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ORIGINAL PAPER

A New Rhodamine-Based "Off-On" Fluorescent Chemosensor for Hg (II) Ion and its Application in Imaging Hg (II) in Living Cells

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Abstract A novel rhodamine derivative (Rh-C), synthesized by the reaction of rhodamine ethylenediamine and cinnamoyl chloride, was evaluated as a chemoselective Hg^{2+} ion sensor. Addition of Hg^{2+} to an ethanol aqueous solution of the Rh-C resulted in a color change from colorless to obvious pink color together with distinctive changes in UV–vis absorption spectrum and fluorescence spectrum. However, other common alkali-, alkaline earth-, transition- and rare earth metal ions induced no or minimal spectral changes. The interaction of Hg^{2+} and sensor Rh-C was proven to adopt a 1:1 binding stoichiometry and the recognition process is reversible. The chemosensor displayed a linear response to Hg^{2+} in the range of 0.4–5 μM with a detection limit of 7.4×10^{-8} M. The sensor Rh-C was also successfully applied to the imaging of Hg^{2+} in HL-7702 cells.

Keywords Hg (II) ion · Rhodamine derivative · Fluorescence enhancement · Chemosensor · Fluorescent image · living cells

Introduction

Mercury is a highly toxic and widespread global pollutant [1–4]. The mercuric ion, Hg [II], combines with both inorganic and organic ligands, which can readily penetrate through biological membranes even at very low concentrations [5, 6]. A wide variety of symptoms, including central nervous system, kidney, and endocrine system diseases result from a series of biological effects [7–9]. Thus, much attention has been focused on developing Hg^{2+} fluorescent chemosensors with excellent sensitivity and selectivity, quick response time and easy signal detection [10–16].

The rhodamine-based chemosensors can react specifically with specific metal ions to induce a concomitant change of their photochemical properties (excitation/emission wavelength, fluorescence intensity, and so forth). Alteration in molecular structure between non-fluorescent of spirocyclic and fluorescent ring-open conformations of rhodamine framework is employed as the detection mechanism [17–22].

Since Hg^{2+} is a heavy metal ion with $5d^{10}$ $6s^0$ electronic configuration, the oxygen atom or the nitrogen atom of the imino moiety might be a proper binding site when incorporated into rhodamine fluorophore [23–27]. The increase of the O, N atom has the potential to enhance the selective and sensitivity of the resulting chemosensors toward Hg^{2+}. Herein,we synthesized a rhodamine-based fluorogenic chemosensor (Rh-C), for selective response to Hg^{2+} in aqueous media and in living cells. Further, upon chelation of Hg^{2+}, Rh-C will change to a fluorescent ring-opened form, which could be detected by the naked eye.

Experiments

Materials and Instruments

All the reagents were purchased from commercial suppliers and used without further purification. All the common chemicals were of analytical grade. Solvents were purified by standard procedures. All reactions were monitored by TLC (thin-layer chromatography) with detection by UV. The solutions of Na^+, K^+, Mg^{2+}, Mn^{2+}, Ca^{2+}, Ba^{2+}, Fe^{3+},

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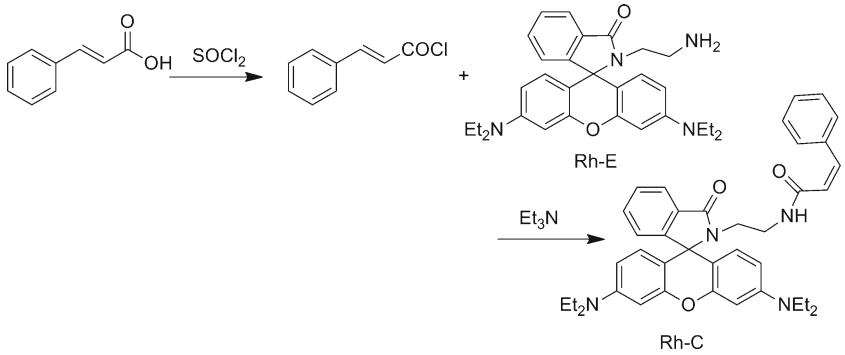
Scheme 1 Synthetic routes of the probe Rh-C



Zn^{2+}, Pb^{2+}, Cu^{2+}, Cd^{2+}, Ag^{+} and Hg^{2+} were prepared from their nitrate salts. Doubly distilled deionized water was used throughout the experiment.

The absorption spectra were acquired on a Purkinje General TU-1901 UV–vis spectrometer. Fluorescence spectra measurements were performed on a Hitachi F-4500 Fluorescence spectrophotometer at room temperature. NMR spectra were measured on a Bruker 300 MHz spectrometer with chemical shifts reported in ppm (in $CDCl_3$; TMS as internal standard). Electrospray ionization (ESI) mass spectra were conducted by ABI-057-TY4675 instrument. The pH measurements were carried out on a PHS-3W pH meter. The masses of the samples and solvents were determined by a Mettler Toledo AB204-N analytical balance with an accuracy of 0.0001 g. Fluorescence images experiments were carried out with an Olympus IX71 inverted fluorescence microscope.

Synthesis of Probe Rh-C

The newly synthesized rhodamine B derivative (Rh-C) was prepared in high yield (Scheme 1). Compound rhodamine ethylenediamine (Rh-E) was facilely synthesized in high yield according to the procedure as published in the literature [28].

Rhodamine ethylenediamine (Rh-E) (2.52 g, 5.19 mmol) and triethylamine (4 mL) were dissolved in 25 mL dry dichloromethane. After cooling to 0 °C in ice bath, to the solution was added dropwise a solution of acinnamoyl chloride (0.86 g, 5.19 mmol) in 20 mL of dichloromethane over 10 min. The resulting mixture was stirred for 2 h at room temperature. The recation mixture was then evaporated and the crude product was purified by column chromatography (silica gel, petroleum ether: ethyl acetate 1: 1, v/v). The yield was 72 %. ^{1}HNMR($CDCl_3$, 300 MHz): δ=7.98 (dd, 1H), 7.57 (s, 1H), 7.51 (m, 2H), 7.47 (dd, 2H), 7.35 (m, 3H), 7.08 (dd, 2H), 6.47 (d, J=8.9Hz, 2H), 6.30–6.39(m, 5H), 3.18–3.34 (m, 12H), 1.19 (t, J=7.1Hz, 12H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ=170.15, 165.82, 153.85, 153.28, 140.10, 135.18, 132.84, 130.40, 129.32, 128.66, 128.45, 128.21, 127.84, 123.93, 122.83, 121.38, 108.39, 97.89, 65.82, 44.43, 41.24, 40.17, 12.56. IR (KBr, ν/cm^{-1}): 3427, 3006, 2854, 1672, 1615, 1459, 1220, 1118, 979. ESI-MS ($M+H^+$): m/z=614.33 ($C_{39}H_{42}N_4O_3$).

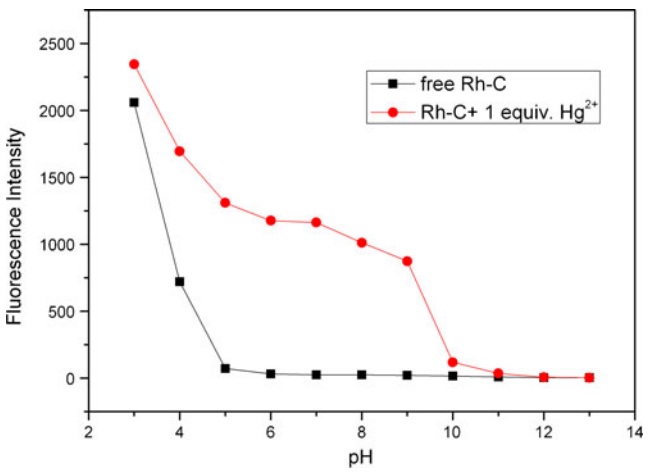


Fig. 1 Fluorescence intensity of Rh-C (10 μM) in the absence and presence of 1 equiv. Hg^{2+} in water -ethanol solution (30:70, v/v) at different pH. The pH modified by adding 75 % $HClO_4$ or NaOH (10 %). Excitation: 535 nm, emission: 586 nm

General UV–vis and Fluorescence Spectra Measurements

2 mM of each inorganic salt ($NaNO_3$, KNO_3, $Mg(NO_3)_2$•$6H_2O$, $Mn(NO_3)_2$•$4H_2O$, $Ca(NO_3)_2$, $Ba(NO_3)_2$, $Zn(NO_3)_2$•$6H_2O$, $Fe(NO_3)_2$•$6H_2O$, $Pb(NO_3)_2$, $Cu(NO_3)_2$•$3H_2O$, $Cd(NO_3)_2$•$2H_2O$, $AgNO_3$ and $HgNO_3$•$0.5H_2O$) was dissolved in distilled water to afford 2×10^{-3} mol•L^{-1} aqueous solution. A 2.0×10^{-3} mol•L^{-1} stock solution of Rh-C was prepared in absolute ethanol. All the measurements were made according to the following procedure. To 10 mL glass tubes containing different amounts of metal ions, proper amounts of the solution of Rh-C was added directly with micropipette, then diluted with buffered (HEPES 20 mM, pH=7.0) water-ethanol (7/3, v/v) to 10 mL, then the absorption and fluorescence sensing of metal ions were run. In selectivity experiments, the test samples were prepared by placing the appropriate amounts of metal ion stock solution into 10 mL solution of Rh-C (10 μM). All samples were prepared at room temperature, shaken for 10 s and waited for 10 min before test. Fluorescence measurements

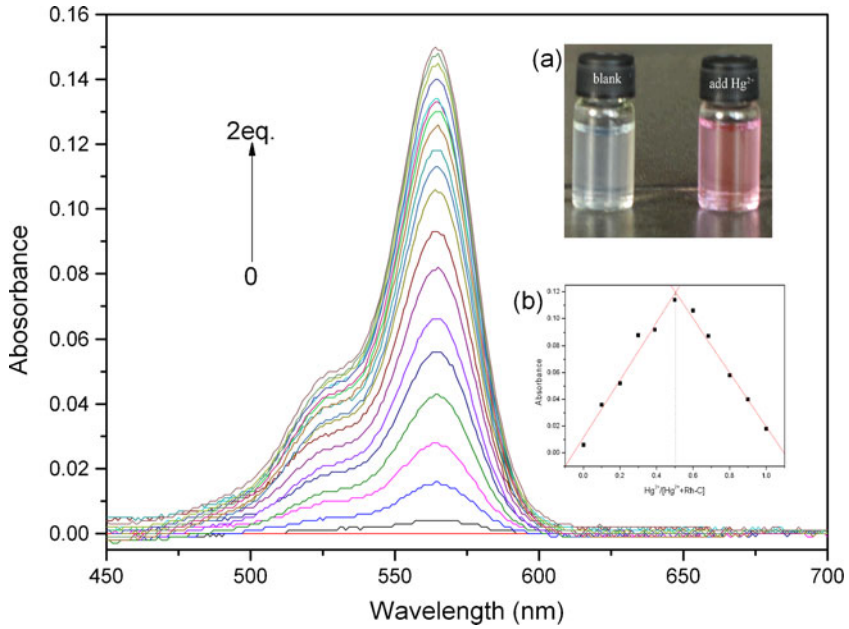


Fig. 2 UV–vis absorption spectra of Rh-C (10.0 μM) obtained during the titration by Hg^{2+} (0-2 equiv.) in buffered (HEPES 20 mM, pH=7.0) water-ethanol (7/3, v/v) at room temperature. Inset: **a** The color change of Rh-C (10 μM)+2 equiv. Hg^{2+} in solution, **b** Job's plot of changes of absorbance at 562 nm, the total concentration of [Hg^{2+}]+[Rh-C] was 20 μM

were carried out with excitation and mission slit width of 10 and 5 nm and excitation wavelength was 535 nm.

Experimental Details for Cell Imaging Experiments

The HL-7702 cells (human hepatocyte cell line) were cultured in DEME (Invitrogen) supplemented with 10 % FBS (Invitrogen). One day before imaging, the cells were seeded in 6-well flat-bottomed plates. The next day, the HL-7702 cells were incubated with 10 μM sensor Rh-C for 0.5 h at 37 °C in humidified environment of 5 % CO_2 and then washed with phosphate-buffered saline (PBS) three times before incubating with 10 μM $Hg(NO_3)_2$ for another 0.5 h, cells were rinsed with PBS three times again, then the fluorescence imaging of intracelluar Hg^{2+} was observed under inverted fluorescence microscope (IX71, Olympus, Japan) with a 40×objective lens (excited with green light). The HL-7702 cells only incubated with 10 μM Rh-C for 0.5 h at 37 °C under 5 % CO_2 was as a blank control.

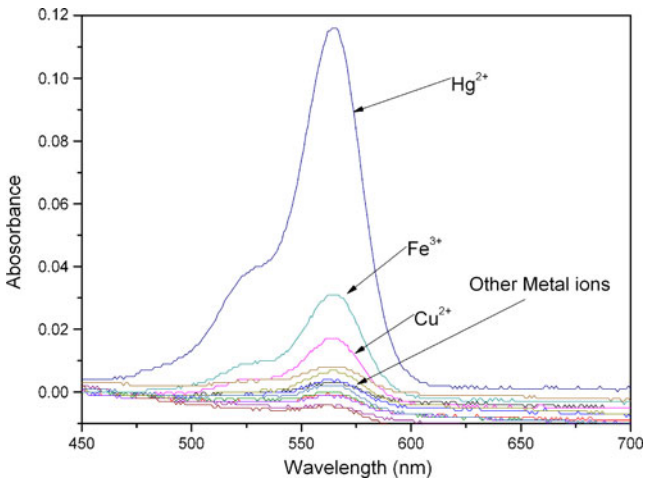


Fig. 3 UV–vis spectra of Rh-C (10.0 μM) upon addition of 2.0 equiv. of Hg^{2+} and 10 equiv. other metal ions (Ag^{+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, Cu^{2+}, Fe^{3+}, K^{+}, Mg^{2+}, Mn^{2+}, Na^{+}, Pb^{2+} and Zn^{2+}) in buffered (HEPES 20 mM, pH=7.0) water-ethanol (7/3, v/v)

Determination of Binding Constants

The binding constant was calculated from the absorption intensity titration curves according to Benesi-Hildebrand equation as follow.

$$\frac{1}{A-A_0}=\frac{1}{K_a(A_{max}-A_0)[c]}+\frac{1}{A_{max}-A_0}$$

A and A_0 is the absorbance of Rh-C solution in the presence and absence of Hg^{2+}; respectively; A_{max} is the saturated absorbance of Rh-C in the presence of excess amount of Hg^{2+}; [c] is the concentration of Hg^{2+} ions added (mol L^{-1}). The association constant values K_a is calculate by the ratio intercept/slope.

Results and discussion

Synthesis and Structural Characterization of Rh-C

Rh-C was facilely synthesized from rhodamine B by acylation reaction, as summarized in Scheme 1. Its structure was confirmed by ^{1}HNMR, ^{13}CNMR and MS data.

A solution of Rh-C in ethanol is colorless and weakly fluorescent, indicating that the spirolactam form of Rh-C

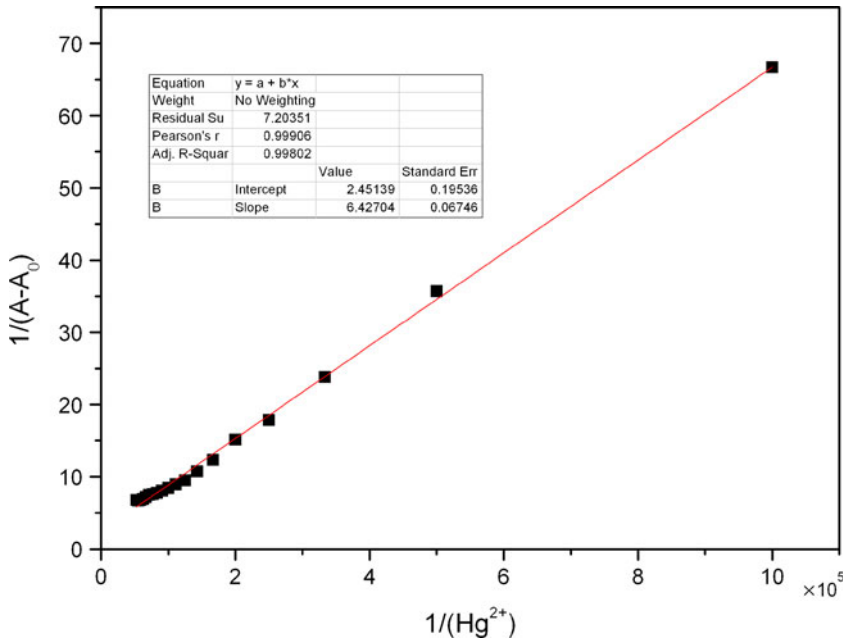


Fig. 4 Benesi-Hildebrand plot (absorbance at 562 nm) of Rh-C using 1:1 stoichiometry for association between Rh-C and Hg^{2+}

exists predominantly. The characteristic peak of the 9-carbon of Rh-C near 66 ppm in the ^{13}CNMR spectrum also supports this consideration [29]. The chemosensing behavior of Rh-C was investigated by UV–vis and fluorescence measurements.

Effect of pH Value

For practical application, the appropriate pH conditions for successful operation of the sensor were evaluated. The effects of pH on the fluorescence response of Rh-C obtained without and with Hg^{2+} in water-ethanol (7/3, v/v) are shown in Fig. 1. For free Rh-C, at acid conditions (pH<5), the ring opening of rhodamine took place because of the strong protonation. No obvious fluorescence emission of Rh-C was observed between pH 5 and 12, suggesting that the compound is insensitive to pH and that the spirolactam form is still preferred in this condition. However,the addition of Hg^{2+} led to the fluorescence enhancement over a comparatively wide pH range (5.0–9.0), which is attributed to opening of the rhodamine ring. The results suggest that Rh-C was insensitive to pH near 7.0 and could work in physiological pH conditions with a very low background fluorescence [30]. Therefore, further UV–vis and fluorescent studies were carried out in buffered (HEPES 20 mM, pH=7.0) water-ethanol (7/3, v/v) at room temperature.

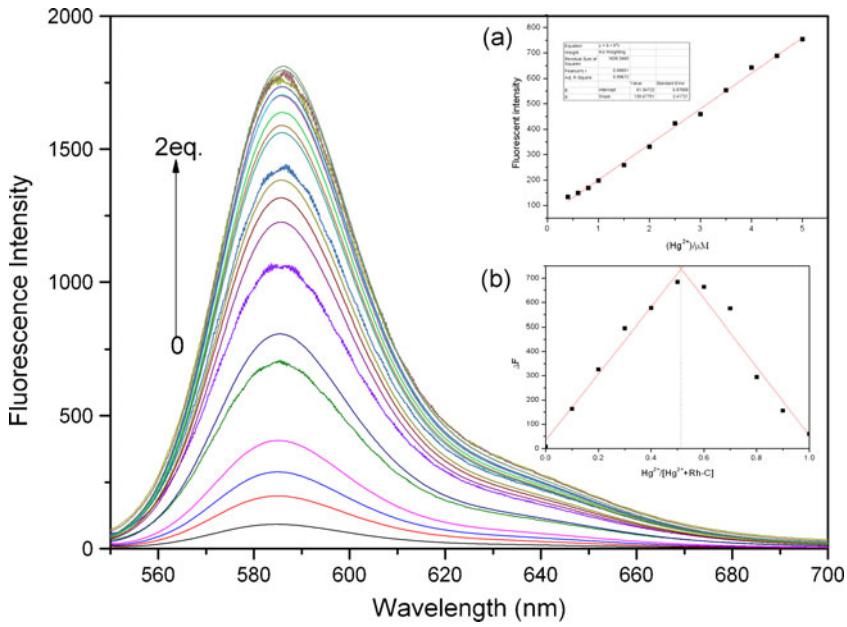


Fig. 5 Fluorescence emission spectra of Rh-C (10.0 μM) in buffered (HEPES 20 mM, pH= 7.0) water-ethanol (7/3, v/v) upon the addition of Hg^{2+} (0–2 equiv.) with an excitation of 535 nm. Inset: **a** fluorescence enhancement at 586 nm as a function of Hg^{2+} concentration, **b** Job's plot of changes of fluorescent intensity at 586 nm, the total concentration of $[Hg^{2+}]$+[Rh-C] was 10 μM

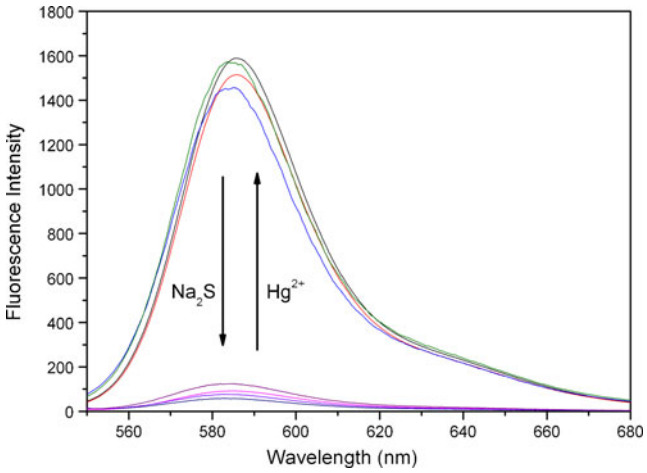


Fig. 6 Reversibility of Hg^{2+} coordination to Rh-C (10 μM) by Na_2S in buffered (HEPES 20 mM, pH=7.0) water-ethanol (7/3, v/v). The top lines represent the fluorescence enhancement that occurs after addition of 1 equiv. of Hg^{2+}. The bottom lines represent the fluorescence intensity of free Rh-C and the fluorescence intensity decrease that occurs after addition 1 equiv. of Na_2S to a solution containing the [Rh-C-Hg^{2+}] species. Four cycles of on/off by Hg^{2+}/Na_2S addition are depicted in this plot.

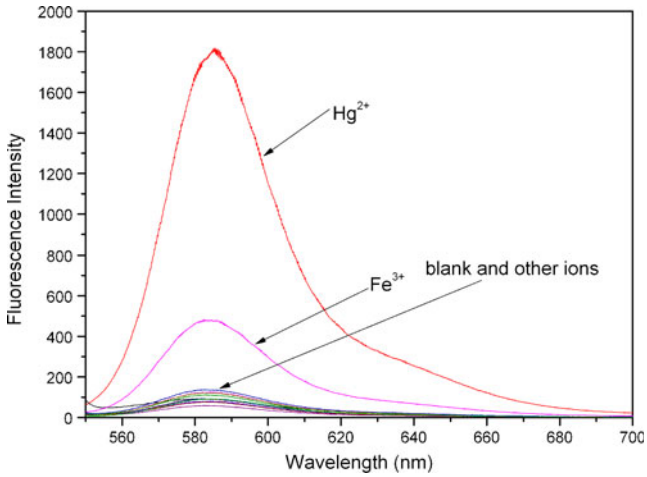
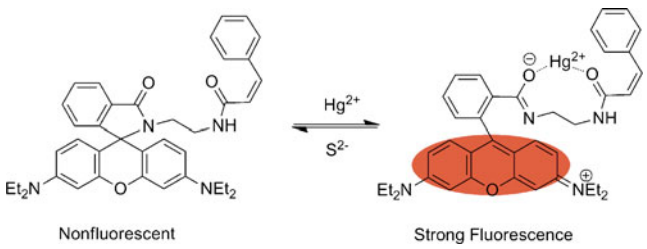


Fig. 7 Fluorescent spectra of Rh-C (10.0 μM) upon addition of 2.0 equiv. of Hg^{2+} and 10 equiv. other metal ions (Ag^{+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, Cu^{2+}, Fe^{3+}, K^{+}, Mg^{2+}, Mn^{2+}, Na^{+}, Pb^{2+} and Zn^{2+}) in buffered (HEPES 20 mM, pH=7.0) water-ethanol (7/3, v/v)

UV–vis Spectral Reponses of Rh-C

The absorption spectra of Rh-C with varying Hg^{2+} concentrations in buffered (HEPES 20 mM, pH=7.0) water-ethanol (7/3, v/v) were recorded, as shown in Fig. 2. Like most of the spirocycle RhB derivatives, When no Hg^{2+} was added to the solution, free Rh-C was colorless and exhibited almost no absorption peak in the visible wavelength range (>400 nm) due to the closed spirolactam ring. In the presence of 2 equiv. of Hg^{2+}, the absorbance was enhanced obviously and a new peak at 562 nm was observed, accompanied by a clear color change from colorless to pink. This enhancement in absorbance can be ascribed to the clear formation of the ring-opened amide form of Rh-C upon Hg^{2+} ions binding [19, 31]. The color of Rh-C also change from colorless to pink (Fig. 2.Inset (a)), indicating that Rh-C can serve as a "naked-eye" sensor for Hg^{2+} in aqueous solution. Other metal ions, such as Ag^{+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, Cu^{2+}, Fe^{3+}, K^{+}, Mg^{2+}, Mn^{2+}, Na^{+}, Pb^{2+} and Zn^{2+} did not show any significant spectral change except Fe^{3+} and Cu^{2+} caused a little bit of enhancement under identical conditions (Fig. 3). These results suggested that Rh-C could serve as a "naked-eye" chemosensor selective for Hg^{2+} in neutral buffered media.

The method of continuous variations (Job's plot) obtained from the Rh-C+Hg^{2+} system in buffered (HEPES 20 mM, pH=7.0) water-ethanol (7/3, v/v), When molar fraction of Hg^{2+} was 0.5, the absorbance at 562 nm got to maximum (Fig. 2.Inset (b)), indicating that forming a 1:1 complex between Rh-C and Hg^{2+}, which was confirmed by the Benesi-Hildebrand method [32].



Scheme 2 Probable complexation mechanism of Rh-C with Hg^{2+}

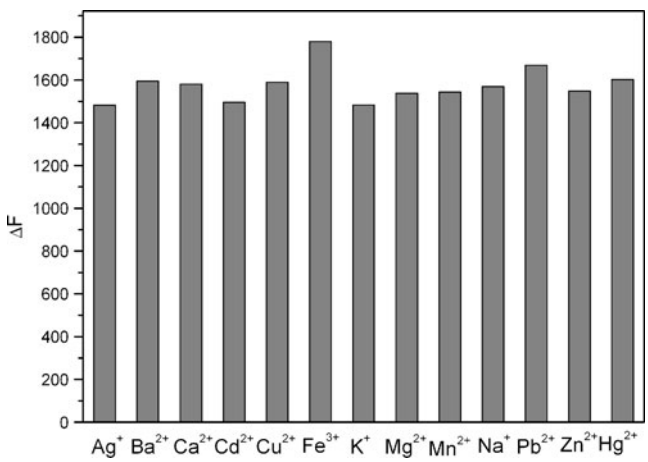


Fig. 8 Competition of Rh-C (10.0 μM) for Hg^{2+} (2equiv.) upon addition of other metal ions (10equiv.) in buffered (HEPES 20 mM, pH=7.0) water-ethanol (7/3, v/v). $\Delta F=F-F_0$, (F_0 and F were the fluorescence intensity of Rh-C in the absence and presence of Hg^{2+}). Excitation: 535 nm, emission: 586 nm

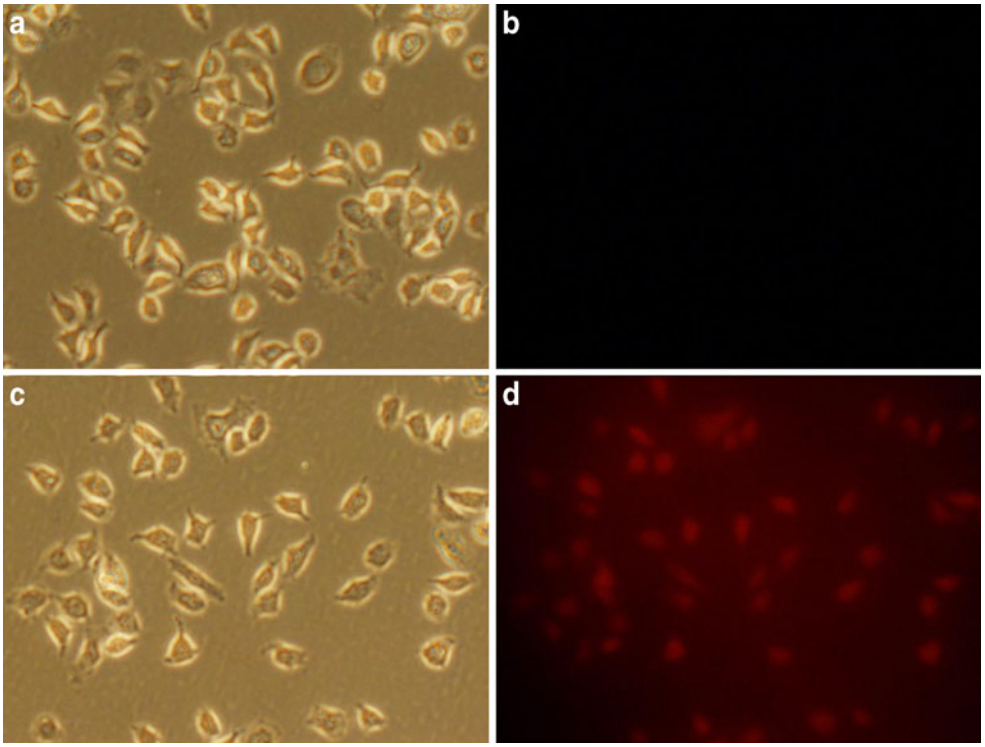


Fig. 9 Fluorescence images of Hg^{2+} in HL-7702 cells with 5 μM solution of Rh-C in Ethanol-PBS (1 : 99, v/v) buffer for 30 min at 37 °C, Bright-field transmission image (**a**, **c**) and fluorescence image (**b**, **d**) of HL-7702 cells incubated with 0 μM, 5 μM of Hg^{2+} for 30 min, respectively

Linear fitting of the titration profiles using Benesi-Hildebrand plot based on a 1:1 binding mode results in a good linearity (Fig. 4), which strongly support the 1:1 binding stoichiometry of Rh-C and Hg^{2+}, and the binding constant was calculate to be 3.81×10^4 M^{-1}[33].

Fluorescence Spectral Responses of Rh-C

Fluorescence titrations of Rh-C with Hg^{2+} in buffered (HEPES 20 mM, pH=7.0) water-ethanol (7/3, v/v) were then performed. As shown in Fig. 5, free Rh-C showed a very weak band in the range 500–700 nm due to the spirocyclic structure. Upon addition gradual increase of Hg^{2+} to the solution of Rh-C (10 μM), a significant enhancement in fluorescence intensity at 586 nm was observed following excitation at 535 nm and gradual increased with Hg^{2+} concentrations. Meanwhile the solution showed an orange fluorescence.

Fitting of the Job's plot evaluated from the fluorescent spectra of Rh-C and Hg^{2+} at 586 nm gave rise to a 1: 1 stoichiometry for the Rh-C-Hg^{2+} complex(Fig 5 Inset (b)), which consistent with the results of absortion. The linear response of the fluorescence intensity toward Hg^{2+} was obtained in Hg^{2+} concentration range of 0.4–5 μM (Fig 5 Inset (a)). And the limit of detect ion (LOD) was obtained of 7.4×10^{-8} M, which was calculated based on 3 δ/k (δ is the standard deviation of the measured intensity of the blank solution and k is the slope of the plot in the inset of Fig. 5). The results demonstrated that the probe can quantitatively determine Hg^{2+} at the environmentally relevant level.

The Proposed Reaction Mechanism

In addition, the Na_2S-adding experiments were conducted to examine the reversibility of this reaction as shown in Fig. 6. Firstly, the addition of Hg^{2+} to the free Rh-C solution makes the fluorescence increase significantly. Secondly, the addition of Na_2S could immediately restore the initial fluorescent intensity and the color of free Rh-C due to the K_d value of 10^{-50} M^2 for Hg^{2+} at a standard condition in the form of $[HgS_2]^{2-}$. The stability of Rh-C has been test by add Hg^{2+} and Na_2S alternately. After four cycles, the fluorescence intensity of Hg-Rh-C quenched a little compared to the original fluorescent intensity. Thus, the probe Rh-C may be used repeatedly by adding Na_2S. It was also confirmed that the response of Rh-C to Hg^{2+} was reversible rather than a cation-catalyzed reaction.

Thus, based on the 1:1 binding mode and the reversible behaviour between Rh-C and Hg^{2+}, a possible coordination mode for Rh-C with Hg^{2+} was proposed (Scheme 2). Rh-C chelates with Hg^{2+} via two O atoms of two carbonyl groups, forming the complexation that requires the opening of the spiroring, which caused the recovery of fluorescence of rhodamine. It is very likely due to the chelation–induced ring opening of rhodamine spirolactam, rather than other possible reactions [25, 34].

Selectivity and Completion

The selectivity of Rh-C for Hg^{2+} was further observed in the fluorescent spectra. As expected, Rh-C exhibited excellent fluorescence selectivity towards Hg^{2+} over all other alkali and alkaline earth metal ions, transition and heavy metal ions except for a little bit of fluorescence enhancement for Fe^{3+} (Fig. 7). And, for reliable application, we can add F^- to mask Fe^{3+} caused the fluorescence. This finding indicated that Rh-C could selectively recognize Hg^{2+} in ethanol aqueous condition.

Then competitive experiments were conducted in the presence of 2.0 equiv. of Hg^{2+} mixed with 10.0 equiv. of various cations in the solution of Rh-C (10 μM), respectively. No significant variation in fluorescence intensity was found by comparison with that without other metal ions besides Hg^{2+} expect for Ag^+ and Zn^{2+} caused a little decrease (Fig. 8). All these indicate that the selectivity of Rh-C for Hg^{2+} over other competitive cations is high.

Bioimaging Applications of Rh-C in HL-7702 Cells

Bioimaging applications of Rh-C for monitoring of Hg^{2+} ions in living cells were then carried out. HL-7702 cells were incubated with Rh-C (5 μM) in culture medium for 30 min at 37 °C, and very weak fluorescence of Rh-C inside the living HL-7702 cells was observed (Fig. 9b). After three times washing with PBS buffer, The cells were then supplemented with 5 μM $Hg(NO_3)_2$ in the growth medium for another 30 min at 37 °C,a bright fluorescence was observed from the intracellular (Fig. 8d). A bright-field transmission image of cells treated with Rh-C and Hg^{2+} confirmed that the cells were viable throughout the imaging experiments (Fig. 9a, c). It is proved that Rh-C is cell-permeable and primarily little toxic to the cell culture. These results demonstrated that Rh-C may be used for detecting Hg^{2+} in biological samples.

Conclusion

In summary, we have developed a novel easily available turn-on fluorescent sensor Rh-C based on a rhodamine conjugate. It selectively responds to Hg^{2+} by chromo- and fluorogenic changes and also facilitates "naked-eye" detection of Hg^{2+}. The background metal ions showed small or no interference with the detection of Hg^{2+} ion. The chemosensor displayed a linear response to Hg^{2+} in the range of 0.4–5 μM with a detection limit of 7.4×10^{-8} M. Moreover, fluorescence microscopy experiments establish that Rh-C can be used for detecting Hg^{2+} within living cells.

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