# A thiophen-thiooxorhodamine conjugate fluorescent probe for detecting mercury in aqueous media and living cells†

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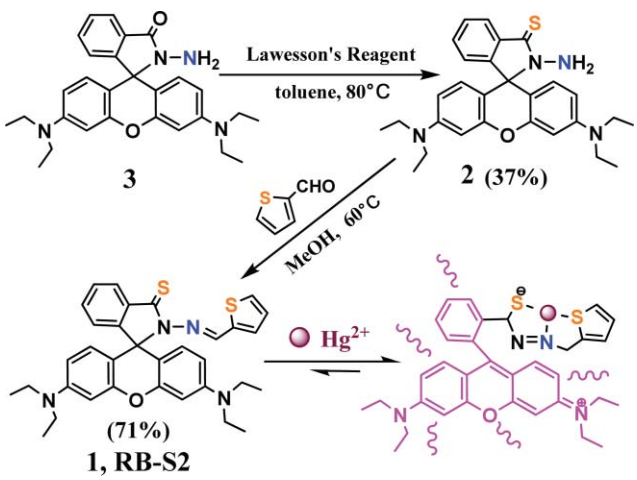
**A rhodamine-based sensor RB-S2 was designed and synthesized by combination of the thiospirolacton chromophore and the thiophen ring block with high affinity to $Hg^{2+}$. Probe RB-S2 exhibits high selectivity and excellent sensitivity in both absorbance and fluorescence detection of $Hg^{2+}$ in aqueous solution. In addition, fluorescent imaging of $Hg^{2+}$ in MCF-7 cells is also successfully demonstrated.**

The fluorescent chemosensors are powerful tools to monitor *in vitro* and/or *in vivo* biologically relevant species such as metal ions because of the simplicity and high sensitivity of fluorescence.[1] Mercury is considered as a prevalent toxic and dangerous heavy metal element because of its high affinity for thiol group in proteins and enzymes, leading to the dysfunction of cells and consequently causing many health problems in the brain, kidney, central nervous, mitosis and endocrine system.[2] Generally, $Hg^{2+}$ is known to cause fluorescence quenching of the fluorophores *via* the spin–orbit coupling effect.[3] This is reflected in the *turn-off* fluorescence response reported in most instances, and the sensors with fluorescence enhancement (*turn-on*) response are still rare.[4] Fluorescent probes show fluorescence enhancement on binding to the cation are preferred due to the lower detection limit and high-speed spatial resolution *via* microscopic imaging.[5] Therefore, development of fluorescence *turn-on* type response for monitoring the level of $Hg^{2+}$ in environmental and biological samples is necessary and indispensable.[6]

Rhodamine-based dyes are used extensively as a fluorescence labeling reagent for their excellent photophysical properties of large absorption coefficients, high fluorescence quantum yields, long absorption and emission wavelength.[7] When guests interact with the sensors, the spirocyclic form of **RhBs** is converted to the opened-cyclic form, which changes from colorless and nonfluorescent to pink and strongly fluorescent. Recently, it has been proposed that several rhodamine-based chemosensors acted as *turn-on* fluorescent sensors for HTM ion.[8] Although these reported chemosensors have demonstrated reasonable selectivity for HTM ion over other type probes, fluorescent chemosensors of better sensitivity and reactivity for $Hg^{2+}$ are still required in biological imaging applications.[9]

In the present case, our strategy for designing a multichannel molecular system is combination of the thiospirolacton chromophore and the thiophen ring block. The **RB-S2** receptor contained the thiospirolacton fragment, which is a well-known specific and reversible binding receptor of $Hg^{2+}$ due to the thiophilic nature of mercury and has been used in a fluorescein-based sensor. Therefore, we speculated that the introduction of the thiophen ring to the thiooxorhodamine-based probe based on the following considerations: (i) increase the affinity to $Hg^{2+}$ ions in competitive aqueous media; (ii) change the spatial effects within one molecule; (iii) realize the real-time detection (quickly induce the fluorescent and color responses); (iv) improve the sensitivity of $Hg^{2+}$ ions. In addition, many important sulfur enzymes and electron transfer proteins incorporate a wide range of metals,[10] which have attracted considerable attention in the synthesis and structural investigations of sulfur-containing chemosensor.

The synthesis of **RB-S2** was shown in Scheme 1. Reaction of Rhodamine B hydrazide with Lawesson's Reagent in toluene afforded the compound **2** with 37% yield. **RS-S2** was prepared by the condensation of 2- thiophenecarboxaldehyde with **2** in MeOH for 12 h (71% yield). This Schiff base was stable in EtOH–water solutions and solid state for over 3 months. The acid–base titration experiments revealed that **RB-S2**/**RB-S2**-$Hg^{2+}$ remained unaffected between pH 5.58 and 9.21 (excitation at 515 nm) in fluorescence intensity (Figure S1, ESI†), suggesting that it was insensitive to pH near 7.0 and could work in approximate physiological conditions.




**Scheme 1** Synthesis of rhodamine probe **RB-S2**.

Fig. 1 shows the fluorescence spectra ($\lambda_{ex}$ = 515 nm) of probe **RB-S2** (1 μM) in the presence of various metal ions, such as alkali metal ions ($K^+$, $Na^+$), alkali-earth metal ions ($Mg^{2+}$, $Ca^{2+}$), and heavy/transition metal ions ($Fe^{2+}$, $Mn^{2+}$, $Ni^{2+}$, $Co^{2+}$, $Cu^{2+}$, $Zn^{2+}$, $Cd^{2+}$, $Ag^+$, $Pb^{2+}$ and $Cr^{3+}$) in aqueous ethanol (HEPES, 50 mM, pH 7.0, 50 : 50, v/v). Introduction of $Hg^{2+}$ to probe **RB-S2** (1 μM)

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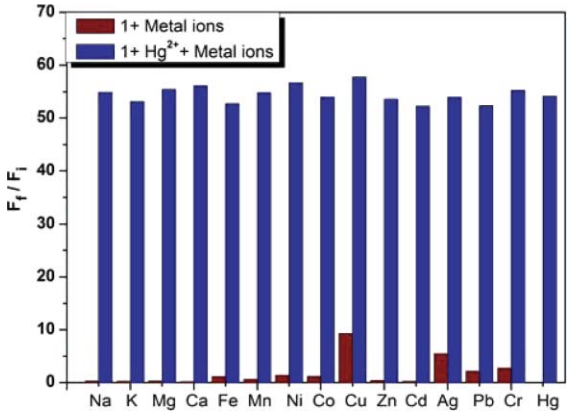


**Fig. 1** Fluorescence responses of 1 μM **RB-S2** to various 10 μM transition-metal ions (1 mM for alkali and alkali-earth metal ions). Bars represent the final ($F_f$) over the initial ($F_i$) integrated emission. Spectra were acquired in aqueous ethanol (HEPES, 50 mM, pH 7.0, 50 : 50, v/v). The red bars represent the addition of the competing metal ion to a 1 μM solution of **RB-S2**. The blue bars represent the change of the emission that occurs upon the subsequent addition of 1 μM $Hg^{2+}$ to the above solution.

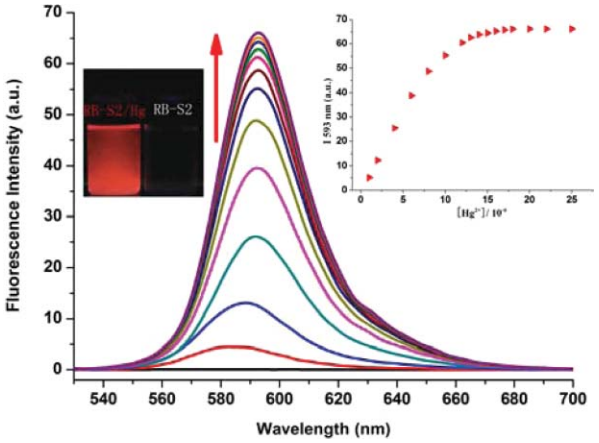


**Fig. 2** Fluorescence spectra of **RB-S2** (1 μM) in aqueous ethanol (50 mM, pH 7.0, 50 : 50, v/v) upon addition of different amounts of $Hg^{2+}$ ion. Excitation was performed at 515 nm. The photograph shows the fluorescent color of **RB-S2** (1 μM) upon addition 2.0 equiv $Hg^{2+}$ in solution. Inset: fluorescence enhancement at 593 nm as a function of $Hg^{2+}$ concentration.

elicited a large fluorescence enhancement. By contrast, alkali and alkali-earth metal ions even at the mM level had almost no influence. In addition, probe **RB-S2** only gave a minimal response to transition-metal ions such as $Fe^{2+}$, $Mn^{2+}$, $Ni^{2+}$, $Co^{2+}$, $Cu^{2+}$, $Zn^{2+}$, $Cd^{2+}$, $Ag^{+}$, $Pb^{2+}$ and $Cr^{3+}$ in neutral conditions. The competitive experiments were conducted in the presence of 1.0 equiv of $Hg^{2+}$ mixed with 10.0 equiv of various cations. No significant variation in fluorescence intensity was found by comparison with $Hg^{2+}$ ions added in **RB-S2** solution. Furthermore, the visual response of probe **RB-S2** to various species demonstrates that the probe can be employed conveniently for $Hg^{2+}$ detection by simple visual inspection (Figure S5, ESI†). These findings indicated that **RB-S2** was a $Hg^{2+}$-specific fluorescent sensor. In addition, the $Na_2S$-adding experiments were conducted to examine the reversibility of the **RB-S2**/$Hg^{2+}$ species (Figure S4, ESI†). The addition of 0.2 ml $Na_2S$ could restore the initial value of free probes due to the $K_d$ $10^{-50}$ $M^2$ value of $[HgS_2]^{2-}$, the color changed from pink to colorless and the fluorescence was turned off. Thus, the probes could be revived by addition of $Hg^{2+}$.

Fluorescence titrations of **RB-S2** with $Hg^{2+}$ in aqueous ethanol (HEPES, 50 mM, pH = 7.0, 50 : 50, v/v) were performed then. As shown in Fig. 2, free probe **RB-S2** showed almost no fluorescence due to the spirocyclic structure. However, the treatment with $Hg^{2+}$ induced a dramatic change at 593 nm in the fluorescence spectra (442-fold) which was much higher than the result obtained with the early reported rhodamine-based $Hg^{2+}$ sensors (<200 times).[11] The recognition interaction was completed immediately after the addition of $Hg^{2+}$ within 1 min, compared to its analogues (which needed equilibrium time before detection).[12] Therefore, **RB-S2** could be used in real-time determination of $Hg^{2+}$ in environmental and biological conditions. The U.S. EPA (Environmental Protection Agency) standard for the maximum allowable level of inorganic Hg in drinking water is no more than 2 ppb.[13] The $Hg^{2+}$ interaction with **RB-S2** at the low concentration region ($0.5 \times 10^{-8}$ M ~ $3.14 \times 10^{-8}$ M) showed nearly linear relation (Figure S3, ESI†). The $I/I_o$ was proportional to the amount of $Hg^{2+}$ added in ppb level with a detection limit of $1.72 \times 10^{-9}$ M. The association constant ($K$a) of **RB-S2** with $Hg^{2+}$ is $1.86 \times 10^6$ $M^{-1}$ obtained by a nonlinear curve fitting of the fluorescence titration results.[14]

The absorption spectra (Fig. 3) of **RB-S2** in 50% EtOH solution exhibited a very weak band above 500 nm, which was indicated that the spirolactam **RhB** form was the predominant species. Upon the addition of 1.5 equiv $Hg^{2+}$ ions, a new strong absorption band centered at 567 nm was formed (217-fold) and led to the color change from colorless to pink, suggesting the formation of the spirolactam ring-opened **RB-S2**. Other metal ions had little interference. Only $Ag^{+}$ ions and $Cu^{2+}$ ions displayed a 15-fold and 39-fold enhancement at the same concentration (Figure S6, ESI†). Stoichiometry for the complexes formed for $Hg^{2+}$ ions was evaluated on the basis of the Job's plot and was found to be 1 : 1. The corroborative evidence for the 1 : 1 Stoichiometry was confirmed by ESI-MS (Figure S7, ESI†).

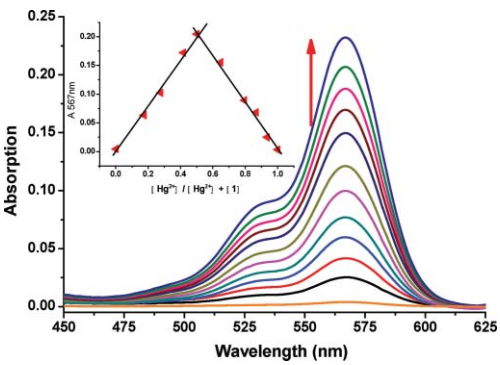


**Fig. 3** Absorption spectra of 5 μM **RB-S2** in aqueous ethanol (50 mM, pH 7.0, 50 : 50, v/v) upon addition of different amounts of $Hg^{2+}$ ions. Inset: the Job's plot; the total concentration of ([$Hg^{2+}$] + [RB-S2]) was 10 μM.

Without $Hg^{2+}$ ions, the peak $m/z$ 567.14 corresponds to **[RB-S2+H]**$^+$. When 2.0 equiv of $Hg^{2+}$ was introduced to the **RB-S2** ethanol solution, a new peak appeared at $m/z$ 867.1 and was assigned to single-charged complex **[RB-S2+$Hg^{2+}$+$ClO_4^-$]**$^+$ (calculated value 867.1).

 

We then tested the ability of **RB-S2** to track $Hg^{2+}$ levels in living cells using a model for respiratory $Hg^{2+}$ exposure (Fig. 3). The MCF-7 cells (human breast carcinoma cell line) were incubated with **RB-S2** (5μM) of $Hg^{2+}$-AM for up to 0.5 h at 37 °C showed very weak negligible intracellular fluorescence (Fig. 4b). However, the cells loaded with **RB-S2** (5 μM) displayed a significant red fluorescence from the intracellular area (Fig. 4c). Bright-field measurements confirmed that the cells after treatment with $Hg^{2+}$ and **RB-S2** were viable throughout the imaging experiments (Fig. 4a). The overlay of fluorescence and bright-field images reveals that the fluorescence signals are localized in the perinuclear area of the cytosol (Fig. 4f). Furthermore, the fluorescence microscopy images of MCF-7 cells contained with **RB-S2**/$Hg^{2+}$ and Hoechst 33342 (Fig. 4d), a well-known fluorescent probe for the cell nucleus,[15] merged well with the $Hg^{2+}$ exposure image of perinuclear area (Fig. 4g). This result not only indicated that **RB-S2** had a good cell-membrane permeability, but also revealed that the $Hg^{2+}$ predominantly existed in the perinuclear area.

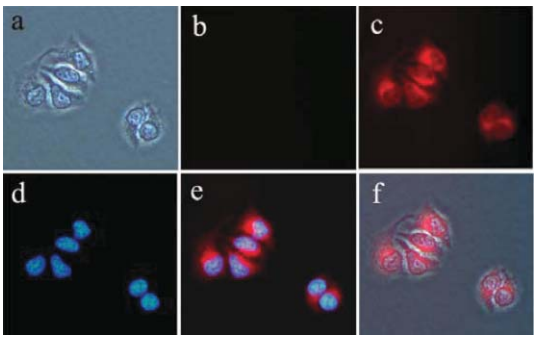


**Fig. 4** (a) Brightfield image of cells shown in panel. (b) Cells incubated with **RB-S2** (5 μM) in ethanol/PBS (1 : 49, v/v) buffer for 30 min. (c) and then further incubated with 5 μM RB-S2 for 30 min. (d) cell nucleus labeled with Hoechst 33342 (1 μM). (e) The overlay image of (c) and (d). (f) The overlay image of (a) and (c).

In recent years, there are many reports about $Hg^{2+}$-induced cell and against $Hg^{2+}$-induced cell death applied to cultured cells.[16] We employed MTT assay to investigate cytotoxicity of $Hg^{2+}$ and **RB-S2** to the MCF-7 cell lines (Fig. 5). The cell viability declined by 79.4% upon $Hg^{2+}$ (25 μM) treatment (24 h), while the viability of the $Hg^{2+}$-poisoned cell increased by 11.7% with the addition of **RB-S2**. At the lower concentration (0.2~2 μM), the addition of **RB-S2** did not change the cell viability exposure to $Hg^{2+}$-poisoned cells. These cytotoxicity tests suggest that RB-S2 may be a good candidate to reduce the cytotoxicity of $Hg^{2+}$ due to sulfur atom compatible with the biological system.

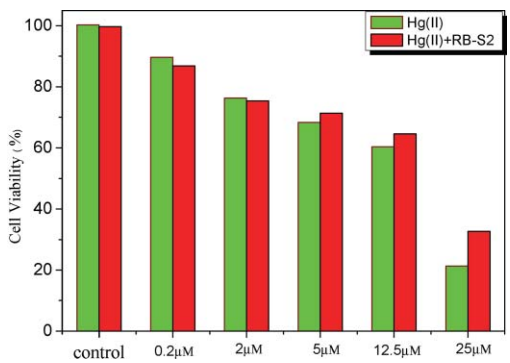


**Fig. 5** Cell viability was quantified by the MTT assay (MCF-7, 24 h).

In summary, we have developed a thiophen-thiooxorhodamine conjugate chemosensor for $Hg^{2+}$, which is a dual-responsive colorimetric and fluorescent $Hg^{2+}$-specific sensor in aqueous solution with high selectivity and excellent sensitivity. The significant changes in the fluorescence color could be used for naked-eye detection. Moreover, fluorescence imaging shows **RB-S2** can be used for low cytotoxicity detecting changes in $Hg^{2+}$ levels within living cells. The molecular design presented here may contribute to the development of more efficient and more useful chemosensors based on the rhodamine platform.

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