The influences of different substituents on spectral properties of Rhodamine B based chemosensors for mercury ion and application in EC109 cells

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Abstract: Six rhodamine-based "Turn-on" fluorescence chemosenors($L_{1\sim6}$) with different substituents for Hg^{2+} were readily synthesized and investigated, which displayed high selectivity and chelation enhanced ratiometric fluorescence change and colorimetric change with Hg^{2+} among the metal ions examined. Based on UV spectral data and fluorescence spectral data, the effect of different substituents on spectral properties of the probes were presented and discussed. The detection limit of Hg^{2+} to probe L_1 was as low as 50 nM, which attribute to its electron donating group. Theoretical calculation also support the process of reaction. Confocal laser scanning microscopy experiments showed that probe could be usedto detect Hg^{2+} in living cells.

Keywords: Rhodamine B; Fluorescent chemosesor ; Sensing mercury ion; Living cells.

1.Introduction

Various transition-metal ions are essential to the life of creatures. Mercury is a dangerous and hazardous toxic which has posed a great threat to our environment[1-4]. Mercury is heavy metal, which can be dispersed in the atmosphere, the ocean, and the soil. Different speciation mercury can penetrate our environment by various ways, such as methyl mercury produced by aquatic microbes which bioaccumulates through the food chain and oxidation of mercury vapor in atmosphere to water-soluble Hg^{2+} ions[5-7]. After long-term exposure in the environment, mercury ion accumulate in organisms which can cause nausea, vomiting, abdominal pain, renal dysfunction and other diseases.

At present, UV spectrophotometry, electrochemical methods, ionization coupled plasma mass spectrometry, ionization coupled plasma atomic emission spectrometry, atomic absorption spectroscopy and other analytical methods can be used to identify and detect metal ions[8-12]. However, these methods have some disadvantages in the application, such as high detection costs, narrow range of applications, and can't achieve real-time detection. In contrast, the fluorescent molecular probe has no

damage to the sample, and gives its priority to easy operability and high sensitivity, so fluorescence analytical methods are effective and efficient ways to detect ions[13-15].

Compared with other fluorescent dyes, rhodamine dyes have some excellent photophysical properties, such as high fluorescence quantum yield, pH insensitive, high molar absorptivity and high extinction coefficient, particularly in its nucleotide and nucleic acid conjugates[16]. Some rhodamine-based chemosensors for Hg^{2+} ions have been reported[17-22], but dual colorimetric and fluorescent chemosensors for mercury ion were still rare[23] and some of them were not efficient enough to be selective toward mercury ion.

Here, we report six rhodamine-based caprolactam derivatives as a sensor for Hg^{2+}, when binding phenomena could be probed through binding-induced changes in an electronic spectral pattern. The difference between these probes are that we selected different substitutional group such as methyl group, nitro group, aldehyde group, halogen group, phenyl group. These rhodamine-based derivatives that behaves as a fluorescent Hg^{2+} sensor with high emission sensitivity and selectivity, which exhibited sensitive detection toward Hg^{2+} via significant fluorescence enhancement in solution, and in the meantime they showed a significant color change from colorless to rose red. We have designed some similar structures to compounds $L_{1\sim6}$ and conduct a series of experiments to explore their properties which have the correlations of nature of substituent group. After comparison of optical properties,probe L_1 showed a good performance in the recognition toward Hg^{2+},which indicated that the different electronic distribution among the senors' structures make an influence on their properties. For this reason, we selected L_1 as a typical example to expound in the following discussion.

2. Experimental

2.1 Apparatus and Materials

Fluorescence spectra measurement were performed on Horiba Jobin Yvon Inc. Fluorolog 3-TSCPC. ^{1}H NMR spectrum was run on a Bruker 300 MHz spectrometer using TMS as the internal standard. Mass spectrum was recorded with a VG ZAB-HS double focusing mass spectrometer. Absorption spectra were measured on a UV-2201

double-beam UV/VIS spectrometer. Melting point was determined using an SGW X-4 digital melting point apparatus.

All the materials for synthesis were purchased from Sinopharm Chemical Reagent Co.,Ltd (Shanghai, China) and used without further purification. Metal solutions were prepared from their analytical grade nitrate salts. The metal ions were prepared as 0.2 mM in water solution.

2.2 Syntheses

As shown in Scheme 1, the compounds L_1~L_6 were prepared by reacting RBH with aromatic aldehyde. RBH was synthesized according to literature.

2.2.1 Synthesis of RBH

As for the synthesis of RBH, sereval different procedures have been reported[24-26]. In this work, RBH was synthesized by a modified method according to Xiang[27]. m/z: 457.2265 ($[M+H]^+$); M^+ calculated 456.2265. IR(KBr,cm^{-1}): ν = 3428, 2987，2926, 1689, 1614, 1514, 1380, 1218, 1117, 818, 767. 1H NMR (300 MHz, $CDCl_3$, δ ppm), δ (ppm):1.17 (t, 12H), 3.34 (q, 8H), 3.62 (bs, 2H), 6.29 (dd, 2H), 6.43(d, 2H),6.45 (d, 2H), 7.11 (dd, 1H), 7.42 (d, 1H), 7.44(d, 1H), 7.93 (dd, 1H). (Fig.S1~S3)

2.2.2 Synthesis of L_1

RBH (0.5 mmoL, 0.24 g) was dissolved in 30 mL ethanol, and then p-tolualdehyde (0.5 mmoL, 0.12 g) was slowly added. The mixture was stirred and refluxed for 12 h at 80 ℃. After distillation in vacuum, the residue was recrystallized with methanol and water to give the final product L1 in yield of 79.8%. m/z: 559.3020($[M+H]^+$); M^+calculated 558.3020. ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 8.64 (s, 1H),7.97 (m, 1H), 7.49 (d, 2H), 7.45 (m, 2H),7.26 (d, 1H), 7.09 (m, 2H), 6.54 (d, 2H), 6.43 (s, 2H), 6.26 (d, 2H), 3.33 (q, 8H), 2.29 (s, 3H), 1.15(t, 12H). (Fig.S4~S6)

2.2.3 Synthesis of $L_{2\sim6}$

Compound $L_{2\sim6}$ was prepared using a general procedure which is essentially similar to that used for L_1. (Fig.S7~S21)

3. Results and Discussion

The structure of compounds $L_{1\sim6}$ were characterized by ^{1}H NMR, HR-MS. The results were in good agreement with the structure showed in Scheme 1. Fluorescence

and UV–vis studies were performed using a 20 μM solution of $L_{1\sim6}$ in a CH_3CN/H_2O (1:1, v/v) solution with appropriate amounts of metal ions.

3.1 Comparison of six rhodamine B based senors

Under the same conditions,we explore the sensitivity measurement of ion probes. As shown in Fig.1, rhodamine B derivatives with methyl exhibits excellent sensitivity towards mercury ion. Derivatives with halogen group also show good sensitivity owing to its electron donating groups. Based on these phenomena, it suggested that chelation-enhanced fluorescence appears when compounds with electron donating groups are complexed with Hg^{2+}. In order to further explore the affinity capacity between probes and metal ions, association constants were calculated to verify the experiment we have conducted. It is indicated in the Scheme 2, the numerical size of association constants minished in order, which in accordance with the results of fluorescence intensity when different substituents with rhodamine B binds to mercury.

After comparison between these probes, we can come to conclude that methyl group, halogen group and phenyl group belong to electron donating group while nitro group, aldehyde group belong to electron-attracting group. When interacting with mercury icon, electron donating group provide electron to coordination site which make it easier to combine. On the contrary, electron-attracting group make it more difficult for probe to interact with mercury icon. So, we prefer to select electron withdrawing substituents to detect metal ions which reveals high sensitivity. To investigate photoelectric properties of these probes further,we selected L_1 as an typical example to expound in the discussion.

3.2 Metal ion selectivity and competition experiments

As shown in Fig.2, UV–vis spectrum of compound L_1 (20μM) exhibited only very weak bands over 450 nm, which could be attributed to the presence of a trace amount of the ring-opened form of compounds. On addition of 10 equiv. Hg^{2+} into solution, L_1 immediately resulted in a significant enhancement of absorbance at about 556 nm simultaneously the color changed into rose red. Other metal ions such as Zn^{2+}, Mg^{2+}, Ca^{2+}, Cd^{2+}, Cu^{2+}, Pb^{2+}, Ba^{2+}, Ni^{2+}, Mn^{2+}, K^+, Li^+, Ag^+, Co^{2+}, except for Fe^{3+}, did not show any significant color and spectral change under any significant color and

spectral change under identical conditions. These phenomena suggest that these compounds can serve as a "naked-eye" chemosensor for Hg^{2+}. To further investigate the interaction of Hg^{2+} and the probe, the Hg^{2+} binding stoichiometry of the probe can be determined from titration and the Job plot[28, 29]. As shown in Fig.3, it is obvious that the fluorescence intensity reached a maximum when the ratio was 0.5, which suggesting that a 1:1 stoichiometry of the Hg^{2+} to the probes respectively in the complex. Furthermore, we also conducted the competitive experiments which indicates the background metal ions showed very low interference with the detection of Hg^{2+} in the water solutions (Fig.4.).

3.3 Emission spectra and detection limit of sensor

A fluorescence titration experiment was carried out in order to further explore the interaction of probes and Hg^{2+}. On addition and gradual increase concentration of mercury ions to the L_1 in CH_3CN/H_2O(V:V=1：1), the fluorescence intensity gradually increased and the solutions turned colorless to rose red. Limit detection of metal ions plays an important role in evaluating fluorescence chemosensor. As for probe L_1, the linear response for the fluorescence intensity response of compound L_1 was between 0 and 140 μM (Fig. 5), and the detection limit of Hg^{2+} was measured to be 0.05 μM. The association constant K of the complex L_1–Hg^{2+} was then calculated to be 3.24×10^3 M^{-1} with a linear relationship (Fig. 6) by Benesi–Hildebrand method, Eq. (1)[30, 31]

$$\frac{1}{F-F_0}=\frac{1}{K(F_{Max}-F_{Min})[Hg^{2+}]}+\frac{1}{F_{Max}-F_{Min}} \qquad (1)$$

As shown in the Fig.7, probe L_1 show a weak fluorescence in the absence of metal ions. On the addition of 10 eqiv. metal ions, the fluorescence intensity has changed. It is obvious that when 10 eqiv. Hg^{2+} was introduced into a solution of L_1 in CH_3CN/H_2O (1:1, v/v), the fluorescence intensity increased sharply. Under this conditions, Fe^{3+} induce a mild fluorescence enhancement while Zn^{2+}, Mg^{2+}, Ca^{2+}, Cd^{2+}, Cu^{2+}, Pb^{2+}, Ba^{2+}, Ni^{2+}, Mn^{2+}, K^+, Li^+, Ag^+, Al^{3+}, Co^{2+} and Na^+ did not show remarkable changes in fluorescence intensity and the color.

3.4 Time-dependence and Fluorescent lifetime

Time-dependence for binding of the probe L_1 with Hg^{2+} is given in Fig.8. Following the addition of 10 equiv. Hg^{2+} ion to 20.0 μM probe L_1, the fuorescence intensity of probe L_1 was turn on rapidlly and reached a stable value within 2 min. The fluorescence lifetime was measured at an excitation 460 nm of the NanoLED source. The decays of probes were found to be monoexponential. The lifetime decays in the absence of Hg^{2+} and in the presence of Hg^{2+} are shown in Fig.9. The average lifetime of L_1 was 1.87 ns and 5.00 ns (XSQ = 1.06) while the lifetime of L_1 + Hg^{2+} was 1.91 ns (XSQ = 1.14). Double exponential fitting equation was used to describe the fluorescence lifetime of probe itself , because xanthene was a chromophore which formed a conjugated system. The upper part of structure formed a plane and orthogonalized with the ring of xanthene which also can be seen as a chromophore. After adding metal ions，the upper part including carbanyl group and phenyl group do not conjugate with xanthene,and the stereo-hindrance effect of carbanyl group limits the rotation of the benzene ring. So the major chromophore is still xanthene which used single exponential fitting equation.

3.5 Application

3.5.1 Test papers

To demonstrate the practical application of our sensor, we prepared the test papers of sensor L_1. As depicted in Fig.10, the color of the test paper changed from colorless to purple and deepened gradually with the increasing of Hg^{2+} concentration. These paper-made test kits may be used as a simple tool for detecting Hg^{2+} in environmental samples.

3.5.2 Fluorescence imaging for EC109 cells

Owing to its favourable spectroscopic properties of the response to Hg^{2+}, L_1 should be suited for fluorescence imaging in living cells. Laser scanning confocal microscopy was used to investigate this proposition. As determined by laser scanningconfocal microscope, EC109 cells with a 10μM solution of probe L_1 in $CH_3CN–H_2O$ (1:1, v/v) 30 min at 25 ˚C led to no intracellular fluorescence(Fig.11b.). After washed with PBS three times, the staining cells were then supplemented with 10 μM of Hg^{2+} under the same condition for 30 min, a significant increase of

fluorescence from the intracellular area was observed (Fig.11c).

3.6 Mechanism

To investigate the interaction of probes and metal ions, probe L_1 was selected as an example to explain mechanism. IR spectra of L_1 and L_1+Hg^{2+} were taken in KBr disks (Fig.S22). It can be seen that the peak at 1698 cm^{-1} which relates to the amide carbonyl absorption disappeared upon with the addition of Hg^{2+}. To further investigate the mechanism of the reaction,we selected L_1 as an typical example to explain the phenomena. In addition，EDTA-adding experiment has been conducted to confirm the reversibility of reaction and the result was shown in the Fig.12,when the 30 eqiv. EDTA was added into L_1+ Hg^{2+} in CH_3CN/H_2O solution, the fluorescence intensity was decreased rapidly(green line).Then after adding the 30 eqiv. Hg^{2+}, the fluorescence intensity was recovered（red line）. This phenomenon indicates that the reaction between the compound and the metal ion are reversible.

As shown in the Fig.13, it indicates that these sereval chemosenors exhibit reversible and sensitive detection to Hg^{2+}. While hydrolytic action of compounds might be happen after a long-term interaction with metal ions. Theoretical calculation has been conducted to verify the stability of compound L_7, we assume the structure L_7 was existed after interacting with Hg^{2+}. Frequency calculation successfully provide the evidence for the existence of the stable structure. To investigate the possible mechanism of the absorption and fluorescent emission of L_1, theoretical calculation of L_1 which based on the optimized structures of the ground S0 state and the first excited S1 state were performed in Fig.14. After adding Hg^{2+}, the energy gap between the HOMO and LUMO is greatly decreased[32].

4. Conclusions

In summary, we reported the design and synthesis of six new rhodamine-based derivatives $L_{1\sim6}$ used as sensitive and selective chemosenor, which could specifically recognize Hg^{2+} ion in solutions by naked eyes. These six probe display 1:1 complex formation with Hg^{2+} which could be monitored by the spectral changes as well as color changes. Based on the experiment above, after a series comparison of these probes with different substituent group, L_1 shows better optical properties than others

owing to its electron donating group and excellent properties. Confocal laser scanning microscopy experiments showed that L_1 could beused to detect Hg^{2+} in living cells. Futhermore, these six new probes show a great potential for biotic environmental detection.

Supplementary Information (SI)

The structure of compounds RBH, $L_{1\sim6}$ were characterized by ^{1}H NMR, HR-MS,IR in Supplementary Information. Supplementary Information is available at www.ias.ac.in/chemsci.

Acknowledgements

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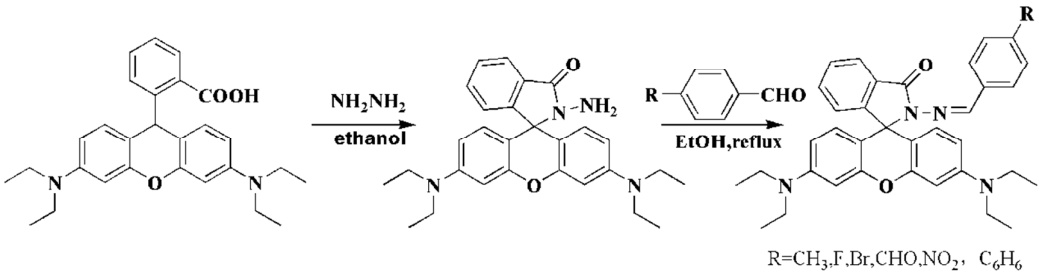

Scheme 1. Synthetic route of target compounds.

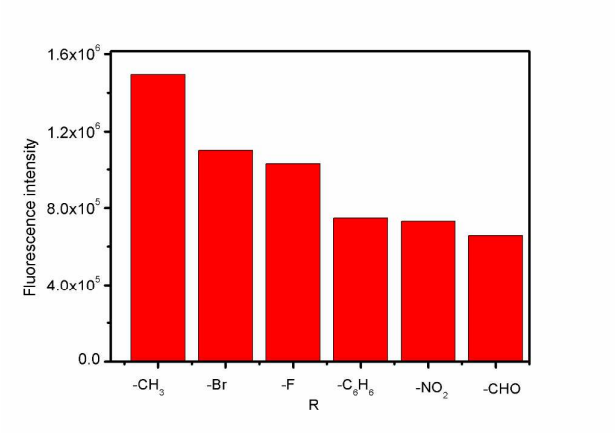

Fig.1. Fluorescence intensity (at 580 nm) of $L_{1\sim6}$ (20μM) in CH_3CN/H_2O (1:1, v/v) with the presence of Hg^{2+} (100 μM) (λex = 500 nm)

Coordination constant

-R	CH_3	Br	F	C_6H_6	NO_2	CHO
Coordination constant/$\mathbf{M^{-1}}$	3.24E3	2.67E3	2.38E3	2.27E3	3.70E2	2.56E2

Scheme 2.The coordination constant of six probes

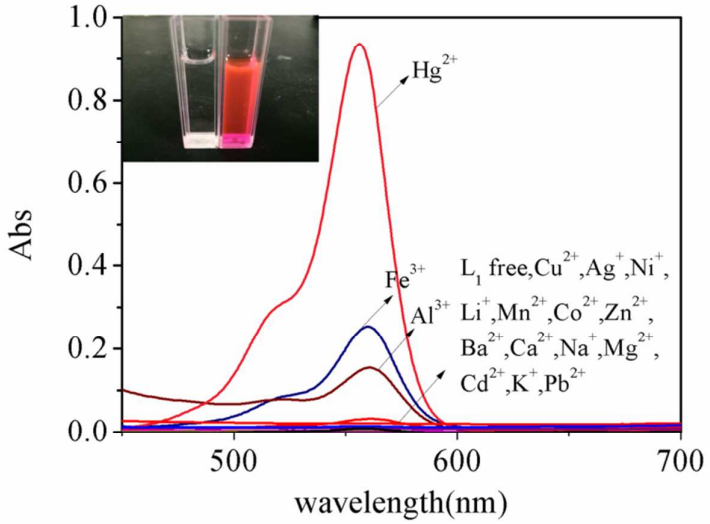

Fig. 2. Absorbance spectra of L_1(20 μM) in CH_3CN/H_2O (1:1,v/v) solutions with the presence of 10 equiv. of various species.

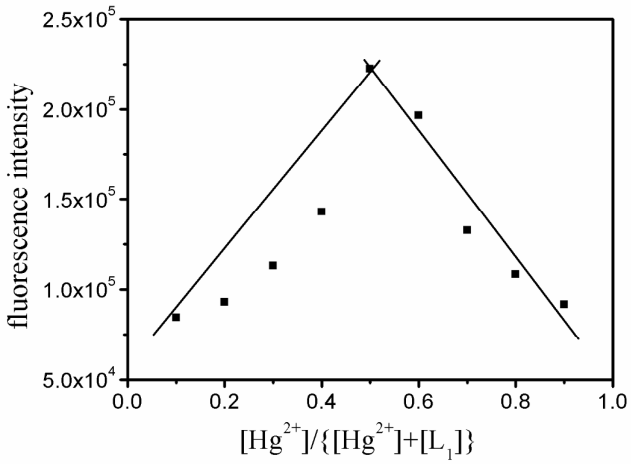

Fig.3. Job's plot of the complexation between L_1 and Hg^{2+}, the total concentration of L_1 and Hg^{2+} is 20.0 μM .

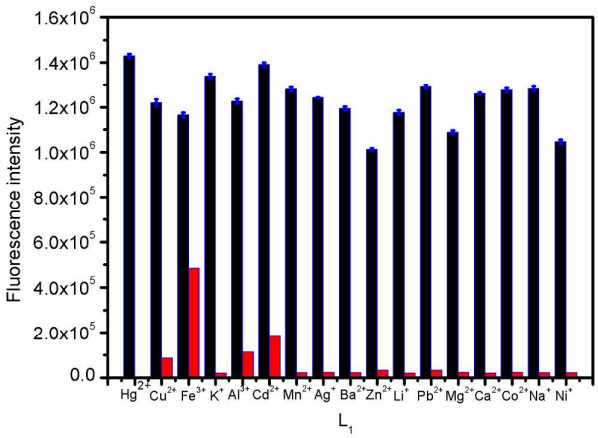

Fig.4. Fluorescence intensity (at 580 nm) of L_1 upon the addition of 20 μM Hg^{2+} in the presence of 20μM background metal ions in CH_3CN/H_2O (1/1, v/v) (λex = 500 nm).

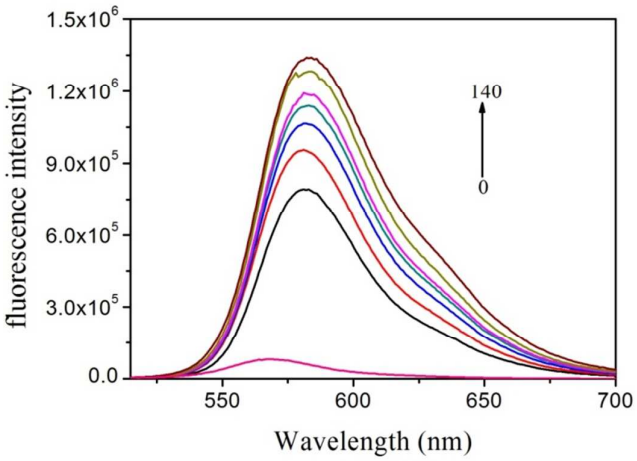

Fig.5(a). The fluorescence intensity (at 580 nm) of compound L_1 (20 μM) as a function of the Hg^{2+} concentration in CH_3CN/H_2O(1:1, v/v) solution (λex = 500 nm, slit = 3 nm)

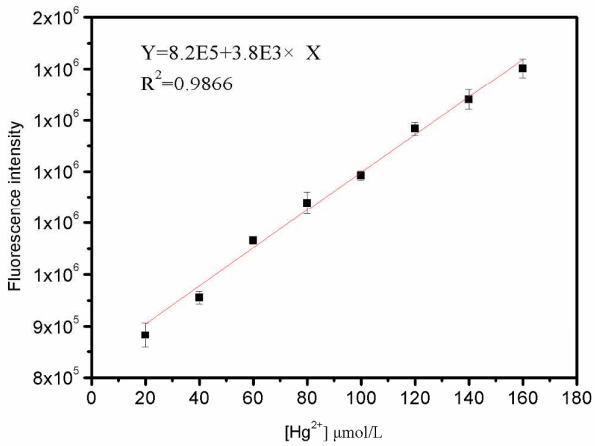

Fig.5(b). Linear relationship of L_1 (20 μM) in CH_3CN/H_2O (1:1,v/v) upon addition of different amounts of Hg^{2+} ions.

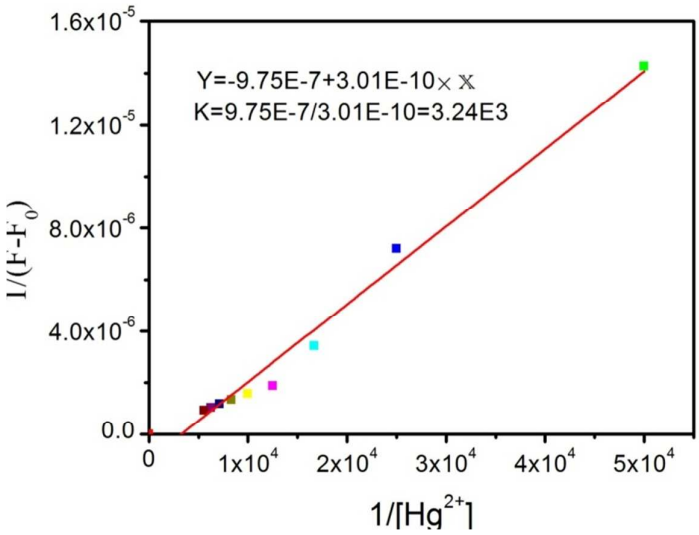

Fig.6. Benesi–Hildebrand plot (λex = 500 nm) of L_1, assuming 1:1 stoichiometry for association between L_1 and Hg^{2+} in the solutions.

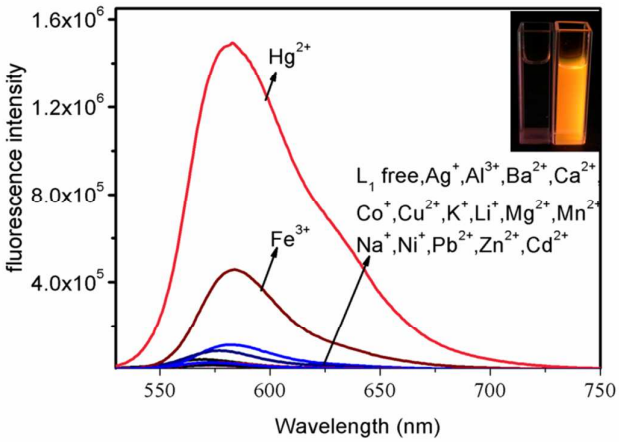

Fig.7. Fluorescence spectra of L_1 (20 μM) in CH_3CN/H_2O (1:1,v/v) with the presence of 10 equiv. of various metal ions (λex =500 nm).

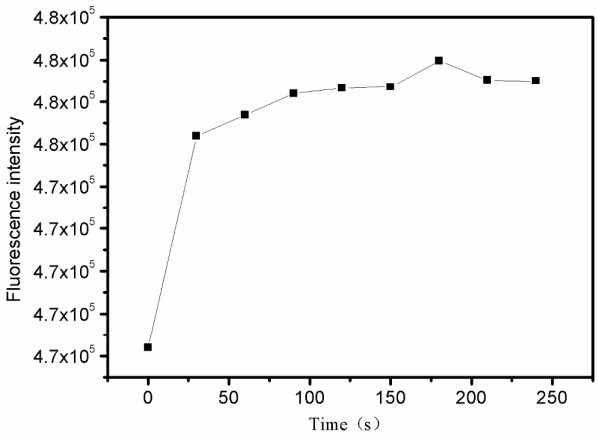

Fig. 8. Effects of time on the reaction of Hg^{2+} with L_1

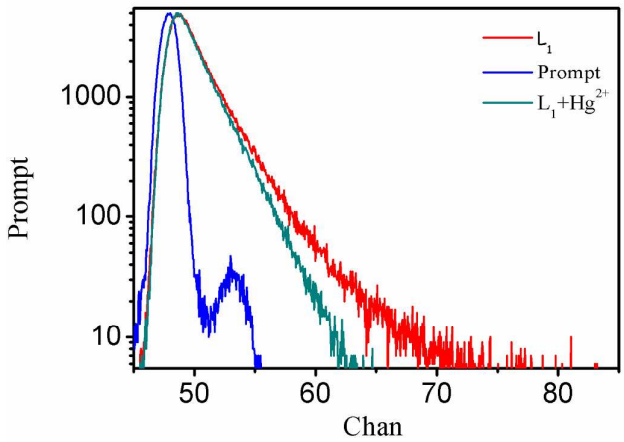

Fig.9. Fluorescence decay curves of L_1 and $L_1 + Hg^{2+}$ in solutions obtained at λ_{ex} =500 nm.

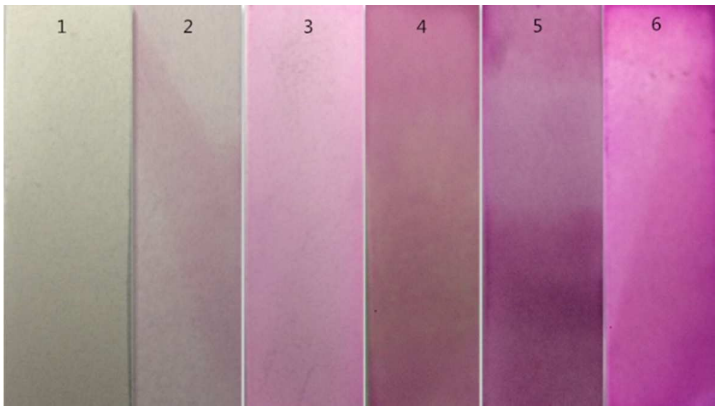

Fig. 10. Photographs of the test kits with L_1 for detecting Hg^{2+} in(CH_3CN/H_2O = 1:1, v/v) solution with different concentrations:(1) 0; (2) 1.0×10^{-5}M; (3) 1.0×10^{-4}M; (4) 1.0×10^{-3}M;(5)1.0×10^{-2}M;(6)1.0×10^{-1}M.

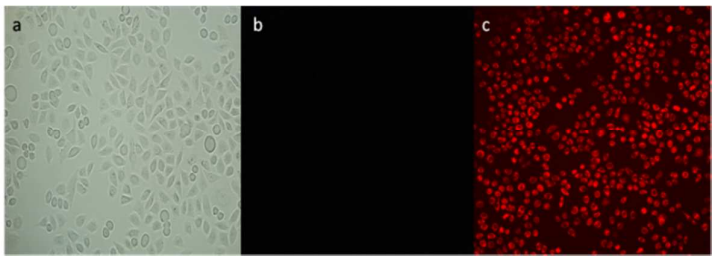

Fig.11. Laser confocal scanning microscopy experiments of EC109 cells: (a) Bright-field transmission image. (b) Cells with 10μM solution of L_1 in $CH_3CN–H_2O$ (1:1, v/v) for 30 min at 25 °C.(c)The staining EC109 cells exposed to L_1 (10μM) for 30 min and then to a $CH_3CN–H_2O$ (1:1, v/v) solution of Hg^{2+} (10μM) for 30 min.

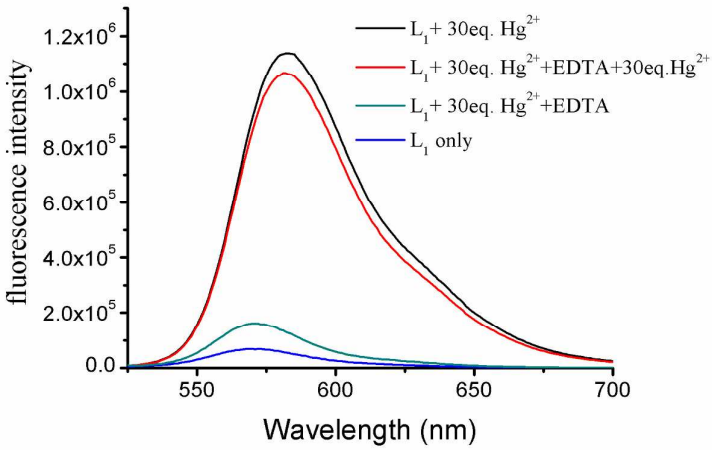

Fig.12. Fluorescence intensity (at 580 nm) of L_1 (20 μM) to Hg^{2+} in CH_3CN/H_2O (1:1,v/v) solutions (1) blue line: 10 μM L_1 only; (2) red line: 20 μM L_2 with 10 equiv. Hg^{2+}; (3) green line: 20 μM L_1 with 10 equiv. Hg^{2+} and then addition of 30 equiv. EDTA; (4) black line: 10 μM $L_1$with 10 equiv. Hg^{2+} and 30 equiv. EDTA then addition of 10 equiv. Hg^{2+} (λex = 500 nm).

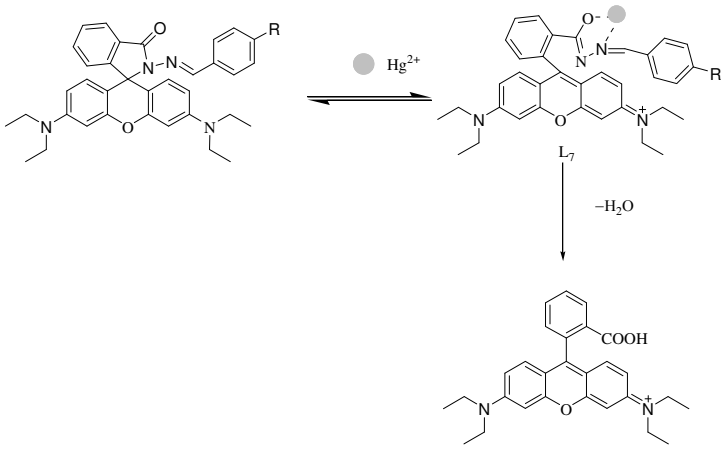

Fig.13. Possible sensing mechanism of $L_{1\sim6}$ with Hg^{2+}

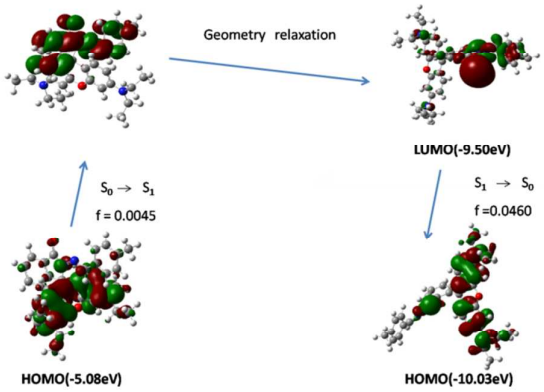

Fig.14. Theoretical molding of the absorption of L_1 without and with Hg^{2+} at the TDDFT level.