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PAPER

Rhodamine-based highly sensitive colorimetric off-on fluorescent chemosensor for Hg^{2+} in aqueous solution and for live cell imaging†

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A novel rhodamine-based highly sensitive and selective colorimetric off-on fluorescent chemosensor for Hg^{2+} ions is designed and prepared by using the well-known thiospirolactam rhodamine chromophore and furfural hydrazone as signal-reporting groups. The photophysical characterization and Hg^{2+}-binding properties of sensor **RS1** in neutral *N*, *N*-dimethylformamide (DMF) aqueous solution are also investigated. The signal change of the chemosensor is based on a specific metal ion induced reversible ring-opening mechanism of the rhodamine spirolactam. The response of the chemosensor for Hg^{2+} ions is instantaneous and reversible. And it successfully exhibits a remarkably "turn on" response toward Hg^{2+} over other metal ions (even those that exist in high concentration). Moreover, this sensor is applied for *in vivo* imaging in Rat Schwann cells to confirm that **RS1** can be used as a fluorescent probe for monitoring Hg^{2+} in living cells with satisfying results, which further demonstrates its value of practical applications in environmental and biological systems.

Introduction

The design and development of detection techniques based on optical-sensing with high selectivity and sensitivity for chemical and biochemical agents is a promising field.[1] An important area within this field is the design of colorimetric and fluorescent chemosensors for heavy-metal ions, such as Hg^{2+} ions. Mercury pollution pervades the globe and remains a danger to human health and the environment because both elemental mercury and ionic mercury can be converted into methyl mercury by bacteria in the environment, which subsequently bioaccumulates through the food chain. Moreover, the extreme toxicity of mercury and its derivatives results from its high affinity for thiol groups in proteins and enzymes, leading to the dysfunction of cells and consequently causing health problems.[2] However, up to date, monitoring heavy-metal ions (such as Hg^{2+} ions) in waste water is mainly based on atomic absorption/emission spectroscopy and inductively coupled plasma-mass spectroscopy (ICP-MS), but the wide utilization of these methods is largely limited due to the expensive instruments and sophisticated analysis program and sample preparation.[3] Therefore, there is an urgent need to develop an innovatory and convenient sensor that is capable of detecting the presence of heavy-metal ions (such as Hg^{2+} ions) both in environmental waste samples and in living cells.

A promising way is to develop optical chemosensors for detecting Hg^{2+} ions, which are based on an indicator that is capable of reporting on the selectivity recognition of Hg^{2+} ions through a variety of optical responses, mainly due to their distinct advantages in sensitivity, selectivity and on-line imaging. Among numerous indicators, rhodamine-based dyes are a kind of excellent candidate for the construction of an off/on-type fluorescent chemosensor due to their excellent spectroscopic properties of large molar extinction coefficients, high fluorescence quantum yields, and long absorption and emission wavelength elongated to the visible region.[4] By virtue of these fascinating properties, excellent examples of rhodamine-based turn-on fluorescent Hg^{2+} sensors have been reported.[5,6] The metal ion sensing mechanism of these sensors is based on the change in structure between the spirocyclic and open-cycle forms. Typically, these sensor molecules prefer their spirolactam ring-closed state, which shows little absorption or fluorescence, whereas ring-opening of the corresponding spirolactam induced by metal ions gives rise to orange fluorescence and a clear color change from colorless to pink. Therefore, most of the reported rhodamine-based chemosensors for Hg^{2+} ions are of colorigenic or fluorogenic type. However, these sensors show insufficient fluorescence intensity enhancement and weak color development to Hg^{2+} ions. Enlightened by the facts that Hg^{2+} is a sulfurphilic ion and that the thiol group possesses strong nucleophilicity, a sulfur-based functional group should be considered and introduced.[6i,j] Bearing those in mind, if the lactone

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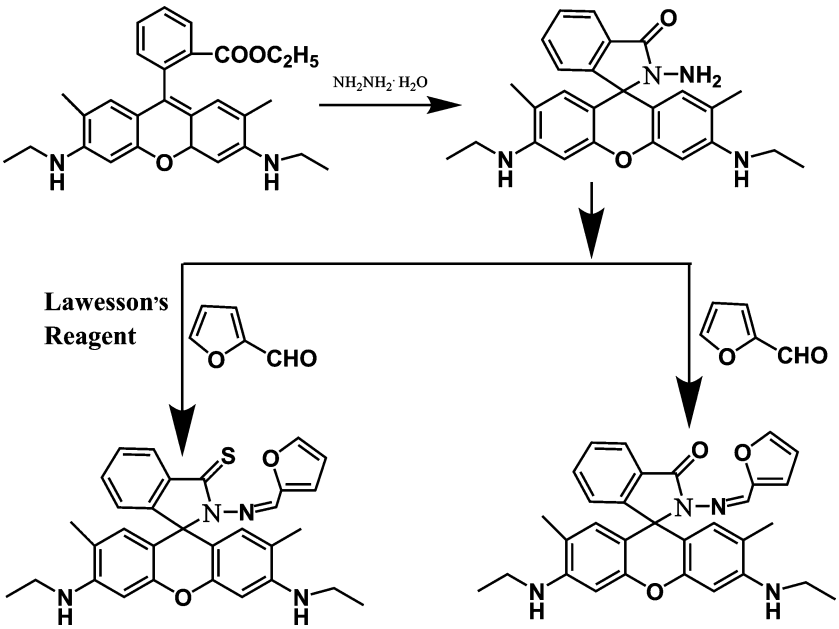


Scheme 1 Synthetic route of sensors **RS1** and **RO1**.

O atom in the rhodamine base is replaced by an S atom, it would be possible to achieve a rhodamine spirolactam based chemosensor effective towards Hg^{2+} sensing *via* color/fluorescence changes.

Herein, we report the systematic investigations of rhodamine based chemosensors **RO1** and **RS1** with different coordination ability and sensing behavior that combine a furaldehyde and a rhodamine chromophore or a thiospirolactam rhodamine chromophore (Scheme 1). Due to the introduction of the thiol atom, the chemosensor **RS1** exhibited prominent absorption and fluorescence enhancements to Hg^{2+} ions with a particular selectivity and excellent sensitivity and could be used for naked-eye detection. Interestingly, this sensor showed a reversible fluorescence response for Hg^{2+} ions and high tolerance to pH, existing in a spirocyclic form within a pH range of 3–9. Furthermore, the sensor could be applied for *in vivo* imaging of Hg^{2+} ions in living cells with satisfying resolution.

Results and discussion

As it is well-known, Hg^{2+} is a soft heavy metal ion, so the replacement of the O atom by a S atom has the potential to enhance the sensitivity of the resulting sensors towards Hg^{2+} ions. In this regard, we designed and synthesized the sensors **RO1** and **RS1** (Scheme 1). Unfortunately Zeng and co-workers have reported the sensor **RO1** in the course of our preparation of this manuscript.[7] The sensor **RS1** was facilely synthesized from rhodamine 6G through a three-step reaction, as summarized in Scheme 1. Its structure was confirmed by ^{1}H NMR, ^{13}C NMR and MS data.

The sensor **RO1** was reported by Zeng *et al.* to show a remarkable Cu^{2+}-induced fluorescence enhancement and color change. And the sensor shows neither the color nor the fluorescence characteristics of rhodamine with the addition of Hg^{2+} ions at low concentration (40 μM). However, in certain environmental samples, some contaminating metal ions (such as Hg^{2+} ions) are more abundant, so selective detection of Cu^{2+} ions in the presence of these metal ions with high concentration is a challenge to the application of sensor **RO1**. Interestingly, after the introduction of the thiol atom, the resulting chemosensor **RS1** exhibits prominent absorption (red color) and strong fluorescence (orange color) to Hg^{2+} ions (Fig. 1). Moreover, the chemosensor **RS1** exhibits high sensitivity and selectivity for Hg^{2+} ions over other commonly coexistent metal ions.

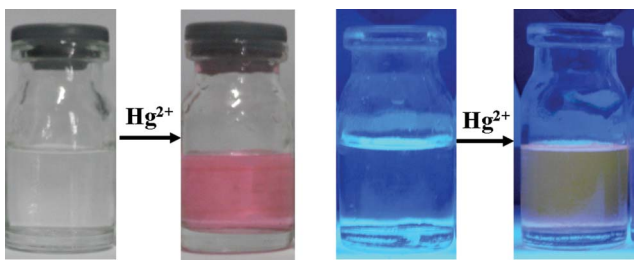


Fig. 1 Change in color (left) and fluorescence (right) of **RS1** (10 μM) in buffered (NaAc-HAc, pH 7.0) water–DMF (1/1, v/v) upon addition of 0.5 mM Hg^{2+} ions.

Absorption spectra were recorded upon the gradual addition of Hg^{2+} ions into buffered (NaAc-HAc, pH 7.0) water–DMF (1/1, v/v). Fig. 2 shows the result of absorption titration of **RS1** with Hg^{2+} ions. When no Hg^{2+} ions were added to the solution of **RS1**, free **RS1**, as expected, exhibited almost no absorption near 450–600 nm, indicating that **RS1** exists as a spirocyclic form. Upon addition of increasing concentrations of Hg^{2+} ions to the solution, a new absorption band centered at 537 nm appeared with increasing intensity, which can be ascribed to the formation of the ring-opened amide form of **RS1** upon Hg^{2+} ions binding.[8] Moreover, the titration solution exhibited an obvious and characteristic color change from colorless to red (Figure S1, ESI†), suggesting that sensor **RS1** can serve as a "naked-eye" indicator for Hg^{2+} ions. To understand the recognition abilities of **RS1** towards Hg^{2+} ions, the Job plot method for the absorbance was conducted to determine the binding stoichiometry of the

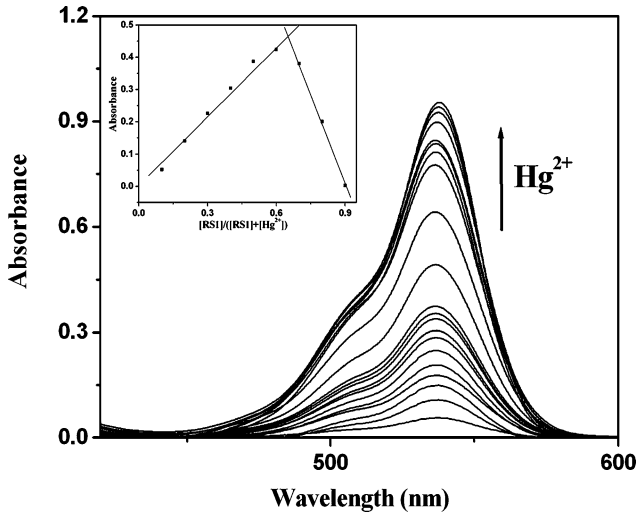
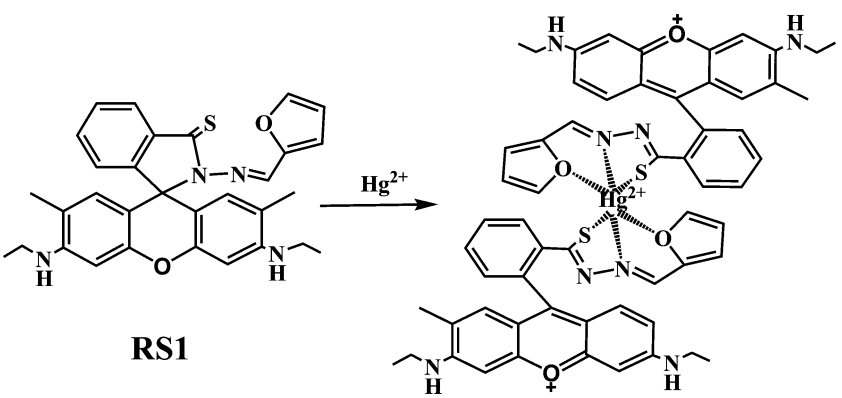


Fig. 2 Absorption spectra of **RS1** (10 μM) in buffered (NaAc-HAc, pH 7.0) water–DMF (1/1, v/v) upon addition of different amounts of Hg^{2+} ions (0–5 equivalents). Inset: Job's plots of the complexation between **RS1** and Hg^{2+} ions (Total concentration of **RS1** and Hg^{2+} ions is 20 μM).

RS1-Hg^{2+} ions complex, by maintaining the total **RS1** and Hg^{2+} ions constant (20 μM) and changing the mole fraction of Hg^{2+} ions from 0 to 1 (Fig. 2, inset).[9] From the Job plot, we can observe that the absorbance went through a maximum at a molar fraction of about 1/3, indicating that a 1 : 2 stoichiometry was most possible for the binding mode of Hg^{2+} and **RS1** [Hg(**RS1**)$_2$]. From the absorption titration experiment, the association constant for Hg^{2+} was estimated to be 5.20×10^5 M^{-2} on the basis of nonlinear fitting of the titration curve assuming 1 : 2 stoichiometry [Hg(**RS1**)$_2$] (Figure S2†). The above results indicate that the sensor **RS1** exhibited a superior binding capability towards the Hg^{2+} ions in comparison with the sensor **RO1** towards Cu^{2+} ions.[7] The strong binding ability of **RS1** towards Hg^{2+} could be ascribed to introducing the S atom. Thus in accordance with the 1 : 2 stoichiometry, the possible binding mode between **RS1** towards Hg^{2+} is proposed in Scheme 2. When no Hg^{2+} ions are added to the solution of **RS1**, the molecules prefer their spirolactam ring-closed state and little fluorescence signal could be observed. However, upon the addition of Hg^{2+} ions, in the **RS1** towards Hg^{2+} system, the thioether S and the imino N atoms of both ligands are involved in the binding of Hg^{2+} ions, forming stable 5-membered rings that requires the opening of the spiro ring of **RS1** to establish the delocalized xanthene moiety that shows long wavelength absorption and fluorescence enhancement.[6i,j]

The absorption spectra changes of **RS1** upon addition of various competitive metal ions with high concentrations (*i.e.*, millimolar level) were also investigated. As illustrated in Fig. 3, we can clearly observe no spectral changes of **RS1** in the presence of Na^+, K^+, Mg^{2+}, Ca^{2+}, Ba^{2+}, Zn^{2+} ions and much smaller spectral changes in the presence of Cd^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Fe^{3+} ions. Unfortunately, the addition of Cu^{2+} ions into the solution of the sensor **RS1**, under the same conditions, can induce the small but significant enhancement of the absorbance at about 537 nm (Fig. 3). The enhancement in absorbance associated with a color change clearly suggests the formation of the ring-opened amide form. However, no obvious fluorescence change of **RS1** could be observed on addition of Cu^{2+} ions, even with high concentration (*i.e.*, millimolar level) (Figure S3†). The quenching of the fluorescence of the open ring form of **RS1** by Cu^{2+} ions could be explained based on the well-known paramagnetic effect of the d^9 Cu(II) system.[5b,10] Because the $dx^2 - y^2$ orbital of Cu^{2+} (d^9) is half-filled, the radiationless deactivation of the excited state of **RS1** may take place through an energy transfer process (electron exchange type), resulting in the fluorescence quenching.[11]

With further investigations, the fluorescence titration of the Hg^{2+} ions was carried out using a solution of **RS1** (10 μM) in buffered (NaAc-HAc, pH 7.0) water–DMF (1/1, v/v). Fig. 4 gives detailed fluorescence changes of **RS1** upon different concentrations of Hg^{2+} ions in the same conditions. Upon the addition of increasing concentrations of Hg^{2+} ions, a significant enhancement of the characteristic fluorescence of rhodamine 6G in a Hg^{2+} ion concentration-dependent way emerges at 564 nm, accompanied with an obvious orange fluorescence enhancement (Figure S4†). With the concentration of Hg^{2+} ions up to 9 equiv of **RS1**, an 120-fold fluorescence enhancement at 564 nm was estimated. All of this supports our expectation that the sensor **RS1** could serve as a highly sensitive fluorescent chemosensor for Hg^{2+} ions. From the fluorescence titration experiment, a linear relationship (R^2 = 0.9979) is observed between the fluorescence intensity of **RS1** and concentration of Hg^{2+} ions from 0.1 μM



Scheme 2 Possible binding mode of **RS1** with Hg^{2+} ions.

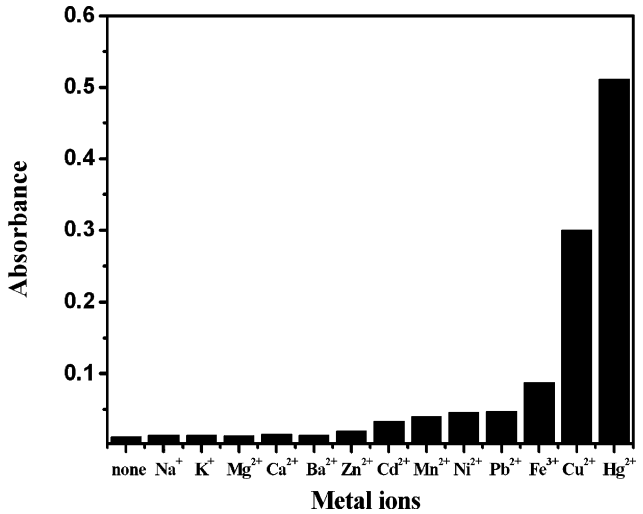


Fig. 3 Absorbances at 537 nm of **RS1** (10 μM) in buffered (NaAc-HAc, pH 7.0) water–DMF (1/1, v/v) in the presence of 0.5 mM of different metal ions.

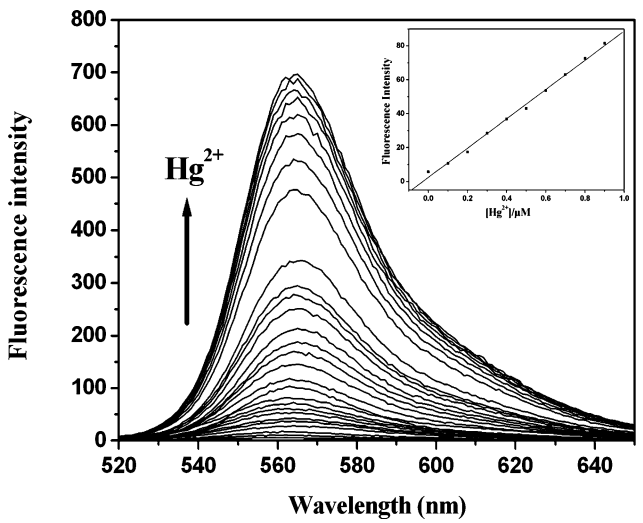


Fig. 4 Fluorescence spectra of **RS1** (10 μM) in buffered (NaAc-HAc, pH 7.0) water–DMF (1/1, v/v) upon addition of different amounts of Hg^{2+} ions (0–9 equivalents). λ_{ex} = 500 nm. Inset: titration curve of I_{564} *vs.* Hg^{2+} concentration (0–1 μM).

to 1 μM (Fig. 4, inset). This clearly indicates that **RS1** enables quantitative Hg^{2+} detection at > 0.1 μM Hg^{2+} ions.

To study the practical applicability, the effects of pH on the fluorescence response of **RS1** in the absence and presence of Hg^{2+} ions were evaluated. A solution of the high concentration of Hg^{2+} ions might cause precipitation of HgO in alkaline conditions, so these experiments were carried out at a pH range from 3.0 to 9.0. Fig. 5 shows the fluorescence responses of **RS1** without and with Hg^{2+} ions as a function of pH. Experimental results show that for free **RS1**, at acidic conditions (pH <5), an obvious off-on fluorescence appeared due to the formation of the open-ring state because of the strong protonation. In the pH range from 4.0 to 9.0, little fluorescence signal (excited at 500 nm) could be observed for free **RS1**, suggesting that the molecules prefer the spirocyclic form. Upon the addition of Hg^{2+} ions, there was an obvious fluorescence off-on change of **RS1** under different pH

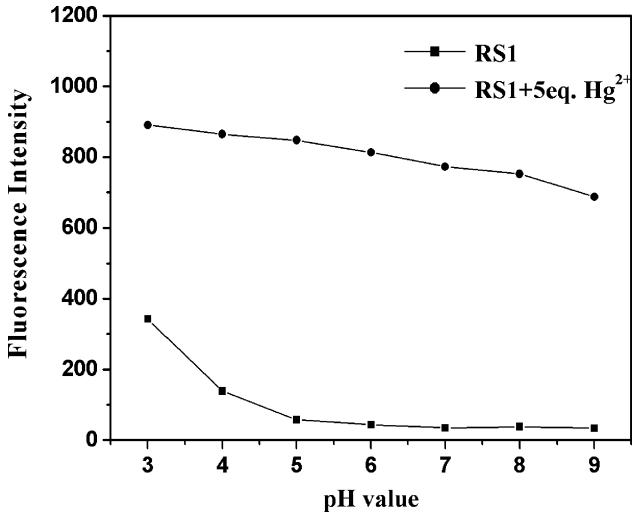


Fig. 5 Fluorescence intensity (λ_{ex} = 500 nm, λ_{em} = 564 nm) of **RS1** (10 Mm) in water–DMF (1/1, v/v) measured with and without Hg^{2+} ions (5 equiv.) as a function of pH.

values. And the pH-control emission measurements revealed that **RS1** could respond to Hg^{2+} ions in the pH range from 5 to 9 with little changes of the fluorescent intensity, suggesting that the **RS1** facilitates quantification of the concentration of Hg^{2+} ions in aqueous solution in a wide pH range. Considering that most samples for Hg^{2+} ions analysis were neutral, therefore, the media for Hg^{2+} ions quantification was then buffered at pH 7.

A short response time and reversible response are also necessary for a fluorescent sensor to monitor Hg^{2+} ions in practical applications. The time dependence of the response of **RS1** to Hg^{2+} ions was investigated and the results revealed that the recognizing event could complete in less than 1 min (Figure S5†). The chemical reversibility behavior of the binding of **RS1** with Hg^{2+} ions was then studied in the buffered water–DMF solution (Fig. 6). In light of the strong binding ability of the sulfur anion toward Hg^{2+} ions, it could be expected that the addition of S^{2-} will liberate Hg^{2+} from the metal–ligand complex, releasing free **RS1**. Upon addition of

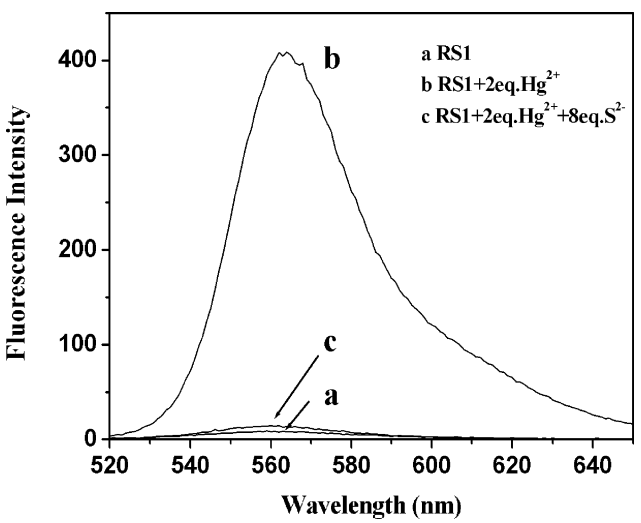


Fig. 6 Fluorescence spectra of **RS1** (10 μM, water–DMF 1/1, v/v, pH = 7.0) in the presence of 2 equiv. of Hg^{2+} and 10 equiv. of Na_2S (λ_{ex} = 500 nm).

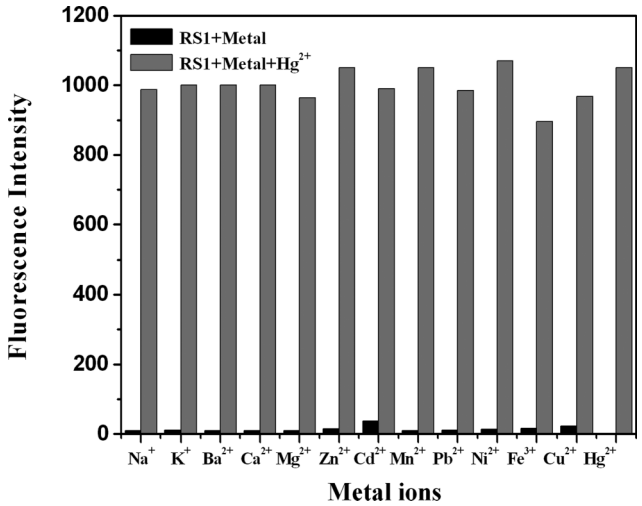


Fig. 7 Fluorescence response of **RS1** (10 μM) to various metal ions (0.5 mM) without and with Hg^{2+} (0.5 mM) in a water–DMF solution (1/1, v/v, pH = 7.0). (λ_{ex} = 500 nm, λ_{em} = 564 nm).

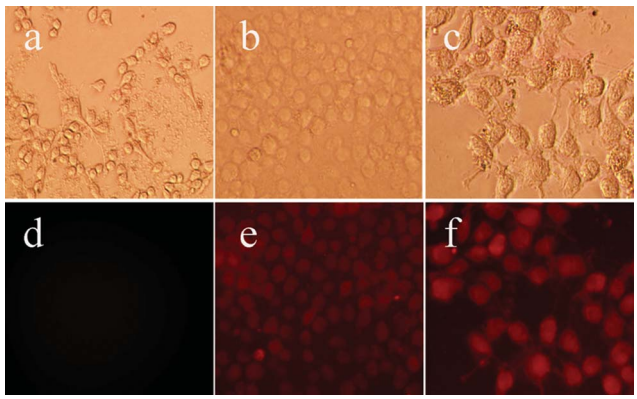


Fig. 8 Fluorescence images of Hg^{2+} ions in Rat Schwann cells with **RS1** (10 μM). Bright-field transmission image (a–c) and fluorescence image (d–e) of Rat Schwann cells incubated with 0 μM, 10 μM and 50 μM of Hg^{2+} ions for 30 min, respectively (Excited with green light).

excess S^{2-}, the color of the metal–ligand mixture changed from red to colorless and fluorescent emission intensity of the system was quenched, indicating that sulfur anion sequestered Hg^{2+} of the metal–ligand mixture, liberating the free **RS1**. Thus, **RS1** can be classified as a reversible chemosensor for Hg^{2+} ions.

Achieving high selectivity toward the analyte over the other competitive species coexisting in the sample is a very important feature to evaluate the performance of a fluorescence chemosensor. Therefore, the selectivity and competition experiments were extended to various metal ions, including alkali, alkaline earth, and transition-metal ions. In certain environmental samples, such as river and seawater, the concentrations of some prevalent toxic metal ions are significantly higher, therefore competition experiments with high concentrations of the above-mentioned metal ions (0.5 mM) were carried out (Fig. 7). The miscellaneous competitive cations did not induce any obvious fluorescence changes (Fig. 7, the black bar portion). Moreover, the competition experiments revealed that the Hg-induced fluorescence intensity was not influenced by the subsequent addition of miscellaneous competitive cations (Fig. 7, the gray bar portion). It is significant to observe that competitive Cu^{2+} ions may not compromise the Hg-induced fluorescence due to the high thiophilic character of Hg^{2+} ions. Obviously, all of these results confirmed that our proposed chemosensor **RS1** has remarkably high selectivity towards Hg^{2+} ions over other competitive cations in the water medium.

In view of the above-mentioned advantages of the sensor **RS1**, bioimaging applications of **RS1** for monitoring of Hg^{2+} ions in living cells were then carried out. After Rat Schwann cells were incubated with **RS1** (10 μM) for 10 min at 37 °C, and then followed by the addition of different concentrations of Hg^{2+} ions from 0, 10, 50 μM, their fluorescence images were recorded with a fluorescence microscope (Fig. 8). In the absence of Hg^{2+} ions, free **RS1** showed no detectable fluorescence signal in living cells. After incubation with Hg^{2+} ions, a bright fluorescence was observed in living cells. Moreover, these fluorescence images display Hg^{2+} concentration dependence: the stronger fluorescence images of Rat Schwann cells are those treated with the higher concentration of Hg^{2+} ions. These results demonstrate that **RS1** can applied for *in vitro* imaging of Hg^{2+} ions in living cells and potentially *in vivo*.

Conclusion

In summary, we have synthesized a simple and easy-to-prepare rhodamine-based optical chemosensor **RS1** for the detection of Hg^{2+} ions. The chemosensor **RS1** displayed 2 : 1 complex formation with Hg^{2+} ions in a broad pH range. Hg^{2+}-specific binding enabled the opening of the spirolactam ring and consequently successfully exhibited a reversible absorption and fluorescence enhancement response toward Hg^{2+} ions over other metal ions (even those that exist in high concentration). Thus the chemosensor **RS1** behaves as a highly sensitive and selective fluorescent probe for Hg^{2+} ions detection with a broad pH range 3–9. Fluorescence imaging experiments of Hg^{2+} ions in living cells demonstrate its value in practical applications in biological systems.

Experimental section

Materials

Rhodamine 6G, 2-furaldehyde, hydrazine hydrate and Lawesson's Reagent were purchased from Alfa Aesar. All the reagents and inorganic metal salts were analytical grade (Shanghai Chemical Reagents Co. China) and used without further purification. Doubly distilled water was used throughout. NaAc-HAc buffer solutions of different pH were prepared using proper amounts of NaAc and HAc (analytical grade) under adjustment by a pH meter.

Characterization

Fluorescence spectra were measured with a Hitachi F-4500 fluorescence spectrophotometer with a 10 mm quartz cuvette. The excitation and emission wavelength bandpasses were both set at 10 nm. All pH measurements were carried out on a Mettler–Toledo Delta 320 pH meter. ^{1}H and ^{13}C NMR spectra were recorded using a mercury-300BB spectrometer (Varian, USA) operated at 300 MHz with tetramethylsilane (TMS) as internal

standard. Mass spectra were performed on Agilent 1100 MS series and AXIMA CFR MALDI/TOF (Matrix assisted laser desorption ionization/Time-of-flight) MS (COMPACT). All of the measurements were operated at room temperature at about 298 K.

Cell culture and imaging

The Rat Schwann cells (RSC 96) were provided by Norman Bethune College of Medicine Jilin University (China). Cells were grown in H-DMEM (Dulbecco's Modified Eagle's Medium, High Glucose) supplemented with 10% FBS (Fetal Bovine Serum) in an atmosphere of 5% CO_2, 95% air at 37 °C. Cells were plated on 6-well plate at 5 × 106 cells per well and allowed to adhere for 12 h. Fluorescence imaging was performed with an Olympus fluorescence microscope (BX51, Olympus, Japan). Immediately before the experiments, the cells were washed with phosphate-buffered saline (PBS) and then incubated with 10 μM of **RS1** (in the culture medium) for 10 min at 37 °C. Experiments to assess Hg^{2+} uptake were performed in the same media supplemented with 0, 10, 50 μM $Hg(ClO_4)_2$ for 0.5 h.

Synthesis of RO1

Rhodamine 6G hydrazide was synthesized according to the literature method.[12] It was then reacted with 2-furaldehyde.[7]

Synthesis of RS1

Rhodamine 6G hydrazide was synthesized according to the literature method.[12] Thiooxorhodamine 6G hydrazone was synthesized according to the literature method and identified by NMR and mass data.[6j] To a boiling solution of thiooxorhodamine 6G hydrazone (0.25 mmol, 0.11 g) and 2-furaldehyde (2.5 mmol, 0.24 g) in ethanol (10 mL) was added 3 drops of acetic acid. After the addition was complete then the mixture was refluxed for 12 h. After the mixture had been left to cool to room temperature, the solution was filtered off and washed with ethanol. After drying over P_2O_5 under vacuum, the pure **RS1** was obtained (0.13 g, yield: 90%).^{1}H NMR ($CDCl_3$, 300 MHz), δ (ppm): 1.28–1.33 (t, 6H), 1.90 (s, 6H), 3.16–3.23 (q, 4H), 3.47 (b, 2H), 6.28–6.30 (dd, 2H), 6.48–6.50 (dd, 1H), 6.61 (s, 2H), 6.87–6.88 (d, 1H), 7.04–7.07 (m, 1H), 7.39–7.42 (m, 2H), 7.53–7.54 (d, 1H), 8.09–8.13 (m, 1H), 8.46 (s, 1H). ^{13}C NMR ($CDCl_3$, 400 MHz), δ (ppm): 15.21, 17.18, 38.82, 63.92, 96.66, 111.00, 112.54, 115.34, 118.27, 122.66, 127.46, 128.14, 130.78, 132.71, 133.44, 135.07, 147.28, 148.06, 150.47, 150.55, 156.36,172.46. MS *m/z*: calc. for $C_{31}H_{30}N_4O_2S$, 522.2; found, 522.9. ^{1}H, ^{13}C NMR and MS charts are shown in Figure S6–8 (see ESI†).

Acknowledgements

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