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A simple and pH-independent and ultrasensitive fluorescent probe for the rapid detection of Hg^{2+}

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ABSTRACT

Development of fluorescent probes for Hg^{2+} has become a hot topic in modern chemical research due to its high toxicity. In this paper, we for the first time report the synthesis and application of a thioether spirocyclic rhodamine B derivative (**TR**) as an efficient fluorescent probe for Hg^{2+}. **TR** was synthesized using a simple procedure under mild condition. By employing a thioether spirocycle instead of classic spirolactam as recognition unit, our proposed probe **TR** is acidity-insensitive, and exhibits a pH-independent and ultrasensitive response to Hg^{2+}. The probe works well within a wide pH range from 3.5 to 11.5, and exhibits a 350-fold fluorescence enhancement upon 0.5 equiv of Hg^{2+} triggered, with a detection limit of 2.5 nM estimated for Hg^{2+}. In virtue of the strong thiophilic characteristic of Hg^{2+}, the response of the probe to Hg^{2+} is instantaneous and highly selective, which make it favorable for cellular Hg^{2+} imaging applications. It has been preliminarily used for highly sensitive monitoring of Hg^{2+} level in living cells with satisfying resolution, demonstrating its value of the practical applications in biological systems.

1. Introduction

It is well-known that mercury is one of the most toxic heavy metal elements, and a very small amount of mercury ions could cause serious damage to the central nervous and endocrine systems [1,2]. Therefore, efficient monitoring of trace amount of Hg^{2+} in biological samples with high spatial resolution is remarkably important for human health. Fluorescence method is highly sensitive, non sample-destructing or less cell-damaging, and can offer fast analysis with spatial resolution. These unique features make it favorable for both detection and imaging of Hg^{2+} in biological samples [3–11]. As a consequence, the design and synthesis of fluorescent probes, in particular turn-on type probes for Hg^{2+} [7–11], has become a hot topic in modern chemical research, since such probes are more suitable for bioimaging applications than those showing Hg^{2+}-induced fluorescence quenching responses.

Rhodamine dyes possess several excellent spectroscopic properties, such as large molar extinction coefficient and high fluorescence quantum yield, and have been widely applied to construct fluorescence turn-on probes for various analytes by employing different fluorescence signal transduction strategies [12–19]. Quite a few rhodamine-based probes have also been developed for fluorescence turn-on detection of Hg^{2+} in the past decade [20–31]. Some of them show high sensitivity towards Hg^{2+} with detection limit located at nM level [30,31], unfortunately, most of these probes are based on the Hg^{2+}-triggered ring-opening reaction of rhodamine spirolactam derivatives (see Fig. 1), which are acidity sensitive and result in pH-dependent response of the probes to Hg^{2+}, and are not convenient for detection of Hg^{2+} in practical diversified samples. It might also result in a poor affinity of the probes for Hg^{2+} under physiological neutral conditions. Few of probes which show pH-independent response to Hg^{2+} have also been reported [22,31], however, they suffered slight interference from other metal ions such as Ag^{+} and Zn^{2+}. Therefore, the design of pH-independent rhodamine probes with high sensitivity and selectivity for Hg^{2+} is desired if these probes are to be used in complex biological or environmental samples.

Since the ether bond is more stable in acidic condition than that of amide bond, and Hg^{2+} exhibits a strong thiophilic affinity, we envisioned that a more acidic stable and sensitive probe might be achieved if we optimized the molecular structure of the rhodamine probe by using a more simple thioether spirocycle instead of classic spirolactam as recognition unit. Herein we reported the design, synthesis and application of a novel thioether

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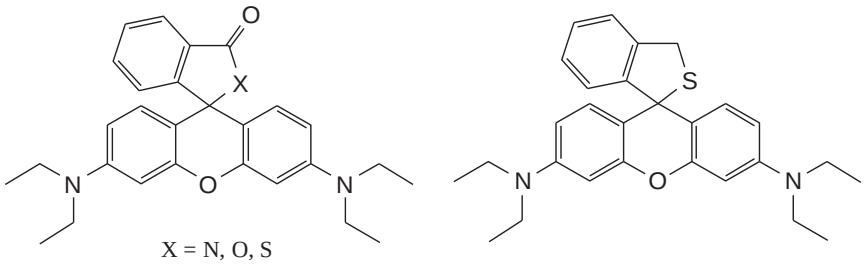


Spirolactam Rhodamine B Thioether spirocyclic Rhodamine B

Fig. 1. The structures of spirolactam rhodamine B and thioether spirocyclic rhodamine B.

spirocyclic rhodamine B derivative **TR** (see Fig. 1) as a fluorescent probe for Hg^{2+} with improved recognition performance. In our new designed probe molecule, a more simple and stable thioether spirocycle was chosen as the Hg^{2+} recognition module. It was synthesized using a simple procedure under mild condition. Such a structure-optimized molecular probe shows pH-insensitive, turn-on fluorescent response to Hg^{2+} in aqueous solution. The probe responses well to Hg^{2+} within a wide pH range from 3.5 to 11.5, exhibits a 350-fold fluorescence enhancement upon 0.5 equiv of Hg^{2+} triggered, and exhibits high sensitivity for Hg^{2+} with a response concentration range from 1.0×10^{-8} to 1.0×10^{-6} M, and a detection limit of 2.5 nM for Hg^{2+}. Owing to the strong thiophilic characteristic of Hg^{2+}, the probe also shows a high selectivity toward Hg^{2+} with a very fast response time. It has been applied for highly sensitive imaging of Hg^{2+} in living cells with satisfying results.

2. Experimental

2.1. Apparatus

Hitachi F-4500 fluorescence spectrometer was used for the determination of the fluorescence with both excitation and emission slits set at 5.0 nm. Shimadzu MultiSpec-1501 UV–visible spectrophotometer was used for the determination of UV–vis absorption spectra. ^{1}H and ^{13}C NMR spectra were obtained on a Varian INOVA-400 spectrometer operating at 400 MHz, 100 MHz respectively, with tetramethylsilane as an internal reference. The pH value of the solution was measured by the Mettler-Toledo Delta 320 pH meter.

2.2. Chemicals

2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (DDQ), PBr_3, 95% $LiAlH_4$, phenyl isothiocyanate and rhodamine B were all purchased from Shanghai Sinopharm Reagent Company. Stock solutions of Fe^{3+}, Al^{3+}, Hg^{2+}, Cu^{2+}, Mn^{2+}, K^{+}, and Ca^{2+} were prepared from their chloride salts; solutions of Zn^{2+}, Cd^{2+}, Pb^{2+}, Co^{2+}, Ni^{2+}, Ag^{+}, and Mg^{2+} were prepared from their nitrate salts with distilled water. These solutions were then diluted with HEPES buffer solution (pH 7.4) for detection. Column chromatography was conducted over silica gel (100–200 mesh) and thin layer chromatography (TLC) was carried out using silica gel 60 F254, both of which were obtained from the Qingdao Ocean Chemicals (Qingdao, China). Water used in all experiments was double distilled and purified by a Milli-Q system (Millipore, USA).

2.3. Spectrophotometric Experiments

A 20 μM stock solution of Probe **TR** was prepared by dissolving an appropriate amount of **TR** in absolute ethanol, which was protected from light and kept at 4 °C for further use. $HgCl_2$ solution was diluted stepwise with HEPES buffer solution (pH 7.4) to give the standard solution of the Hg^{2+} (8×10^{-4} M). 100 μL of **TR** and 900 μL of Hg^{2+} standard solution were combined to afford 1 mL complex solution of Hg^{2+} and **TR**, which contained 2×10^{-6} M of probe **TR** and 5.0×10^{-6}–1.0×10^{-8} M of Hg^{2+}. Blank solution of **TR** was prepared under the same conditions without Hg^{2+}. For all measurements of fluorescence spectra, excitation was fixed at 520 nm, and emission spectra were recorded within the wavelength range of 530–650 nm. Complex systems were allowed to stand for 10 min to ensure complete formation of metal–ligand complex.

2.4. Cell culture and imaging experiments

HeLa living cells for experiment were offered by Biomedical Engineering Center of Hunan University. Initially, the cells were first washed with phosphate buffered saline (PBS), incubated for 30 min with 2×10^{-6} M probe **TR** (1% DMSO, HEPES, pH 7.4) at 37 °C, washed with PBS three times to wash away the free probe, incubated with 1×10^{-6} M of Hg^{2+} (HEPES, pH 7.4) at 37 °C for 30 min, and last washed with PBS three times. The HeLa living cells were then used for fluorescence imaging experiments with an Olympus FV500-IX70 confocal laser microscope.

2.5. Synthetic details

Compound **3**, **2**, **1** were synthesized according to the literature methods [32].

2.5.1. Synthesis of compound *4*

Phenyl isothiocyanate (270 mg, 2 mmol) in DMF was added dropwise into excessive ethylenediamine at room temperature. After stirring for 6 h, the solution was diluted with CH_2Cl_2 and washed with water (50 mL × 3), then dried over anhydrous $MgSO_4$, filtered and the solvent was removed under reduced pressure to give a yellow oily crude product. At last, the crude product was recrystallized from acetonitrile to give a yellow solid (145 mg, yield: 50%). ESI-MS: $[M]^{+}=195.9$.

2.5.2. Synthesis of compound *3*

To a stirred solution of rhodamine B (5.0 g, 10.3 mmol) in absolute tetrahydrofuran (150 mL), 95% $LiAlH_4$ (0.8 g, 20.1 mmol) was added carefully under nitrogen, and the resulting mixture was then stirred for one day at room temperature. Water (50 mL) was carefully added to quench the reaction. The solution was extracted with CH_2Cl_2 and washed with water (100 mL × 3), brine, dried over anhydrous $MgSO_4$, filtered, concentrated to give crude product which was subjected to flash chromatography (silica gel, CH_2Cl_2/EtOH, 100:1, v/v) to yield pure product as a light pink foamy solid (2.2 g, yield: 44%). ESI-MS: $[M+H]^{+}=431.2$.

*2.5.3. Synthesis of compound **2***

Compound **3** (1.0 g, 2.3 mmol) and DDQ (0.52 g, 2.3 mmol) were mixed and dissolved in 100 mL of CH_2Cl_2, and stirred for 4 h at room temperature. Added an appropriate amount of CH_2Cl_2, the mixed solution was washed with water (100 mL × 3), dried, filtered over anhydrous $MgSO_4$, gave a purple crude product under reduced pressure, subjected to flash chromatography (silica gel, CH_2Cl_2/EtOH, 50:1, v/v) to give a mauve solid (0.8 g, yield: 80%). ESI-MS: $[M]^+$ = 429.3.

*2.5.4. Synthesis of compound **1***

To a stirred ice-water bath $CHCl_3$ solution (30 mL) of Compound **2** (0.6 g, 1.14 mmol), PBr_3 (0.4 g, 1.47 mmol) was added dropwise to react for 2 h. The reaction of the mixed solution was continued for 2 h at room temperature, 10% Na_2CO_3 aqueous solution was then added dropwise to remove excess PBr_3 to stop the reaction. The solution was added into 100 mL $CHCl_3$, washed with water (100 mL × 3), brine, dried over anhydrous $MgSO_4$, filtered. The solvent was removed under reduced pressure to give a purple solid, which was used directly to the next reaction without purification.

*2.5.5. Synthesis of compound **TR***

Compound **1** (300 mg, 0.57 mmol) and Compound **4** (195 mg, 1.0 mmol) were combined in absolute ethanol (50 mL) and stirred overnight at 60 °C and cooled to room temperature. The solvent was removed under reduced pressure to give crude product, subjected to flash chromatography (silica gel, petroleum ether/ethyl acetate, 10:1, v/v) and gave colorless solid (63 mg, yield: 25%, and 8.8% total yield from rhodamine B). ^{1}H NMR (400 MHz, CDCl3) δ (ppm): 1.148 (t, J=14.0 Hz, 12H), 3.317 (q, J=21.2 Hz, 8H), 4.485 (s, 2H), 6.306 (q, J=4.0 Hz, 3H), 6.334 (d, J=2.0 Hz, 1H), 6.818 (d, J=9.2 Hz, 2H), 6.450 (d, J=7.2 Hz, 1H), 7.179 (t, J=14.8 Hz, 1H), 7.248 (t, J=14.2 Hz, 1H), 7.336 (d, J=7.6 Hz, 1H). ^{13}C NMR (400 MHz, CDCl3) δ (ppm): 12.643, 29.678, 37.574, 44.303, 63.039, 97.285, 107.958, 114.694, 124.276, 126.854, 127.594, 130.440, 140.106, 147.757, 149.421, 151.206. ESI-MS: $[M+H]^+$ = 445.2.

3. Results and discussion

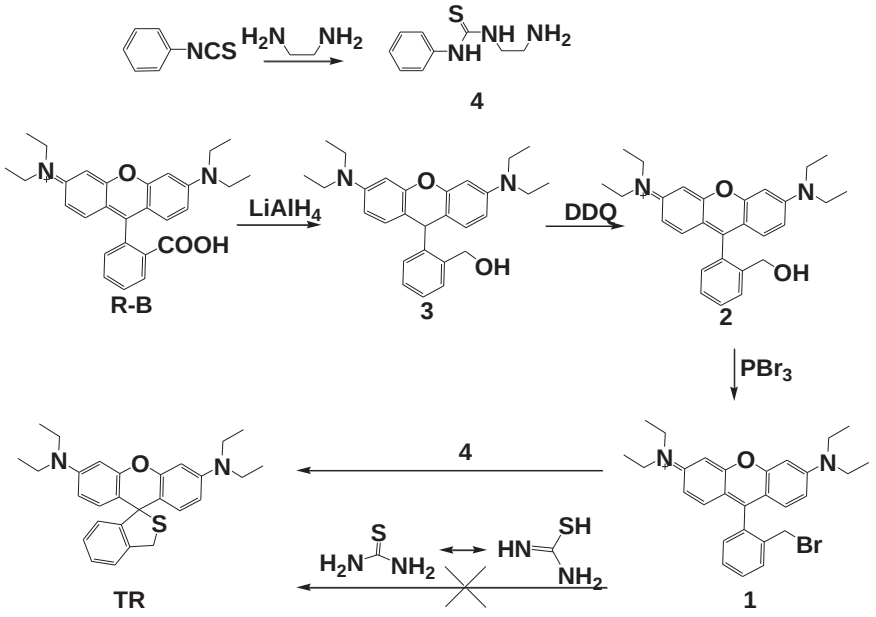
3.1. Optimized design and synthesis of TR

As discussed in the introduction section, most of previously reported rhodamine fluorescent probes for Hg^{2+} show pH-dependent response to Hg^{2+}. It is well known that the ether bond is more stable in acidic condition than that of amide bond, and Hg^{2+} exhibits a strong thiophilic affinity [22]. Therefore, to obtain a pH-independent and more sensitive rhodamine-based Hg^{2+} probe, we try to optimize the structure of the previously reported probes by using a simple thioether spirocycle instead of classic spirolactam as the Hg^{2+} recognition unit. The structure and synthetic route for the novel thioether spirocyclic rhodamine derivative **TR** are shown in Scheme 1.

To synthesize probe **TR**, benzyl bromide derivative of rhodamine B (compound **1**) was first synthesized via a three steps reaction by using rhodamine B as a starting material. Thiourea was first chosen as a thiol source, as it usually exists in two forms in solution (thiourea and isothiourea form). However, the reaction of compound **1** with thiourea (in isothiourea form) following a previously reported procedure did not afford target compound **TR** [26]. An opened-cyclic isothiourea salt of rhodamine B instead of **TR** might be produced in this reaction. N'-amino-ethylbenzene thiourea (compound **4**) was then synthesized as a thiol source to react with compound **1,** which afford target **TR** as a colorless solid in 25% yield. It was characterized using ^{1}H, ^{13}C NMR and Mass spectra, which agreed well with the proposed structure for **TR**. Similar to rhodamine spirolactam derivatives, **TR** forms nearly colorless and fluorescence inactive solutions in either organic solvent (ethanol) or aqueous solution, indicating that the spirocyclic form exists predominantly.

3.2. The effect of acidity on the response of the probe

Due to the poor acidic stability, most of previously reported rhodamine spirolactam-based probes usually show pH-dependent response to Hg^{2+}, which might result in a poor affinity of the probe to Hg^{2+} under neutral condition, and limit their applications in physiological or environmental samples. Our new designed probe employs a simple and more stable thioether spirocycle instead of



Scheme 1. The synthesis of compound TR.

classic spirolactam as the recognition unit, which should afford pH-independent response to Hg^{2+}.

To verify this hypothesis, the effects of acidity on the fluorescence response of the probe **TR** to Hg^{2+} were first investigated. The experiments were carried out at a pH range from 3.5 to 11.5, with concentration of **TR** fixed at 2 μM, and Hg^{2+} at 0.7 μM, respectively (Fig. 2). Experimental results show that for both free **TR** and **TR**+Hg^{2+}, the emission intensity almost did not vary with the pH value in a wide range from 3.5 to 11.5, suggesting that the response of our new designed probe to Hg^{2+} is pH-independent, indicating the success of our optimized design. Such a probe is convenient for practical applications in determination of Hg^{2+} in complex biological or environmental samples, as it is no need for strict control of the pH value of the sample solution for determination of Hg^{2+}.

3.3. Fluorescent sensing performance of the probe to mercury ions

To investigate the fluorescent sensing performance of the probe, the fluorescence titration of the Hg^{2+} ion was then carried out using a solution of 2 μM probe **TR** in buffered (0.01 M HEPES, pH=7.4) water/C_2H_5OH (9: 1, v/v), with results given in Fig. 3a. It can be found that free **TR** shows no characteristic fluorescence emission peak of rhodamine B, demonstrating that it mainly exists in thioether spirocyclic form. Upon the addition of Hg^{2+}, however, a characteristic fluorescence emission of rhodamine B at about 576 nm was observed for **TR**, indicating the configuration transformation of the probe molecule from the spirocyclic form to a ring-opened form. The fluorescence intensity of the probe increased with increasing concentrations of Hg^{2+} added, with the concentration of Hg^{2+} up to 0.5 equiv of **TR**, a 350-fold fluorescence enhancement was observed for the probe, and further addition of Hg^{2+} did not induce obvious increase of the fluorescence intensity of the probe. The fluorescent response of **TR** towards Hg^{2+} ion was calculated to cover a linear range from 1.0×10^{-8} to 1.0×10^{-6} M (Fig. 3b), with a detection limit of 2.5 nM for estimated by $3\sigma_b$/slope (σ_b, standard deviation of the blank samples), which is superior to that of most previously reported rhodamine spirolactam-based probes. Therefore, our proposed probe was sensitive enough to detect Hg^{2+} in environmental water samples, even in drinking water, which has a limit of 10 nM defined by the US Environmental Protection Agency. Such an improved sensitivity of our probe might be ascribed to the strong thiophilic affinity of Hg^{2+}, as well as the simple thioether

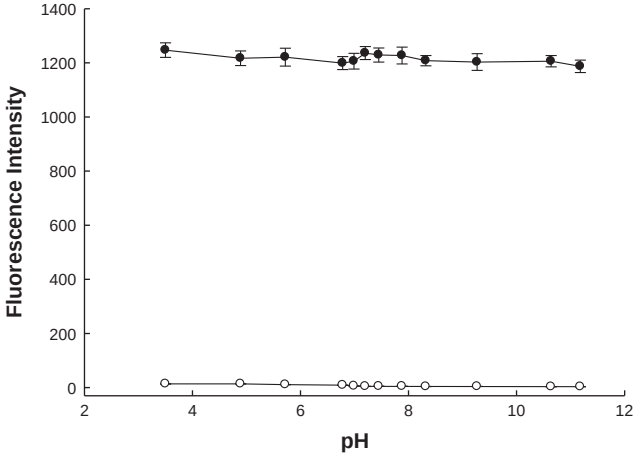


Fig. 2. Effect of acidity on the emission intensity of probe **TR** at 576 nm (2.0 μM) in the absence (dashed line); and presence of 0.7 μM of Hg^{2+} (solid line). The error bars indicated the standard deviations of three independent experiments.

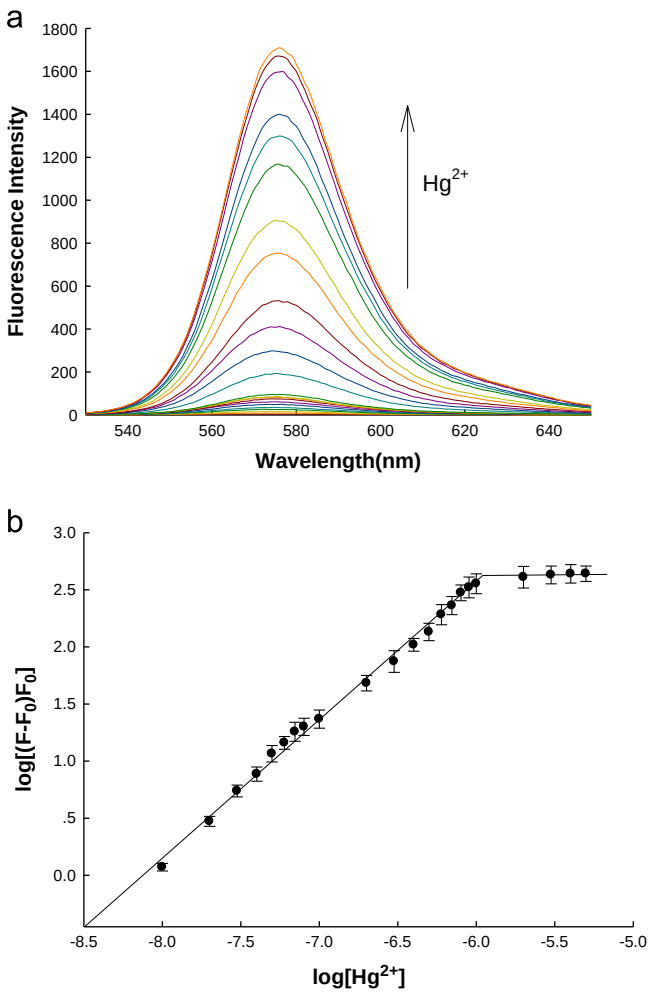


Fig. 3. (a) Fluorescence spectra of probe **TR** (2 μM) upon titration with different concentrations of Hg^{2+} (from 1×10^{-8} M to 5×10^{-6} M). (b) The calibration curve of **TR** for Hg^{2+}. The error bars indicated the standard deviations of three independent experiments. F_0: fluorescence intensity of free **TR**, F: fluorescence intensity of **TR** in the prescence of Hg^{2+}. λ_{ex}=520 nm; λ_{em}=576 nm.

spirocyclic recognition unit, which might exhibit less steric hindrance effects on Hg^{2+} than that of spirolactam recognition unit.

Since Hg^{2+} exhibits strong thiophilic affinity, our proposed thioether spirocycle-based probe TR should theoretically show a high selectivity towards Hg^{2+}. The selectivity experiments for TR were then extended to various metal ions, including Ag^{+}, Cu^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cr^{3+}, Al^{3+}, Fe^{3+}, K^{+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+}. The selectivity was first tested with excitation fixed at 520 nm and recorded the fluorescent response to abovementioned 14 competing metal ions (Fig. 4, black bar portion). The addition of 1 μM of Hg^{2+} could induce a significant fluorescence enhancement of probe, while 100 μM of other metal ions did not give obvious fluorescence increase, indicating that our proposed probe exhibits high selectivity to Hg^{2+} over other metal ions. In certain environmental samples, such as river water and sea water, the concentrations of some other contaminating metal ions, such as Mg^{2+}, Zn^{2+}, or Cu^{2+} are significantly higher than that of Hg^{2+}; selective detection of Hg^{2+} in the presence of these metal ions with high concentration is a challenge to many fluorescent probes. To test the practical applicability of our fluorescent probe for Hg^{2+}, competition experiments were also carried out. Hundred times concentration of abovementioned metal ions (100 μM) are added to 1 μM of Hg^{2+} in buffered (0.01 M HEPES, pH=7.4) water/C_2H_5OH (9: 1, v/v), and the fluorescence responses of the probe are recorded, and then compared with that of buffered solution

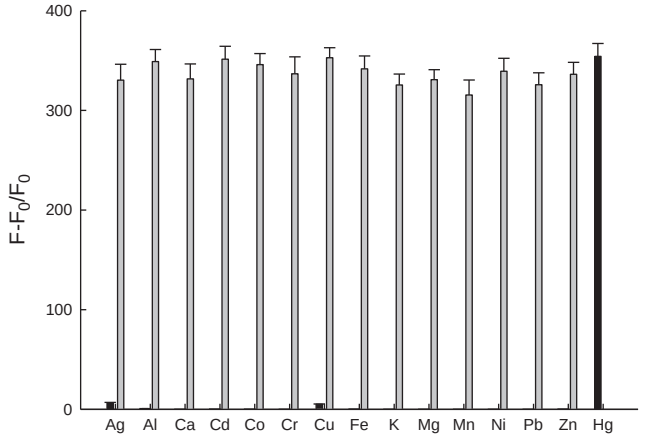


Fig. 4. The black bar portions: fluorescence response of **TR** (2 μM) to 1×10^{-6} M of Hg^{2+} or 1×10^{-4} M of other metal ions. The gray bar portions: fluorescence response of **TR** (2 μM) to mixture of 50 μM of other metal ions with 1.0 μM of Hg^{2+}. The error bars indicated the standard deviations of three independent experiments. λ_{ex}=520 nm; λ_{em}=576 nm.

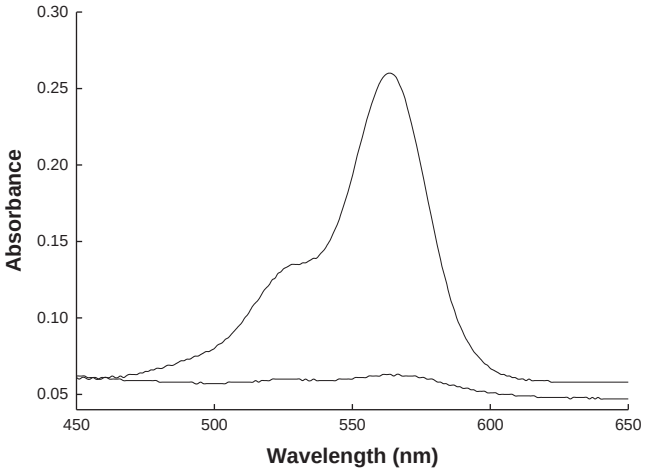


Fig. 5. A change in the absorption spectra after Hg^{2+} was added in a buffered solution (0.01 M HEPES, pH 7.4, 10% ethanol as a co-solvent).

containing only 1 μM of Hg^{2+}. As shown in Fig. 4 (the gray bar portion), the fluorescence responses of the probe to Hg^{2+} are almost unchanged before and after the addition of other interfering metal ions. All these selective results indicate that our proposed probe could meet the selective requirements for biomedical and environmental applications.

The probe also exhibits a fast response to Hg^{2+}. The reaction of 2 μM of **TR** and 2 μM of Hg^{2+} was completed within 1 min (data not shown). Moreover, an instantaneous response towards Hg^{2+} was observed with its concentration less than 1 μM, indicating that our fluorescent probe could meet the response time requirements for real-time and dynamic monitoring of Hg^{2+} in practical samples.

3.4. Response mechanism.

Similar to previously reported rodamine spirolactam-based probes, the fluorescence enhancement response of **TR** to Hg^{2+} is most likely the result of the spirocycle-opening mechanism. To verify the proposed mechanism, the change of the UV–vis spectra for **TR** upon the addition of Hg^{2+} was first investigated, with results shown in Fig. 5. It can be seen that the free **TR** shows no obvious absorption band, which demonstrated that the rhodamine conjugated system of **TR** was destroyed due to the existence of the thioether spirocycle. However, the characteristic absorption band of rhodamine B was observed at 565 nm upon the addition of Hg^{2+}. This result together with the fluorescence titration data indicated that Hg^{2+} could induce the configuration transformation of the probe molecule from the spirocyclic form to a ring-opened form, demonstrating that the sensing of Hg^{2+} with the probe is indeed through a spirocycle-opening mechanism.

Job's method for the absorbance was then applied to determine the stoichiometry of the **TR**–Hg^{2+} complex, by keeping the sum of the initial concentration of mercury ion and **TR** at 3.0×10^{-6} M, and the molar ratio of mercury ion changing from 0 to 1. The absorbance of **TR** in the absence (A_0) and presence (A) of mercury ion was determined respectively. A plot of $(A–A_0)/A_0$ versus the molar fraction of mercury ion is provided in Fig. 6a. It shows that the $(A–A_0)/A_0$ value goes through a maximum at a molar fraction of about 0.33, indicating a 1:2 stoichiometry of the Hg^{2+} to **TR** in the complex, which means that one Hg^{2+} ion could bind with two **TR** molecules through the S atom to form a stable sandwich structure. The proposed binding model of **TR** with Hg^{2+} was shown in Fig. 6b.

3.5. Intracellular imaging of Hg^{2+}

The proposed probe TR provides a pH-independent, turn-on fluorescent response to Hg^{2+} with a large signal-to-background ratio (up to 350), and high sensitivity and selectivity, which should benefit for imaging of Hg^{2+} level in biological samples with high

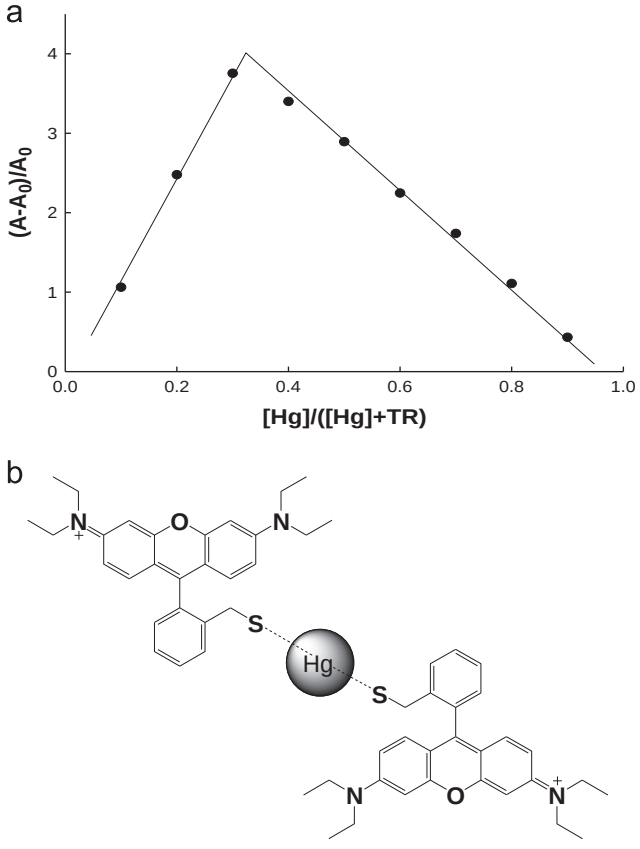


Fig. 6. (a) Job plot for determining the stoichiometry of **TR** and Hg^{2+}. The total concentration of TR and Hg^{2+} was 3.0×10^{-6} M. Molar fraction was given by $[Hg^{2+}]/([Hg^{2+}]+[TR])$. (b) Proposed binding model of **TR** with Hg^{2+}.

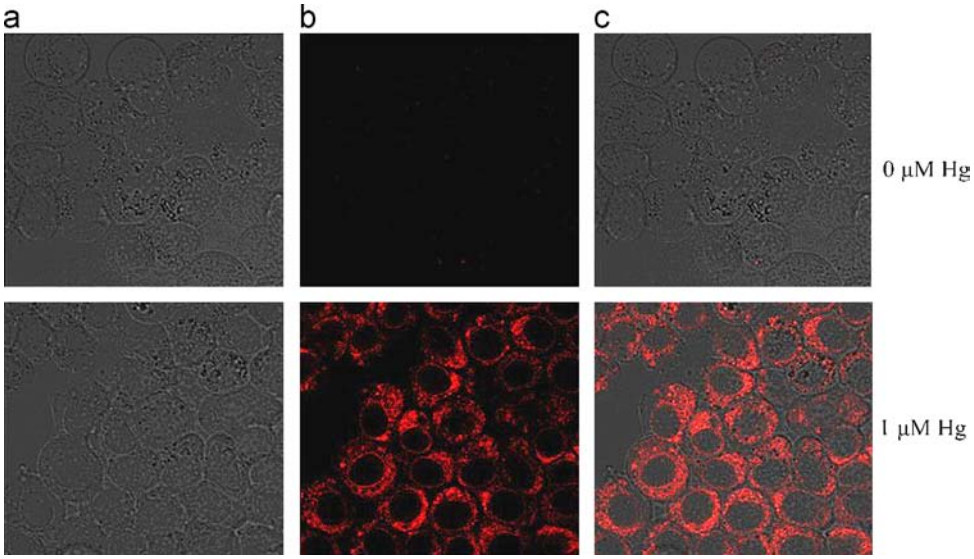


Fig. 7. Fluorescence imaging of Hela cells incubated with 2 μM **TR** and then further treated with Hg^{2+} (1 μM). (a) Bright field image; (b) fluorescence image; and (c) merged bright field/fluorescence images. The emission is centered at 590 ± 10 nm.

sensitivity. Confocal fluorescence imaging experiments were then carried out for intracellular Hg^{2+} on an Olympus FV500-IX70 laser fluorescence microscope. Fig. 7 showed the single-channel confocal fluorescence images for Hg^{2+} in living Hela cells at (590 ± 10) nm. Hela cells were first incubated with **TR** (2 μM) for 0.5 h, which showed no observable fluorescence signal in living cells, indicating free **TR** maintaining its spirocyclic form in living cells. When the Hela cells pre-incubated with **TR** were further treated with 1 μM of Hg^{2+} for 0.5 h and washed, a bright red fluorescence was then, suggesting that the new designed probe was cell permeable, and could be applied for in vitro imaging of Hg^{2+} in living cells with high sensitivity.

4. Conclusions

In summary, we have developed a new thioether spirocyclic rhodamine B based fluorescence probe **TR**. By employing a simple thioether spirocycle instead of classic spirolactam as the Hg^{2+} recognition unit, the proposed probe exhibits a pH-independent and ultrasensitive response to Hg^{2+}. It responses well to Hg^{2+} within a wide pH range from 3.5 to 11.5, and exhibits a 350-fold fluorescence enhancement upon 0.5 equiv of Hg^{2+} triggered. Moreover, its fluorescence intensity enhanced in a linear fashion with Hg^{2+} concentration cover from 1.0×10^{-8} to 1.0×10^{-6} M, with a detection limit of 2.5 nM. Most importantly, the response of the probe is fast and highly selective for Hg^{2+}, with the fluorescence changes of the probe are remarkably specific for Hg^{2+} in the presence of other metal ions (even coexisting in high concentration), which meet the response speed and selective requirements for biomedical and environmental monitoring application. The living cell imaging experiments further demonstrate its value in the practical applications in biological systems.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.09.033.

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