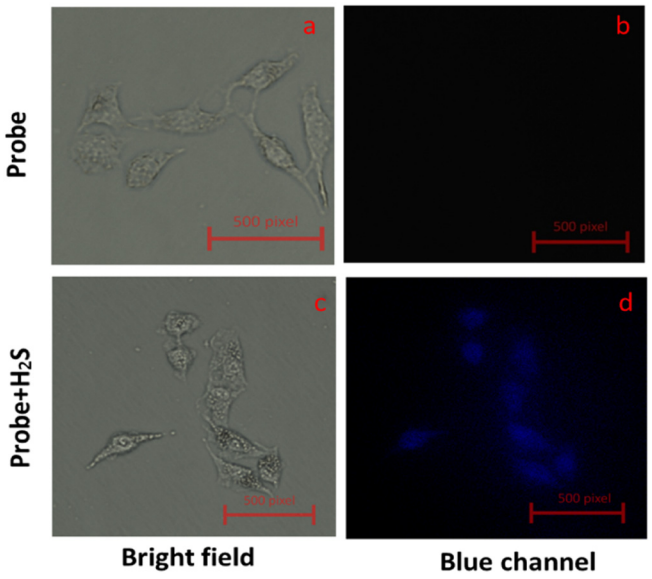


Fig. 6. Fluorescence images in A549 cells. Fluorescence image of A549 cells incubated with YQ-1 (10 μM) for 30 min (a, b); Cells pretreated with YQ-1 and then incubated with 50 μM NaHS for 30 min (c, d).

Acknowledgments

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Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.saa.2017.08.002.

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